Characterization and biological activities of polyphenol and polysaccharide extract from *Phellinus linteus* and *Phellinus igniarius*

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Characterization and biological activities of polyphenol and polysaccharide extract from *Phellinus linteus* and *Phellinus igniarius*

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INTRODUCTION

Mushrooms have been appreciated for their flavors, economic value, ecological value, and medicinal properties for many years [1]. Many therapeutic effects have been reported for medicinal mushrooms such as anti-inflammatory [2], antitumor [3-5], anticancer and immunomodulatory effects [6-7]; stimulating macrophage activity and anti-hepatitis B virus activity [8]; and anti-oxidative activities [9-12]. *Phellinus linteus* (*P. linteus*) and *Phellinus igniarius* (*P. igniarius*) (L.) Quel. are basidiomycetous fungi belonging to the Hymenochaetaceae. Extracts of both were found to have anti-inflammatory activity *in vitro* as well as *in vivo* [13-16]. *P. igniarius* is a rich source of secondary metabolites like triterpenoids and polyphenols [17].

Polyphenol containing extracts from *P. igniarius* were found to be strongly anti-oxidant [18-21]. Little or no attention has ever been paid to their effects on stroke, which is a neurodegenerative disorder in whose pathogenesis reactive oxygen species (ROS) [22] such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) play an important role. Oxidative stress might be pathogenic at the early stage in the disease, and it can exacerbate it during later stages.

Iodoacetic acid (IAA) is an alkylating agent that reacts with cysteine residues of proteins. It is an irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is an enzyme of the glycolytic pathway. It was reported that IAA-treated neuronal cells die following depletion of intracellular ATP, mitochondrial dysfunction, and production of reactive oxygen species (ROS) [23-26]. These observations are similar with those of *in vivo* ischemic stroke [23].

Acrolein is a common environmental pollutant that is associated with respiratory disease, aberrant platelet aggregation and increased thrombosis [27]. Kazuei Igarashi's group suggested that the toxicity of acrolein (CH_2=CHCHO) is more severe than that of H_2O_2 and nearly equal to that of •OH [28]. Acrolein is spontaneously formed from spermidine and spermine by amine oxidase [29]. Once cells are damaged, polyamines are released from RNA [30], and acrolein is produced by polyamine oxidizing enzymes. Acrolein is toxic and will cause further cell damage and increase the levels of protein-conjugated acrolein (PC-Acro) as observed in stroke [31]. Furthermore, Igarishi's group presented that the toxicity of H_2O_2 was reduced by polyphenols [32]. Based on this we have investigated the effect of polyphenol extract from *P. igniarius*, and it prevented toxicity from acrolein and ROS in FM3A cells, Neuro-2a cells and photo-induced thrombosis model mice. Furthermore, we identified *P. igniarius* polyphenols by liquid chromatography mass spectrometry (LCMS) and proton nuclear magnetic resonance spectroscopy (NMR).

Polysaccharide extracts from fruiting bodies of several mushroom species have shown various biological activities such as free radical scavenging activity [33], antitumor activity [34], anti-inflammatory activity [35] and they have successfully been used in immunochemotherapy of cancer in Japan [36]. A major polysaccharide component of mushrooms is cell wall derived (1→3;1→6)-β-D-polysaccharide [37-39], of which the most common biological effect is immunomodulation [40-41].

Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are known to trigger pro-inflammatory immune responses [42]. CLRs specifically bind to glycans, such as mannans, GlcNAc, GalNAc, lNAc, and glucans from different mushrooms that all have a different structure and conformation and could induce different immunomodulatory effects [43-47].

Lipopolysaccharide (LPS) is an endotoxin consisting of a lipid-carbohydrate component from the outer membrane of gram-negative bacteria, such as *E. coli*. LPS is recognized by TLR4 which activates the innate immune system and promotes the secretion of proinflammatory cytokines including tumor necrosis factor (TNF-α) by monocytes and macrophages [48]. In addition, TNF-α is often induced together with Interleukin-6 (IL-6) that plays a major regulatory role in acute local and systemic inflammatory responses such as those elicited by either local lung or systemic exposure to endotoxin [49]. IL-6 is thought to have both pro- and anti-inflammatory effects. In the background of obesity and insulin resistance a low and persistent level of chronic inflammation can be found with IL-6 as one of the suggested mediators [50]. IL-6 has been implicated in the release of triglycerides and free fatty acids, downregulation of lipoprotein lipase, insulin resistance, and increased production of reactive oxygen species and decreased nitric oxide generation [50]. Interleukin-10 (IL-10) is a monokine that is not only produced in monocytes and macrophages but also in Th1 cells, B cells and Tr1 cells [51-55]. IL-10 plays a suppressing role in inflammatory responses. It downregulates the expression of TNF-α in monocytes and macrophages. IL-6 and TNF-α cytokines induced by LPS were both found significantly inhibited by IL-10 in macrophage cell lines [56] In this manuscript, the structure and composition of (1→3; 1→6)-β-D-polysaccharides from *P. linteus* and *P. igniarius* were studied by HPLC, GC-MS and NMR (1-H, 13-C, COSY, NOESY and TOCSY). Furthermore, the effects of (1→3; 1→6)-β-D-polysaccharides were examined on the production of IL-6, IL-10 and TNF-α by the Abelson leukemia virus transformed monocyte/macrophage cell line (RAW 264.7) that was treated by LPS.

RESULTS AND DISCUSSION

CHAPTER 1

Characterization and identification of polyphenol extracts from *Phellinus linteus* and *Phellinus igniarius*

P. linteus and *P. igniarius* are well-known fungal species of the genus *Phellinus* from the Hymenochaetaceae family which has been used as a source of traditional herbal medicine in oriental countries such as China, Korea, and Japan for many years (Fig. 1). *P. linteus* extract was reported to has anti-angiogenic, anti-oxidant [57-58], anti-inflammation [59], anti-cancer [60] and anti-bacteria effect [61]. It was reported earlier that hispolon, a phenolic compound from *P. linteus* has anti-inflammatory, anti-proliferative and anti-metastatic effects [62]. P. igniarius was also reported that has Antiproliferative and antimetastatic effects [63], anti-tumor effect [64], Antioxidant activity [65] and anti-influenza virus effect [66]. The group of phenolic compounds offers several bioactivities such as anti-oxidant effects [67-68], DPPH radical-scavenging capacity [69] and tyrosinase inhibition [70].

Therefore, we undertook the analysis of the DMSO soluble fraction of the *P.linteus* and *P. igniarius* ethanol extract followed by electron spray ionization LC-MS. The characterization of *P. linteus* polyphenols was confirmed by proton nuclear magnetic resonance spectroscopy (NMR). This study describes the difference in polyphenol composition of these tightly related *Phellinus* species. Whether this difference could be responsible for the intriguing difference in biological activity remains to be solved.

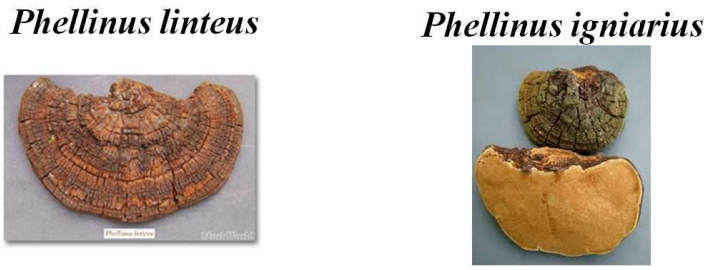

Fig. 1. *Phellinus linteus* and *Phellinus igniarius*

1.1. Total Phenolics Determination

The crude polyphenol extract were measured by the Folin-Ciocalteau method and UV absorbance polyphenol index (*750 nm*) as reference values, with highly significant correlation coefficients of antioxidant effect for 0.9946 by the range of gallic acid from 0 to 500 µg/ml (Fig. 2). The DMSO fraction from *P. linteus* and *P. igniarius* contained 458.73 µg and 323.08 phenol µg per ml respectively. The water extract from *P. linteus* and *P. igniarius* contained 20.18 µg and 55.82 µg phenol per ml respectively indicating that crude polyphenol extract has 23 and 6 times respectively more antioxidant effect than the crude water extract by Folin-Ciocalteau assay.

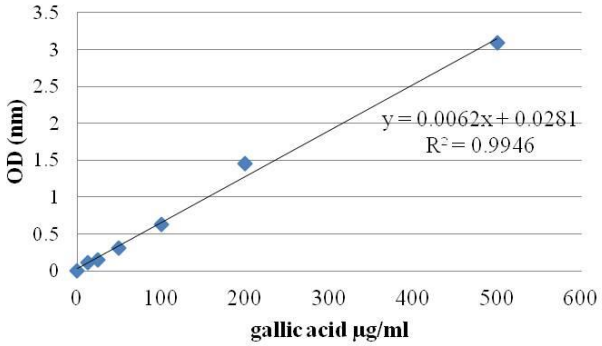

Fig. 2. The coefficients of antioxidant effect by the range of gallic acid from 0 to 500 µg/ml.

1.2 Analysis of polyphenols by liquid chromatography mass spectrometry (LC-MS)

1.2.1 Analysis of polyphenols from Phellinus linteus by LC-MS

The total ion current (TIC) chromatogram of the polyphenol extract from *P. linteus* showed 10 main peaks (Fig. 3). The composition of the chromatograms was analyzed by MASS++ with the MassBank library. To confirm identification, the compounds were identified by comparing their retention time and mass spectral data (Fig. 4). The ESI-MS of compound 1 gave a molecular ion at m/z 136.84 $[M-H]^-$ at 25.0 min (Fig. 4A) whereas the ESI-MS of 3,4-Dihydroxybenzaldehyde gave a molecular ion at m/z 136.90 $[M-H]^-$ at 23.9 min (Fig. 5). The molecular formula of compound 1 was determined as $C_7H_6O_3$ by ^1H NMR spectral data of 3,4-Dihydroxybenzaldehyde (Fig. 6) (Suabjakyong et al., 2015). This strongly suggested that compound 1 is 3,4-Dihydroxybenzaldehyde (Table 1).

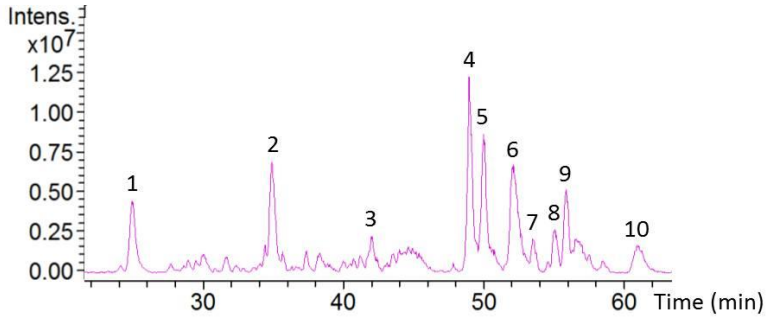

Fig. 3. Total ion current (TIC) chromatogram of polyphenols from *P. linteus*. The peak numbers are the same as in Table 1.

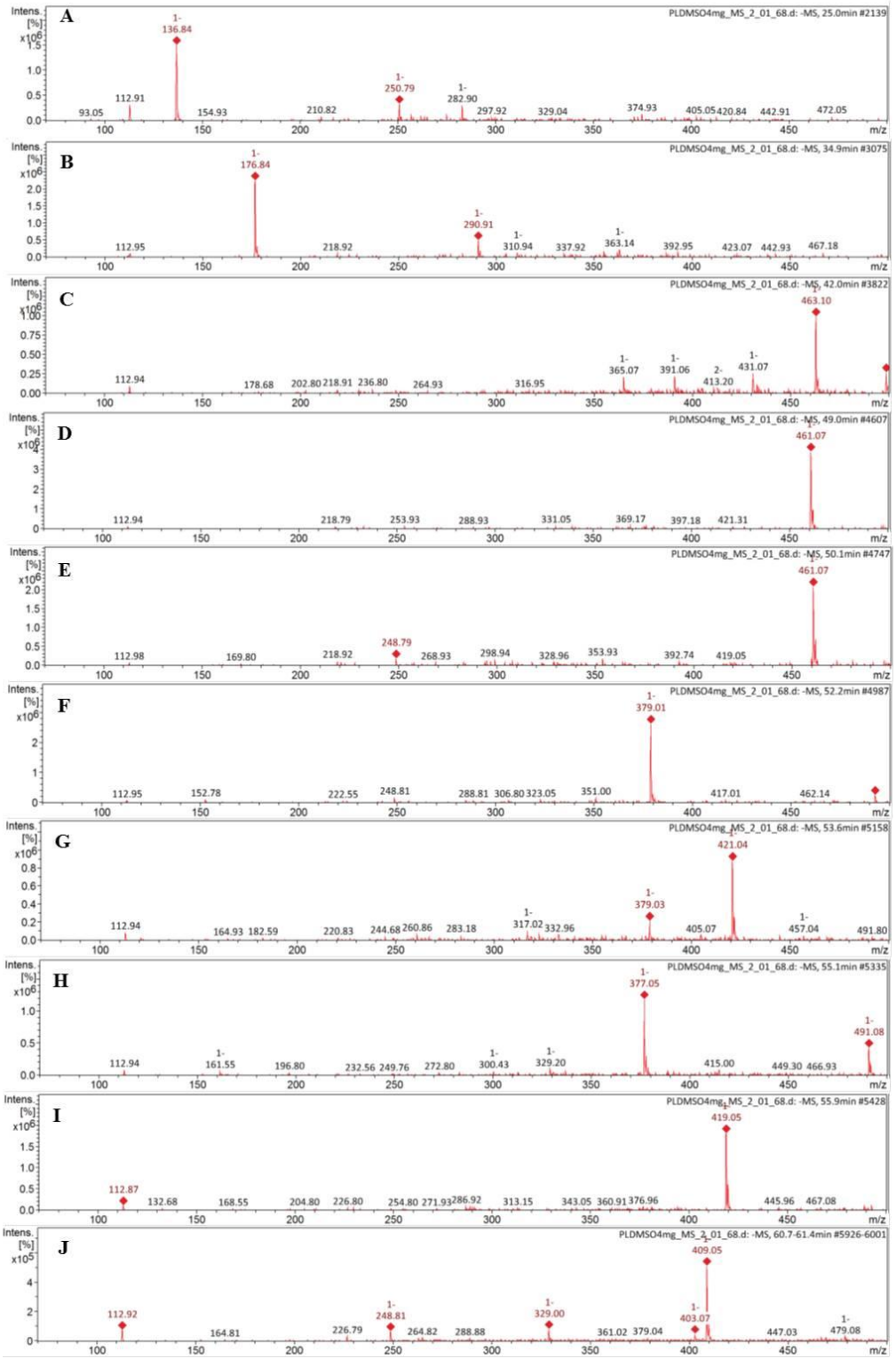

Fig. 4. ESI-MS spectra of *P. linteus* polyphenols from compounds 1-10 (A-J).

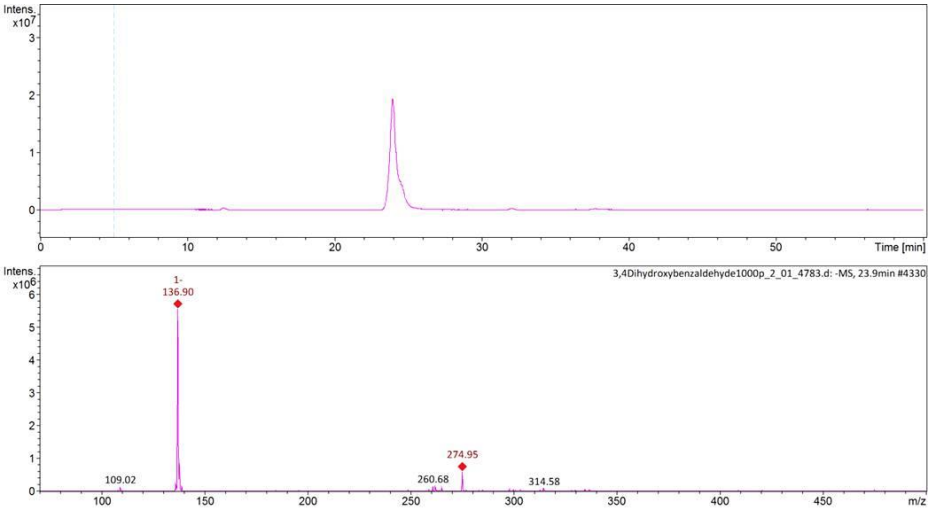

Fig. 5. Chromatogram and ESI-MS spectra of 3,4-dihydroxybenzaldehyde.

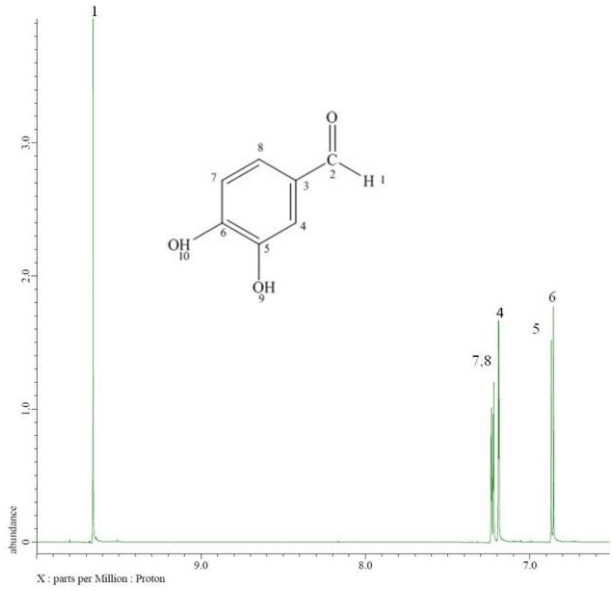

Fig. 6. ^{1}H 600 NMR spectra of 3,4-dihydroxybenzaldehyde in DMSO-d6 with TMS at 25ºC.

The ESI-MS of compound 2 gave a molecular ion at m/z 176.84 $[M-H]^-$ at 34.9 min (Fig. 3B). The ESI-MS of Esculetin and 4-(3,4-Dihydroxyphenyl-3-buten-2one gave a molecular ion at m/z 176.75 at 23.9 min (Fig. 6) and 177 $[M-H]^-$ [71] respectively. 4-(3,4-Dihydroxyphenyl-3-buten-2one has one more carbon atom than Esculetin. It was tentatively suggested that compound 2 is 4-(3,4-Dihydroxyphenyl-3-buten-2one. The ESI-MS of compound 3 gave a molecular ion at m/z 463.10 $[M-H]^-$. Weesepoel (2009) suggested that compound 3 might be Davallialactone or Interfungin A. Proton NMR resonances of compound 3 showed strongly signals of Interfungin A [72] that lack signals of Davallialactone as shown in Table 2. These observations strongly suggest that compound 3 is Interfungin A. The ESI-MS of compound 8 gave a molecular ion at m/z 377.05

$[M-H]^-$. Weesepoel (2009) suggested compound 8 might be Inoscavin E or Phellifuropyranone A. In 2013, Yoon & Paik (2013) showed that *P. linteus* extract contains Inoscavin E [73]. Furthermore, the proton NMR resonances of compound 8 showed strongly signals of Inoscavin E [74] as shown in Table 3. It was strongly suggested that compound 8 is Inoscavin E.

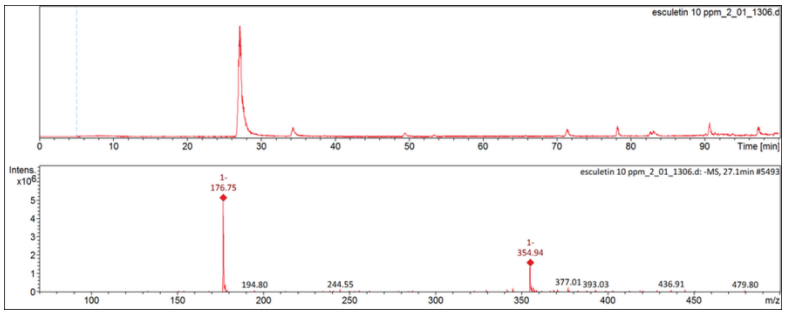

Fig. 7. Chromatogram and ESI-MS spectra of Esculetin.

Table 1. Tentative identification of polyphenol extract from *Phellinus linteus* by UPLC/ESI-MS in negative

Compounds	RT (min)	Tentative identification	MW	$[M-H]^-$ m/z	UPLC/ESI-MS (% base peak)
1	25.0	3,4-dihydroxybenzaldehyde	138.12	136.84	136.84(100), 250.79(21.69), 282.89(20.29), 256.03(7.92), 261.81(6.87), 263.81(5.24)
2	34.9	4-(3,4-dihydroxyphenyl-3-buten-2one	178.19	176.84	176.84(100), 290.91(21.86), 363.11(9.73), 355.10(8.08), 310.96(5.56)
3	42	Interfungin A	464.43	463.10	463.10(100), 431.05(26), 391.06(22.47). 365.05(21.48), 433.09(12.40), 379.12(7.24), 827.55(6.81), 438.95(6.51), 423.06(6.05)
4	49.0	Inonoblin C	462.42	461.07	461.07(100)
5	50.1	Inonoblin C	462.42	461.07	461.07(100)
6	52.2	Phelligridin D	380.31	379.01	379.01(100)
7	53.6	Interfungin B	422.40	421.04	421.04(100), 379.03(24.40), 317.02(11.79), 377.07(6.00), 323.08(5.70), 457.04(5.67), 354.85(5.64)
8	55.1	Inoscavin E	378.34	377.05	377.05(100), 491.08(36.29), 329.21(8.16), 161.55(5.68), 300.46(5.34)
9	55.9	Inoscavin C	420.38	419.05	419.05(100)
10	60.9	Methylinoscavin D	410.38	409.05	409.05(100), 329.00(15.75), 248.81(12.94), 403.07(9.63), 479.07(5.75), 467.00(5.37)

1.2.2 Analysis of polyphenols from *P. igniarius* by LC-MS

The total ion current (TIC) chromatogram of the ethanol extract from *P. igniarius* showed main 9 peaks (Fig. 8). The composition of the chromatograms was analyzed by MASS++ with MassBank library. To confirm identification, the compounds were identified by comparing their retention time and mass spectral data (Fig. 9). The ESI-MS of compound 1 gave a molecular ion at m/z 136.89 $[M-H]^-$ at 24.0 min (Fig. 9A) that ESI-MS of 3,4-Dihydroxybenzaldehyde gave a molecular ion at m/z 136.90 $[M-H]^-$ at 23.9 min (Fig. 5). The molecular formula of compound 1 was determined as $C_7H_6O_3$ by ^1H NMR spectral data of 3,4-Dihydroxybenzaldehyde (Fig. 6). This strongly suggested that compound 1 is 3,4-Dihydroxybenzaldehyde (Table 2).

The ESI-MS of compound 2 gave a molecular ion at m/z 176.81 $[M-H]^-$ at 33.8 min (Fig. 9B). The ESI-MS of Esculetin and 4-(3,4-Dihydroxyphenyl-3-buten-2one gave a molecular ion at m/z 176.75 at 23.9 min (Fig. 7) and 177 $[M-H]^-$ [71] respectively. 4-(3,4-Dihydroxyphenyl-3-buten-2one has one more carbon atom than Esculetin. It was tentatively suggested that compound 2 is 4-(3,4-Dihydroxyphenyl-3-buten-2one. Compound 3 to 7 and 9 were tentative identified by masses of the corresponding $[M-H]^-$ from the M.Sc thesis of Weesepoel [71].

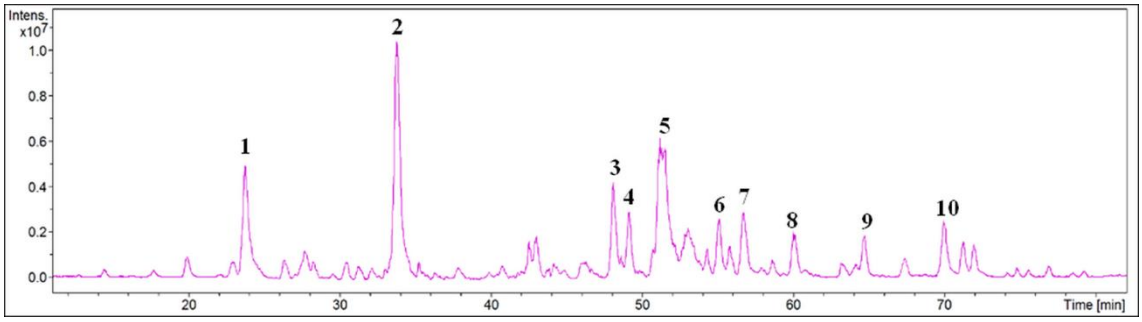

Fig. 8. Total ion current (TIC) chromatogram of polyphenols from *P. igniarius.*

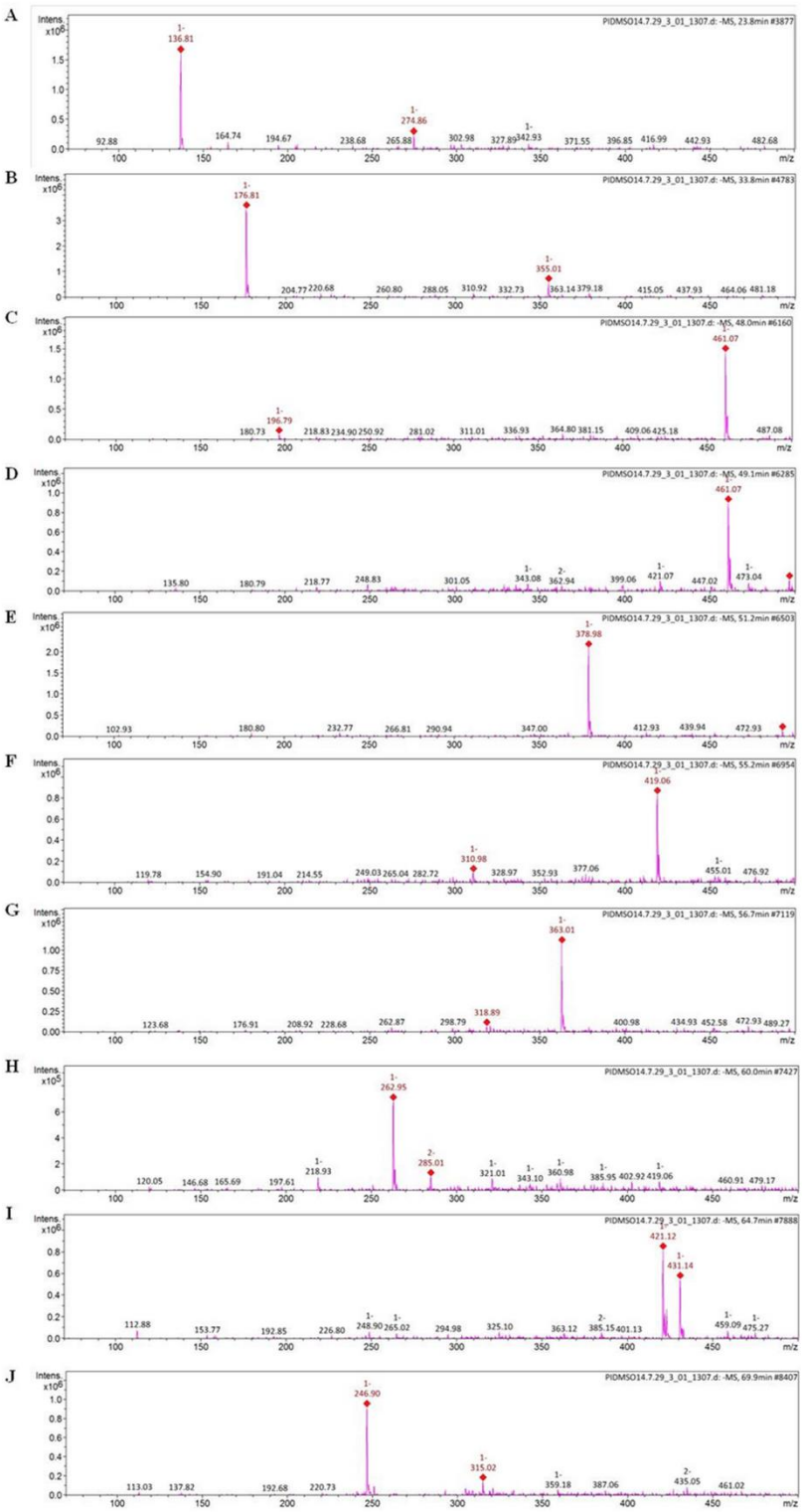

Fig. 9. ESI-MS spectra of *P. igniarius* polyphenols from compounds 1-10 (A-J).

Table 2. Tentative identification of polyphenol extract from *P. igniarius* by UPLC/ESI-MS in negative

Compounds	RT (min)	Tentative identification	MW	$[M-H]^-$ m/z	UPLC/ESI-MS (% base peak)
1	23.8	3,4-dihydroxybenzaldehyde	138.12	136.81	136.81(100), 274.86(14.27), 342.94(5.98)
2	33.8	4-(3,4-dihydroxyphenyl-3-buten-2one	178.19	176.81	176.81(100), 355.01(16.71)
3	48.0	inonoblin C	462.42	461.07	461.07(100), 196.79(6.10)
4	49.1	inonoblin C	462.42	461.07	461.07(100), 196.79(6.10)
5	51.2	phelligridin D	380.31	378.98	378.98(100), 492.97(6.69)
6	55.2	inoscavin C	420.38	419.06	419.06(100), 310.98(11.18), 758.17(2.60), 454.98(6.93), 847.25(6.49), 453.07(6.48), 375.01(6.34), 411.10(5.83), 459.04(5.77), 381.07(5)
7	56.7	phelligridin C	364.31	363.01	363.01(100), 379.00(5.57)
8	60	unknown	n.d.	262.95	262.95(100), 571.02(15.03), 218.93(14.74), 360.98(13.51), 321.02(12.51), 419.05(9.56), 385.95(8.05), 359.11(7.97), 343.06(7.10),379.00(6.56), 250.91(6.46), 364.97(5.78), 751.02(5.76)
9	64.7	interfungin B	422.40	421.12	421.12(100), 431.14(66.72), 459.08(9.10), 248.92(8.02), 265.03(6.06), 475.23(5.78), 771.24(5.77)
10	69.9	unknown	n.d.	246.90	246.90(100), 315.02(15.50), 871.16(9.39), 305.09(7.68), 433.05(5.47), 359.18(5.39), 309.01(5.07)

3.3. Nuclear magnetic resonance spectroscopy (NMR)

All polyphenol compounds were tentatively identified by masses of the corresponding $[M-H]^-$ from the M.Sc thesis of Weesepoel (2009) [71]. The assignment of proton NMR resonances in polyphenols from *P. linteus* indicated the presence of Inonoblin C [75] (compound 4 and 5), Phelligridin D [75] (compound 6) and Inoscavin C [76] (compound 9) in Table 3. Furthermore, Furthermore, ^1H NMR resonances of polyphenols from *P. igniarius* were assigned,

identifying Inonoblin C [75] (compounds 3 and 4), Phelligridin D [75] (compound 5), Inoscavin C [76] (compound 6) and Phelligridin C [76] (compound 7) in Table 4.

Table 3. Compounds identified in polyphenol extract from *Phellinus linteus* by assignment of their proton resonances

Compound	^{1}H NMR chemical shifts and their assignments
3,4-Dihydroxybenzaldehyde **(1)**	9.699 s, 6.918 s, 6.905 s, 7.273 d (2.06), 7.259 d (2.06), 7.236 d (2.06)
Interfungin A **(3)**	6.235 s, 6.693 d (17.18), 7.383 d (17.18), 7.062 d (2.06), 6.813 d (8.25), 7.012 dd (8.25, 2.06), 6.931 d (2.06), 6.686 d (8.25), 6.839 d (8.25), 7.727 s, 7.051 s
Inonoblin C **(4),(5)**	6.176 s, 6.693 d (17.18), 7.297 d (17.18), 7.062 d (2.06), 6.732 d (8.25), 7.012 dd (8.25, 2.06), 6.820 s, 7.099 s, 6.319 brd (2.06), 2.273 s (Me)
Phelligridin D **(6)**	6.725 s, 7.534 s, 8,351 s, 6.693 d (17.18), 7.297 d (17.18), 7.062 d (2.06), 6.732 d (8.25), 7.012 dd (8.25, 2.06)
Inoscavin E **(8)**	6.962 s, 6.832 s, 7.236 d (2.06), 6.839 d (8.25), 7.266 dd (8.25, 2.06), 6.70 d (17.18), 7.297 d (17.18), 7.062 d (2.06), 6.745 d (8.25), 7.012 dd (8.25, 2.06)
Inoscavin C **(9)**	6.846 s, 6.734 s, 7.072 s, 7.236 d (2.06), 6.839 d (8.25)

Table 4. Compounds identified in polyphenol extract from *Phellinus igniarius* by assignment of their proton resonances

Compound	^{1}H NMR chemical shifts and their assignments
3,4-Dihydroxybenzaldehyde **(1)**	9.702 s, 6.923 s, 6.909 s, 7.284 d (2.06), 7.270 d (2.06), 7.242 d (2.06)
Inonoblin C **(3),(4)**	6.161 s, 6.690 d (17.18), 7.330 d (17.18), 6.985 d (2.06), 6.751 d (8.25), 7.009 dd (8.25, 2.06), 6.816 s, 7.091 s, 6.260 brd (2.06), 2.280 s (Me)
Phelligridin D **(5)**	6.730 s, 7.539 s, 8,361 s, 6.690 d (17.18), 7.330 d (17.18), 7.068 d (2.06), 6.789 d (8.25), 7.009 dd (8.25, 2.06)
Inoscavin C **(6)**	6.816 s, 6.758 s, 7.091 s, 7.242 d (2.06), 6.847 d (8.25)
Phelligridin C **(7)**	6.730 s, 7.539 s, 8,361 s, 6.909 s, 6.847d (8.25)

CHAPTER 2

Bioloigical activities of polyphenol extracts from

Phellinus linteus and *Phellinus igniarius*

2.1 The protection of polyphenol extracts on acrolein and H_2O_2 in *in vitro*

To characterize the effects of PI EtOH extract on acrolein treated FM3A cells we first determined the possible cytotoxicity of PI EtOH extract by trypan blue assay. The extract showed no cytotoxicity at concentrations of 2, 1 and 0.5 µg/ml and only very slight cytotoxicity at 5 µg/ml.

The cell toxicity caused by 5 (Fig. 10A) and 2 µM (Fig. 10B) acrolein was strongly prevented by 5 µg/ml PI EtOH extract and also prevented by a lower concentration of PI EtOH extract (Fig. 11). Cell toxicity caused by 100 µM H_2O_2 was prevented by 5 µg/ml PI EtOH extract (Fig. 10C), and it was also prevented by 0.5 µg/ml PI EtOH extract (Fig. 10D). PI EtOH extract strongly prevented the effects acrolein and to a higher extent than the effects of H_2O_2. The results suggest that the reactivity between •OH and PI EtOH extract may be relatively weak compared with the reactivity between acrolein and PI EtOH extract where strong prevention was observed.

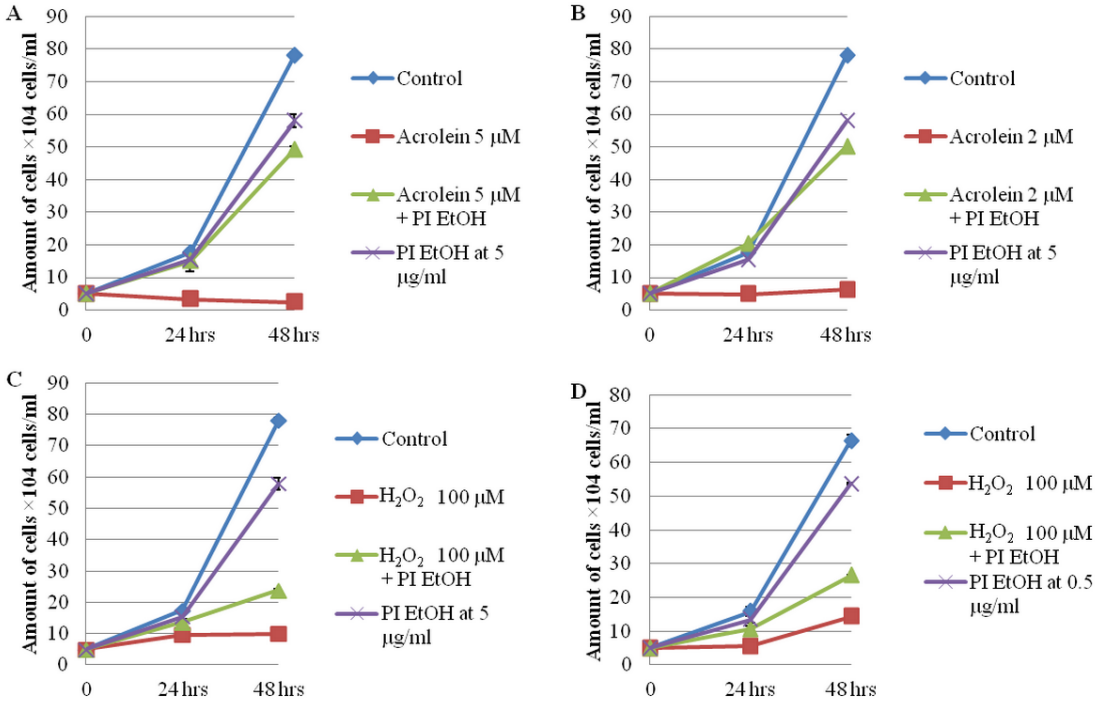

Fig. 10. The cytotoxicity of acrolein and H_2O_2 in FM3A cells. The viable cell number of FM3A cells that were treated with PI EtOH extract at 5 µg/ml plus acrolein at 5 µM (A) or 2 µM (B) or

100 μM H_2O_2 and cultured for 24 and 48 hrs. The number of cells was counted with 0.25% trypan blue. Each value represents the mean ± SD (n=3).

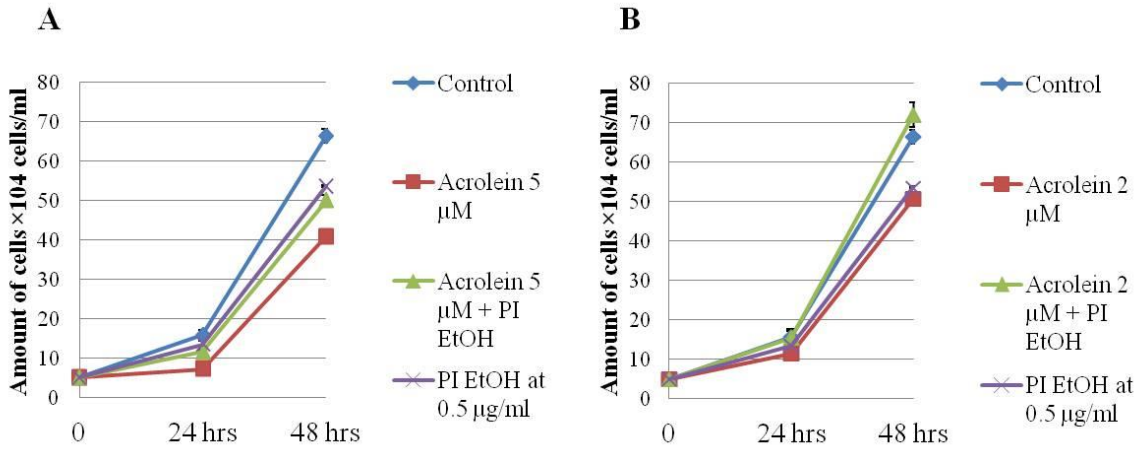

Fig. 11. The cytotoxicity of acrolein in FM3A cells. The viable cell number of FM3A cells that were treated with PI EtOH extract at 0.5 μg/ml plus acrolein at 5 μM (A) or 2 μM (B) and cultured for 24 and 48 hrs. The number of cells was counted with 0.25% trypan blue. Each value represents the mean ± SD (n=3).

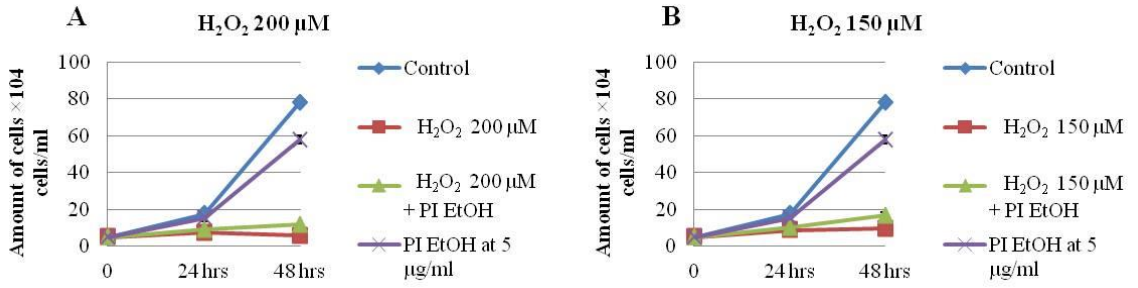

Fig. 12. The cytotoxicity of H_2O_2 in FM3A cells. The viable cell number of FM3A cells that were treated with PI EtOH extract at 5 μg/ml plus 200 μM (A) or 100 μM (B) H_2O_2 and cultured for 24 and 48 hrs. The number of cells was counted with 0.25% trypan blue. Each value represents the mean ± SD (n=3).

We therefore undertook to measure the cytotoxicity of acrolein and iodoacetic acid in Neuro-2a cells instead of the solid tumor derived FM3A cells. The results showed that PL and PI crude aqueous extracts may protect cultured Neuro-2a cells, i.e., the number of cells at the end of

the incubation period, from the deleterious effects of being pretreated with 8 µM acrolein or 5 µM iodoacetic acid, respectively, for 4 hrs. PL ethanol extract may allow cells to recover after a pretreatment with 5 µM iodoacetic acid for 4 hrs (Fig. 13). In Fig. 14, it is shown that crude polysaccharide extract from *P. linteus* and *P. igniarius* provided no protection against iodoacetic acid. On the other hand, 0.5 µM PI EtOH extract and PI- and PL-derived polysaccharide extracts can protect cells from the toxic effects of 8 µM acrolein for 24 hrs (Fig. 14). These results strongly suggest that PI- and PL-derived extracts can protect Neuro-2a cells from acrolein cytotoxicity.

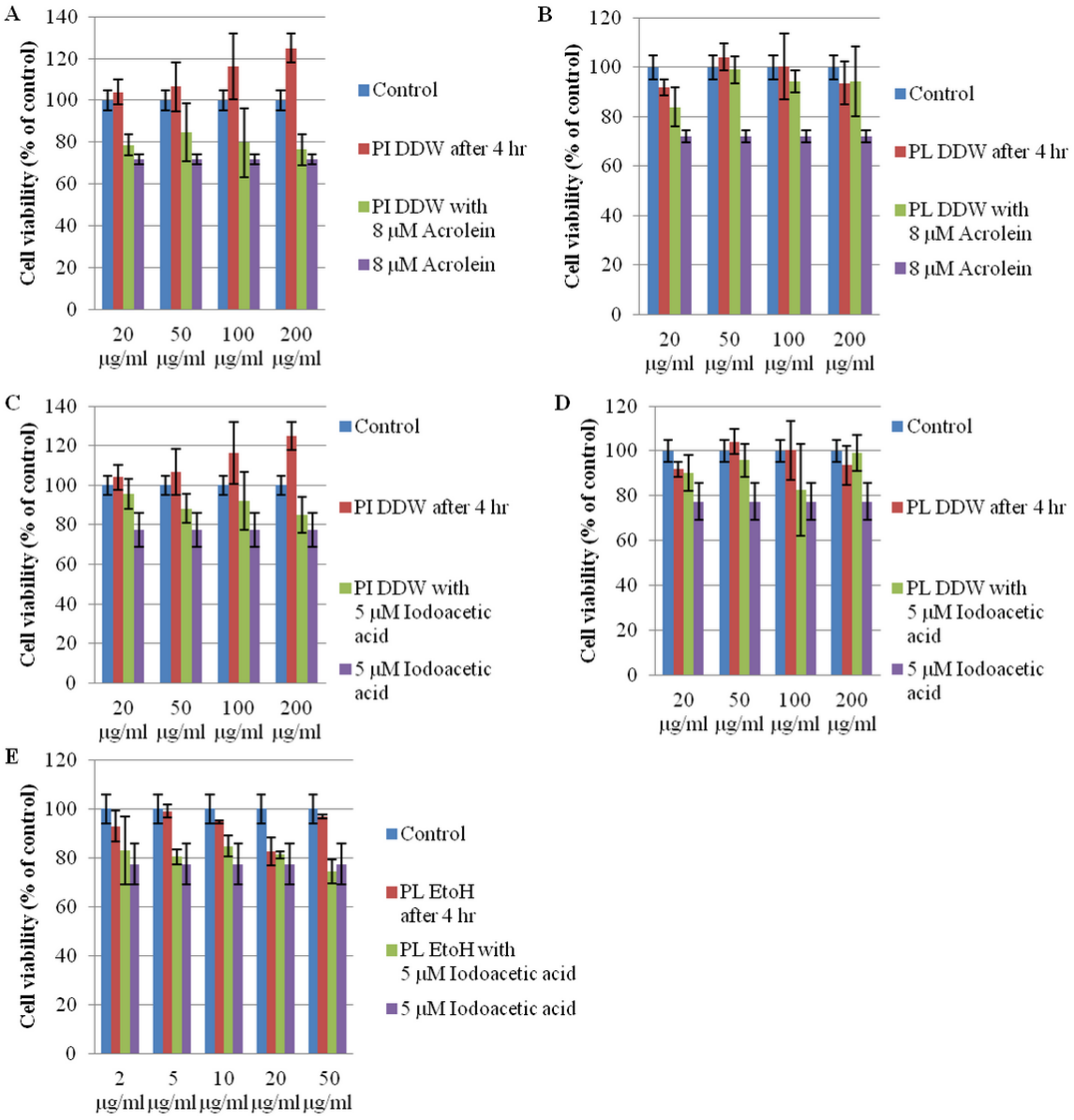

Fig. 13. The effect of *P. igniarius* and *P. linteus* extracts on the cytotoxicity of acrolein and iodoacetic acid. The viable cell number of Neuro-2a cells that were treated with PI (A) or PL (B)

DDW crude extract at 20, 50, 100 and 200 µg/ml after a pretreatment with 8 µM acrolein for 4 hrs. PI (C) and PL (D) DDW crude extract at 20, 50, 100 and 200 µg/ml after a pretreatment with 5 µM iodoacetic acid for 4 hrs. PL EtOH crude extract (E) at 2, 5, 10, 20 and 50 µg/ml after a pretreatment with 5 µM iodoacetic acid for 4 hrs. Cell viability was determined by MTT assay after 24 hrs. Each value represents the mean ± SD (n=3).

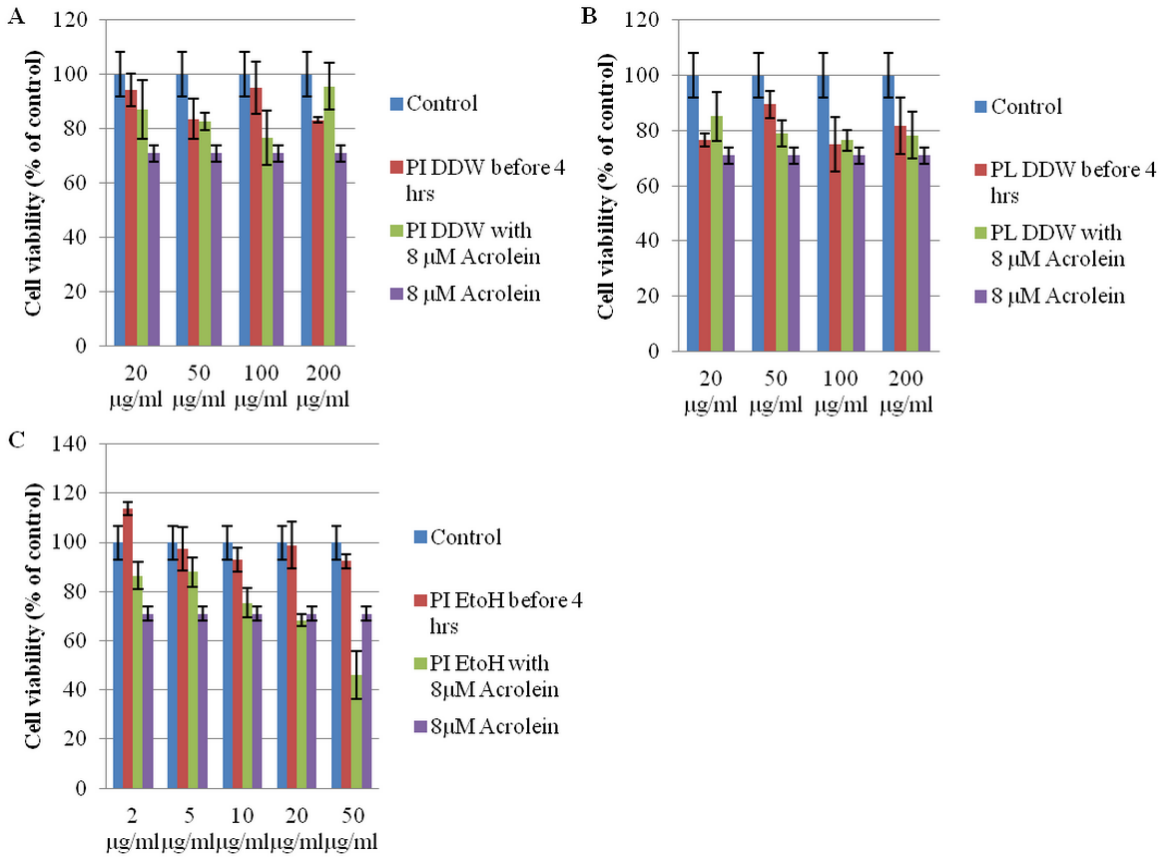

Fig. 14. Effect of pretreatment of Neuro-2a cells with PL and PI extracts on cytotoxicity of acrolein. The viable cell number of Neuro-2a cells that were treated with PI EtOH crude extract (A) at 2, 5, 10, 20 and 50 µg/ml, PI (B) or PL (C) DDW crude extract at 20, 50, 100 and 200 µg/ml for 4 hrs and treated with 8 µM acrolein. Cell viability was determined by MTT assay after 24 hrs. Each value represents the mean ± SD (n=3).

We then did a time study, which is shown in Figs. 14 and 15. Crude polysaccharides of *P. linteus* and *P. igniarius* at 10, 20, 50 and 100 µg/ml, PI ethanol extract dissolved in water at 10, 20,

50 and 100 µg/ml and PI ethanol extract dissolved in DMSO at 0.5, 1, 2 and 5 µg/ml were tested on Neuro-2a cells with acrolein at 2, 5, and 10 µM for 12, 24 and 48 hrs.

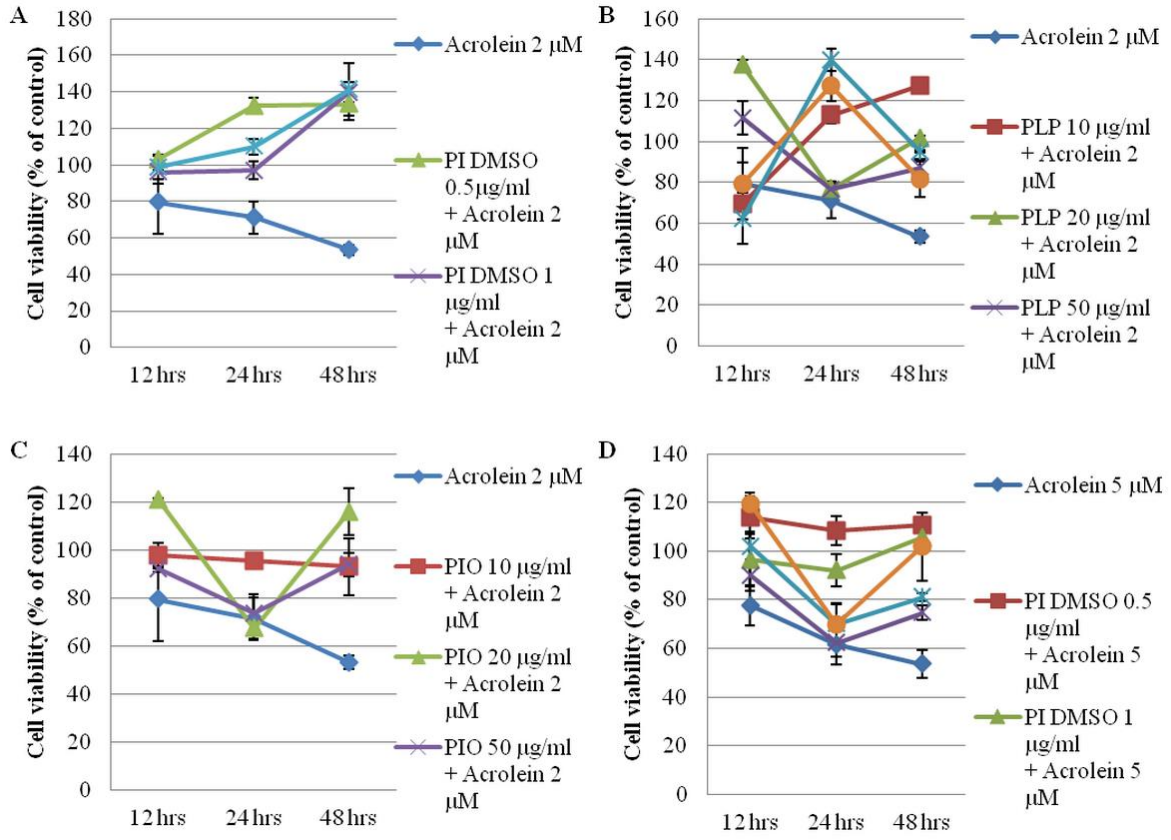

Fig. 15. Matrix assay of ethanol extract. EtOH extract dissolved in DMSO at 0.5, 1 and 2 µg/ml with acrolein at 2 µM (A); glucans of PI at 20 and 50 µg/ml and polysaccharides of PL at 10, 20 and 50 µg/ml with acrolein at 2 µM (B); PI ethanol extract dissolved in DDW at 10, 20 and 50 µg/ml with acrolein at 2 µM (C) or 5 µM (D). Cell viability was determined by MTT assay after 12, 24 and 48 hrs. Each value represents the mean ± SD (n=3).

The results showed that PI ethanol extract dissolved in DMSO at 0.5, 1 and 2 µg/ml protected Neuro-2a cells from the effects of 2 µM acrolein. Also, PL polysaccharides at 10, 20 and 50 µg/ml and PI ethanol extract dissolved in water at 10, 20 and 50 µg/ml may protect cells from 2 µM acrolein. PI ethanol extract dissolved in DMSO at 0.5 and 1 µg/ml may protect cells from 5 µM acrolein. PL polysaccharides at 50 µg/ml may protect cells from 5 µM acrolein (Fig. 15). These observations indicated that the polyphenol extract of *P. igniarius* greatly reduced the growth inhibition of Neuro-2a cells that was caused by acrolein, contrary to earlier results by Kazuei Igarishi's group in which acrolein toxicity was not prevented by polyphenols [32].

2.2 The protection of polyphenol extracts on mice stroke model

In the foregoing *in vitro* experiments we found that PIDMSO (= *P. igniarius* ethanol extract dissolved in DMSO) greatly reduced the inhibition of cell growth induced by acrolein. We therefore undertook to measure the effects *in vivo* using mice with brain infarction photochemically induced by Rose Bengal as a model The effects of PIDMSO on brain infarction were examined to confirm the correlation between brain infarction and PC-Acro (= protein-conjugated acrolein). Mice were injected intraperitoneally with PIDMSO at 200 ng to 20 mg/kg. A brain infarction was photo-induced after injection of Rose Bengal [77]. The volume of the infarction was determined by staining 2 mm thick coronal slices with triphenyltetrazolium. This stains the viable brain tissue red, whereas infarct tissue remains unstained [78]. Under our experimental conditions, in which we measured the average volume of infarction at 24 hrs after induction of stroke, the average volume of infarction decreased from 56.22 mm^3 to 21.33 mm^3 and 30.02 mm^3 from intraperitoneally applied PIDMSO at 20 μg/kg and 20 mg/kg, respectively (Fig. 15A). The stroke-induced mice treated intraperitoneally with 20 μg/kg PIDMSO showed the highest reduction of infarction volume, 62.24%. Furthermore, stroke-induced mice treated intraperitoneally with 200 ng/kg and 2 μg/kg PIDMSO experienced infarction volume reductions of 5.33% and 14.94%, less than the infarction volume reductions with 20 μg/kg to 20 mg/kg PIDMSO (Fig. 17). However, the infarction volume reduction did not change significantly with 20 μg/kg, 200 μg/kg, 2 mg/kg and 20 mg/kg PIDMSO (Fig. 18).

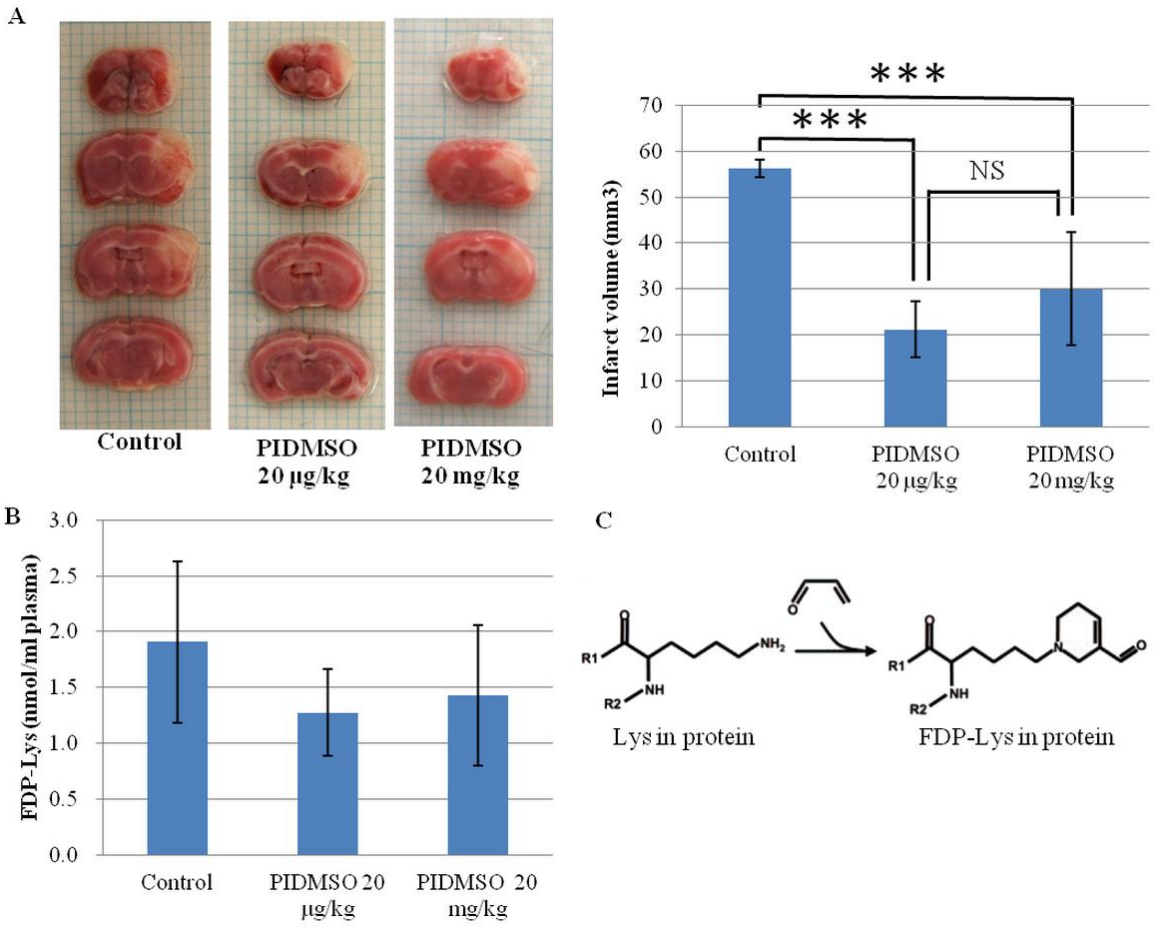

Fig. 16. The effect of PIDMSO on a mouse stroke model. The effect of PIDMSO on brain infarction size (A), the level of PC-Acro in plasma (B) and the formation of FDP-lys from acrolein in protein (C). Experiments were performed using 6 mice in each group as described in Materials and Methods. Data are shown as mean ± SE. ****P<0.001* compared with control mice.

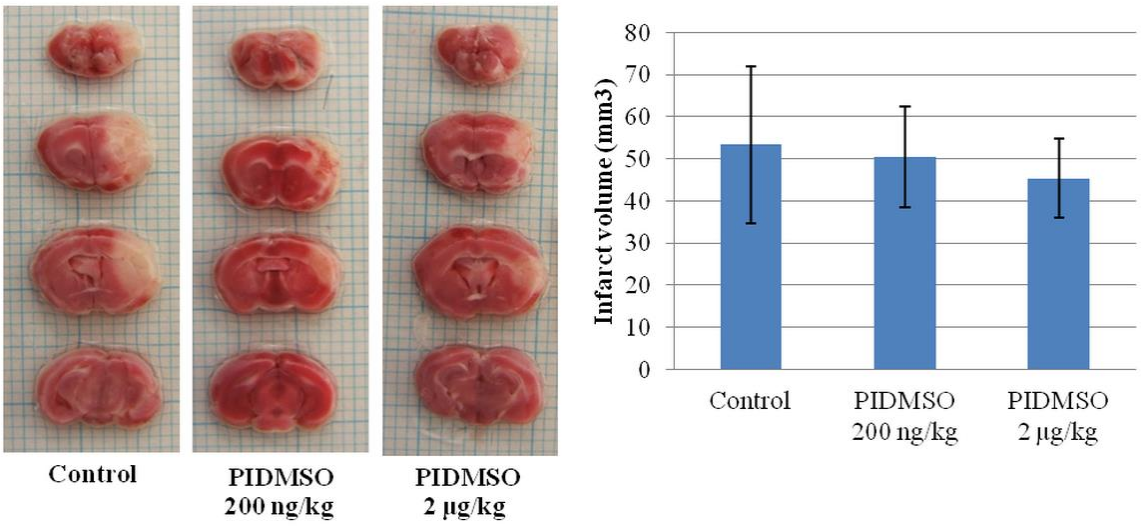

Fig. 17. The effect of 200 ng/kg and 2 µg/kg PIDMSO on brain infarction size.

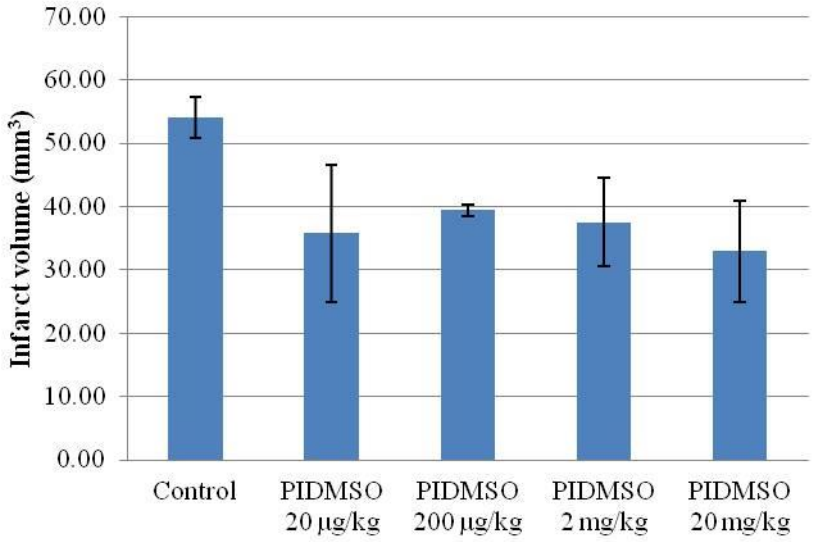

Fig. 18. The effect of 20 µg/kg and 200 µg/kg PIDMSO on brain infarction size.

2.3 Biochemical analysis on mice stroke model

Free acrolein is rapidly converted to PC-Acro through its interaction with lysine side chains in proteins (Fig. 16C) [43]. PC-Acro in plasma was measured by the ACR-LYSINE ADDUCT ELISA system. The level of PC-Acro in plasma decreased greatly (Fig. 16B) concomitantly with a reduction of the infarction. Furthermore, PC-Acro at the locus of brain infarction was measured by Western blotting using an antibody against FDP-Lys. PC-Acro (68 kDa) at the locus of the brain infarction decreased modestly from intraperitoneal PIDMSO at 20 μg/kg and 20 mg/kg (Fig. 19E).

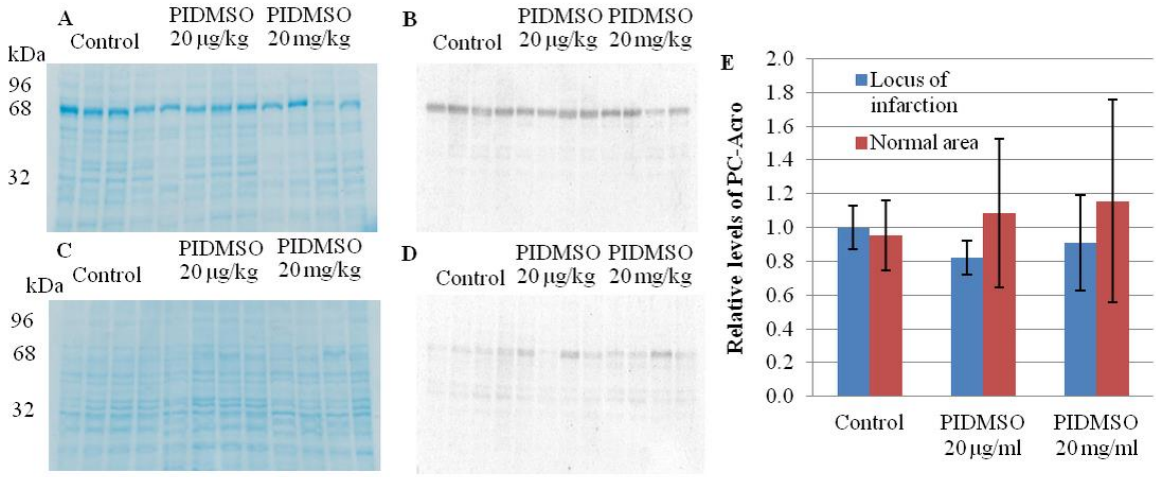

Fig. 19. The levels of PC-Acro and albumin in brain tissue. Total proteins (5 μg) from brain tissues at the locus of infarction (A,B) and a normal area (C,D) were stained with Coomassie Brilliant Blue R250 (A,C), and the relative levels of PC-Acro and albumin were estimated by Western blotting using antibodies against mouse albumin (B,D,E). The results represent the mean ± SD of 6 mice.

CHAPTER 3

Characterization and identification of polysaccharides from *Phellinus linteus* and *Phellinus igniarius*

3.1 Analysis of polysaccharides by High-performance liquid chromatography

The purified polysaccharides from *P. linteus* and *P. igniarius* were semi-quantified for monosaccharide composition by GC-MS. This showed that glucose, galactose and mannose were the major components of both. Monosaccharide composition was more accurately determined by HPLC as is shown in Table 5. The polysaccharides from *P. linteus* and *P. igniarius* contain mainly glucose 78.88% and 57.58% respectively with minor proportions of mannose, galactose, xylose, arabinose and rhamnose (Fig. 20). Furthermore, these polysaccharides were contain 3-*O*-Me-galactose as shown in between peak 1 and peak 2. Yan et al. was reported that *P. igniarius* contain 3-*O*-Me-galactose [80]. Also HPLC analysis showed absence of amino sugar and sulfate groups (data not shown indicating that polysaccharides are not contaminated by protein). For *P. linteus* the findings were in close agreement with those of Kozarski et al. [81] who found glucose content of 84.8 %.

Table 5. Monosaccharide composition of the extracts

Standard	% sugar of *P. linteus*	% sugar of *P. igniarius*
Rhamnose	0.82	1.31
Mannose	8.32	14.51
Arabinose	1.13	2.63
Galactose	8.06	20.65
Xylose	2.80	3.32
Glucose	78.88	57.58

Molecular weight of each of the polysaccharides was determined using HPLC with reference to the calibration curve using pullulan standards of known molecular weight (Fig. 21). Based on this analysis, polysaccharides from *P. linteus* and *P. igniarius* were estimated to be 20,708 Da and 18,518 Da respectively.

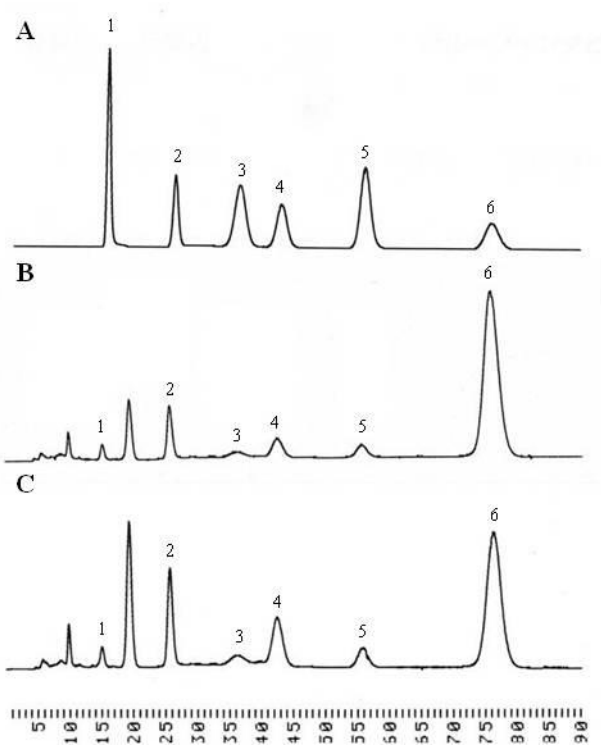

Fig. 20. The HPLC chromatogram of neutral sugar Mixture of six monosaccharide standard (1=Rhamnose, 2=Mannose, 3=Arabinose, 4=Galactose, 5=Xylose, 6=Glucose) (A). The composition of monosaccharides in polysaccharides from *P. linteus* and (B) *P. igniarius* (C).

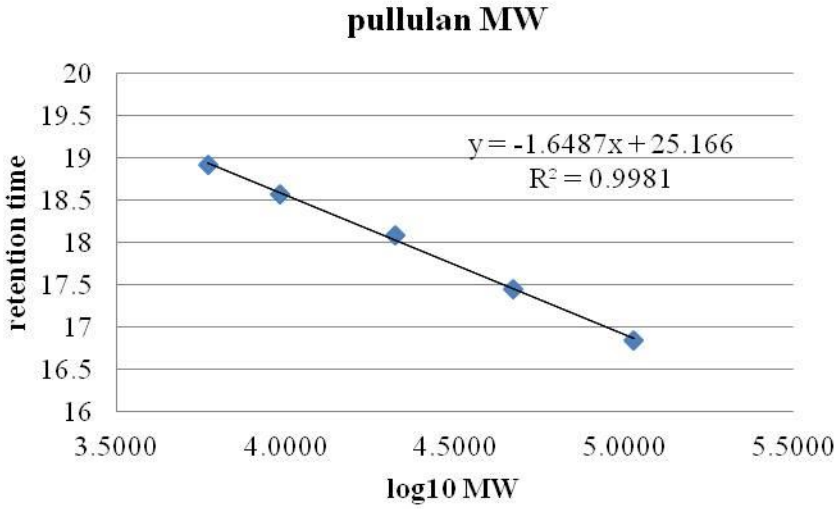

Fig. 21. The calibration curve using pullulan standards

3.2 The analysis of polysaccharides by Gas chromatography–mass spectrometry

The analysis of partially O-methylated alditol acetates (PMAAs) was carried out as described in GC-MS Analysis of Partially Methylated Alditol Acetates [82]. PMAA-GCMS patterns obtained from the polysaccharides of *P. linteus* and *P. igniarius* were very complex as can be seen in Figure 22. The majority of glycosidic linkages are 1→3, 1→6 or 1→ 3,6 from glucose in Table 6-7. Polysaccharides have more terminal glucose residues probably related with a relative small molecular size.

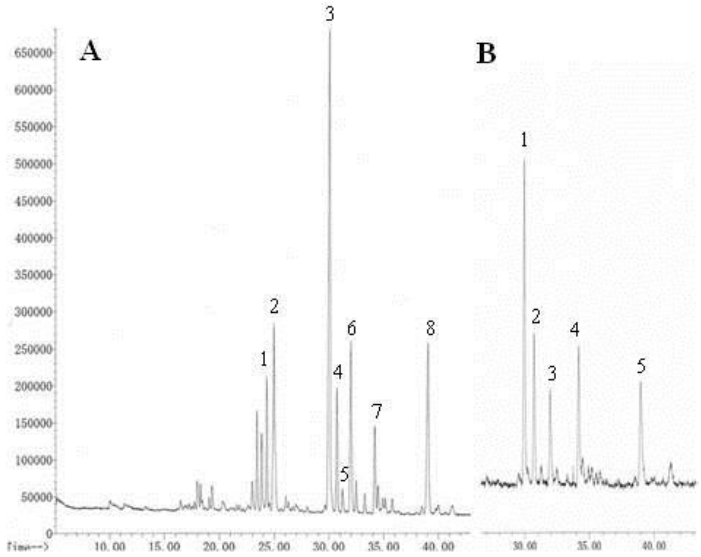

Fig. 22. The PMAAs chromatogram of the methylation analysis of polysaccharides from *P. linteus* (A) and *P. igniarius* (B) obtained by GC-MS.

Table 6. Identification of each peak on GC-MS for methylation analysis of polysaccharide from *P. linteus*

Peak number	O-methylated alditol acetates	t_R (min)	linkage type	Relative expression
1	2,3,4,6-Me_4-Man*p*	24.320	terminal	++
2	2,3,4,6-Me_4-Glc*p*	24.979	terminal	+++
3	2,4,6,-Me_3-Glc*p*	30.090	1, 3	+++++
4	2,4,6-Me_3-Man-*p*	30.737	1, 3	++
5	2,4,6,-Me_3-Gal*p*	31.225	1, 3	+
6	2,3,4,-Me_3-Man*p*	32.006	1, 6	+++
7	2,3,4,-Me_3-Glc*p*	34.177	1, 6	++
8	2,4-Me-Glc*p*	39.057	1, 3, 6	+++

Table 7. Identification of each peak by GC-MS for methylation analysis of polysaccharide from *P. igniarius*

Peak number	O-methylated alditol acetates	t_R (min)	linkage type	Relative expression
1	2,4,6-Me_3-Glc*p*	29.937	1, 3	++++
2	2,4,6-Me_3-Man*p*	30.694	1, 3	+++
3	2,3,4-Me_3-Man*p*	31.926	1, 6	++
4	2,3,4-Me_3-Glc*p*	34.146	1, 6	+++
5	2,4-Me-Glc*p*	38.941	1, 3, 6	++

3.3 Characterization of polysaccharide by Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy experiments were performed in order to elucidate the linkage type of polysaccharides. 1-H and 13-C NMR spectra obtained from each polysaccharide show a quite similar pattern even though the monosaccharide compositional analysis of each polysaccharide showed its heterogeneity. The 13-C NMR spectrum showed six carbon signals at ∂ 105.34 (C-1) ppm, which correspond to β anomeric carbons, as well as at ∂ 86.70 (C-3), 76.50 (C-5), 73.10 (C-2), 68.60 (C-4) and 63.58 (C-6) ppm (Fig. 23) corresponding to β-(1→3)-linked polysaccharides, which is similar to the spectrum of purified Saccharomyces cerevisiae (1→3)-β-Glucan [83]. In addition, there was a small amount of α-glucan present at ∂ 100.55 which was similarly found in the spectrum of Agaricus bisporus extract [84]. Several other low intensity signals were observed for these polysaccharides that indicated mainly polysaccharide and minor mannose content. The 1-H NMR spectra of polysaccharides from P. linteus and P. igniarius are shown in Figure 24. The chemical shifts from 1-H NMR and 2D NMR are presented in Table 8-9. The two-dimensional COSY, NOESY and TOCSY experiments confirmed these polysaccharides have a main chain of →3)-β-D-Glcp-(1→ and →6)-β-D-Glcp-(1→ (Fig. 25-26). In addition, NOESY and TOCSY spectra by a short mixing time of NMR experiments showed clearly all of six protons of glucose that confirmed that these polysaccharides are relatively small molecules as compared with polysaccharide from Tremella fusiformis that was estimated to be 4,600 kDa (data not shown). These observations indicated that the polysaccharides from P. linteus and P. igniarius are (1→3; 1→6)-β-D-polysaccharides containing some neutral sugar as galactose, rhamnose, arabinose, and xylose. In addition, polysaccharide from P. igniarius has a side chain of α-D-Manp-(1→ (Fig. 27b).

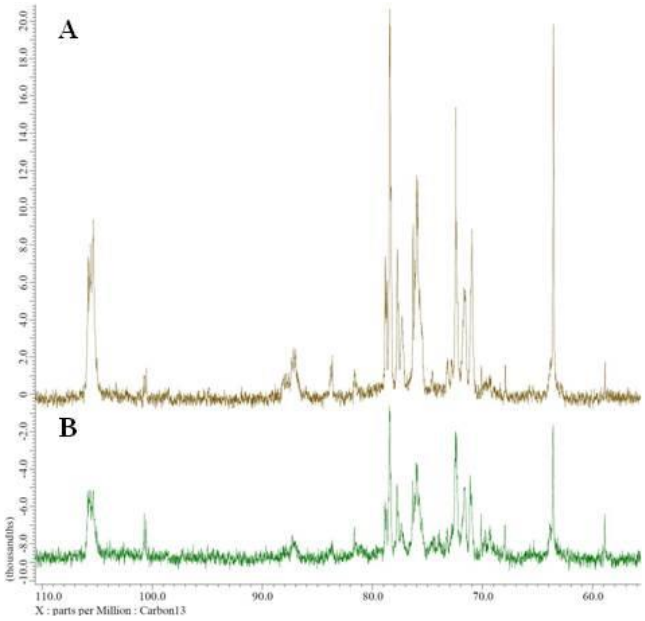

Fig. 23. 13-C NMR spectra of polysaccharides from *P. linteus* (A) and *P. igniarius* (B).

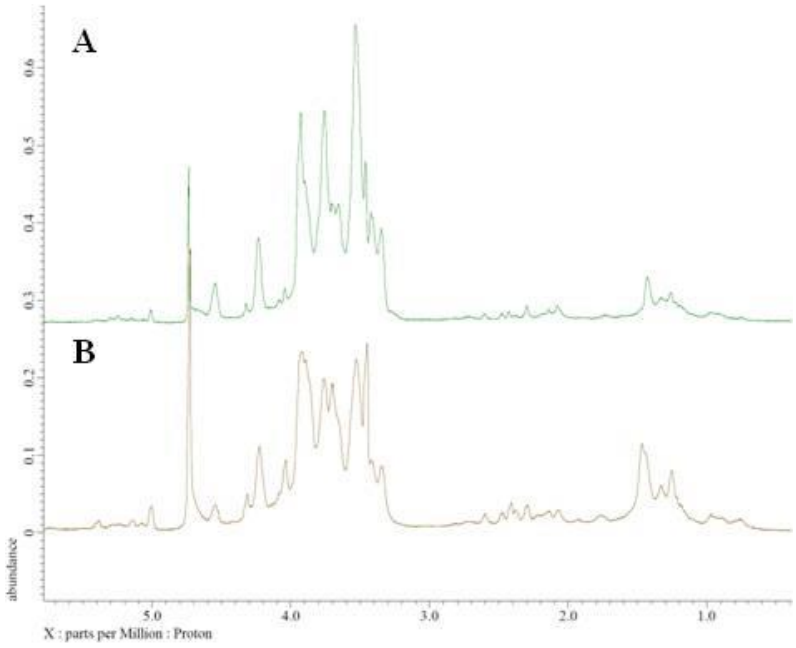

Fig. 24. 1-H NMR spectra of Polysaccharides from *P. linteus* (A) and *P. igniarius* (B) in D_2O at 30ºC.

Table 4. Chemical shifts of Proton and Carbon from *P. linteus* Polysaccharide.

		1	2	3	4	5	6α	6β
	PIP	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6α/ C-6	H-6β
a	3)-β-D-Glc*p*-(1	4.56/ 105.34	3.33/ 73.10	3.52/ 86.70	3.28/ 68.60	3.31/ 76.50	3.72/ 63.57	3.48
b	3,6)-β-D-Glc*p*-(1	4.54/ 105.34	3.35/ 72.80	3.54/ 85.80	3.26/ 68.60	3.52/ 75.00	4.08/ 68.70	3.58
c	β-D-Glc*p*-(1	4.25/ 105.34	3.04/ 73.70	3.23/ 76.30	3.11/ 70.30	3.17/ 76.70	3.71/ 61.20	3.71

Table 5. Chemical shifts of Proton and Carbon from *P. igniarius* Polysaccharide.

		1	2	3	4	5	6α	6β
	PIP	H-1/ C-1	H-2/ C-2	H-3/ C3	H-4/ C-4	H-5/ C5	H-6α/ C-6	H-6β
a	3)-β-D-Glc*p*-(1	4.56/ 105.34	3.33/ 72.90	3.52/ 86.70	3.28/ 68.50	3.31/ 76.40	3.72/ 63.57	3.48
b	3,6)-β-D-Glc*p*-(1	4.54/ 105.34	3.35/ 73.00	3.54/ 85.80	3.26/ 68.25	3.52/ 75.0	4.08/ 68.70	3.58
c	β-D-Glc*p*-(1	4.25/ 105.34	3.04/ 73.40	3.23/ 76.30	3.11/ 70.30	3.17/ 76.6	3.71/ 61.2	3.50
d	α-D-Man*p*-(1	4.98	3.24	3.54	3.26	3.52	n.d.	n.d.

n.d., Not detected

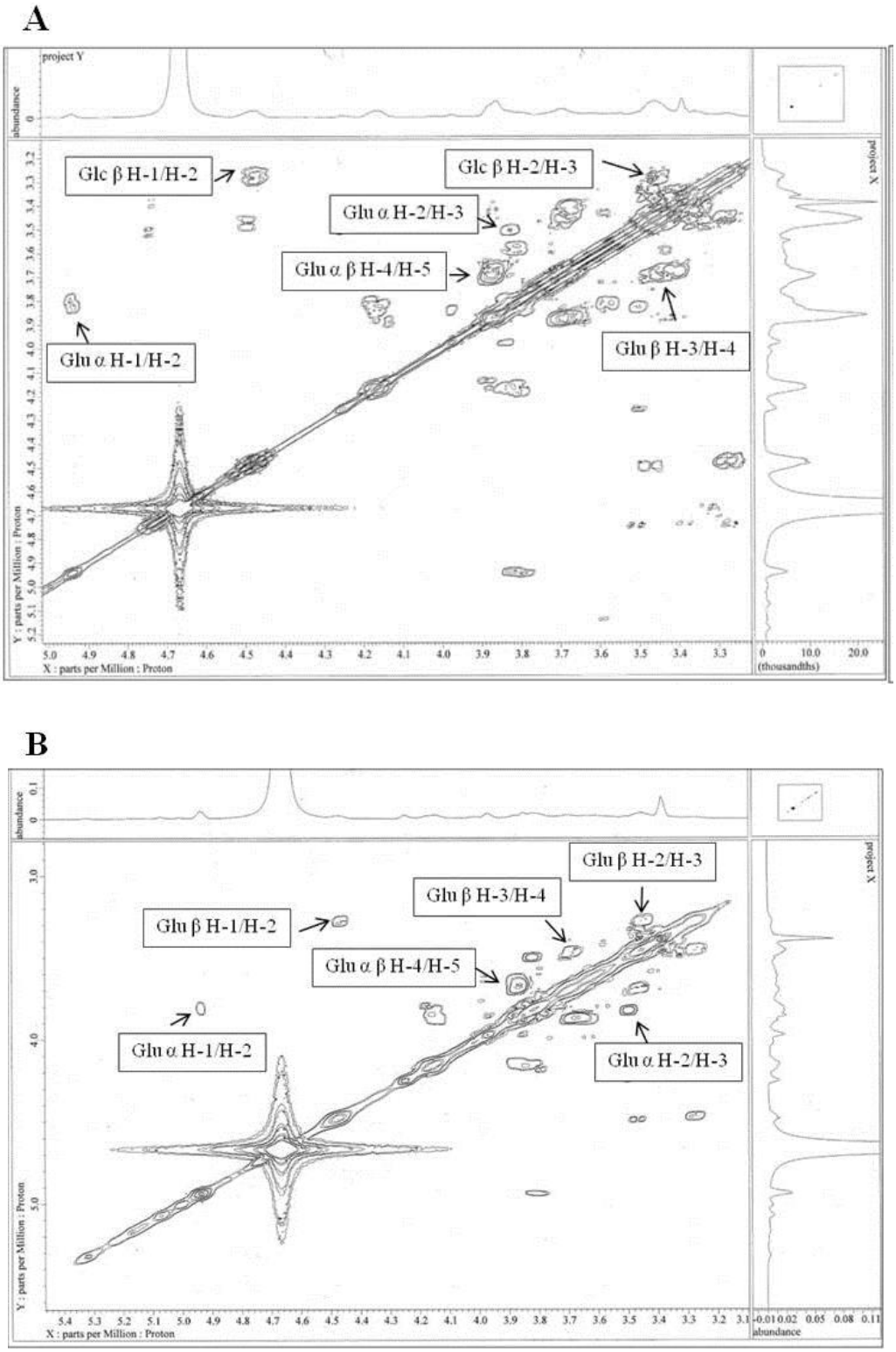

Fig. 25. The two-dimensional COSY spectra of Polysaccharides from *P. linteus* (A) and *P. igniarius* (B) in D_2O at 30ºC

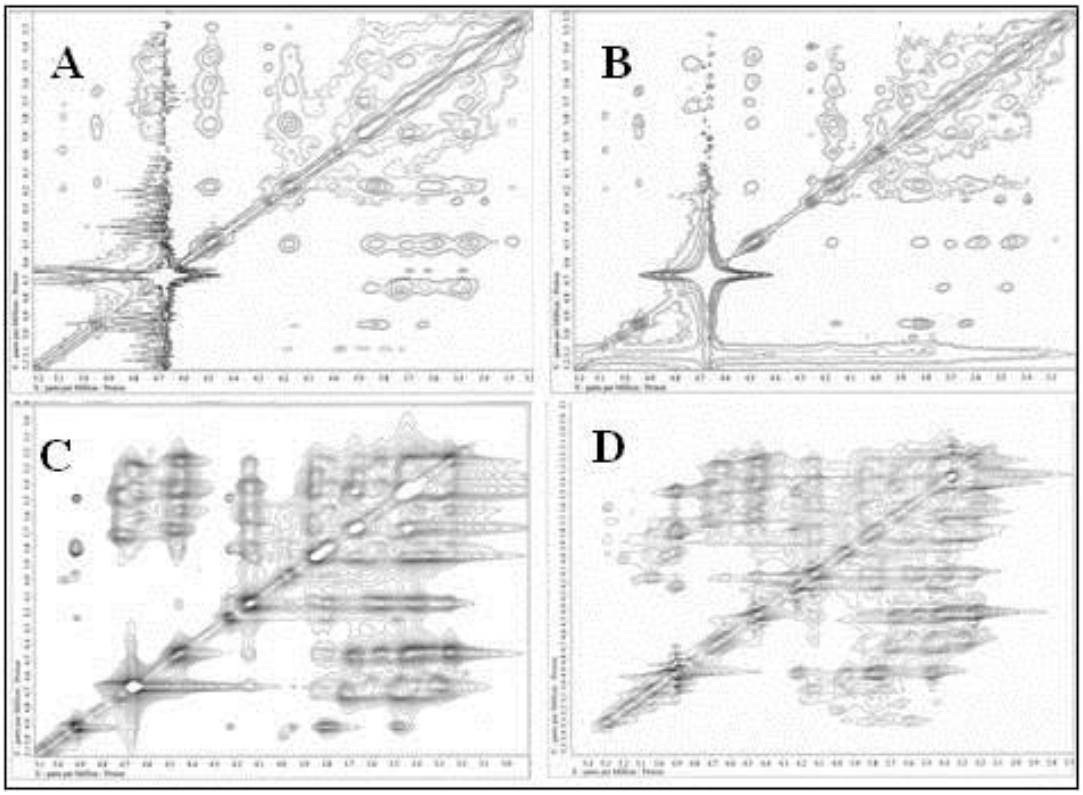

Fig. 26. The two-dimensional NOESY and TOCSY spectra of Polysaccharides from *P. linteus* (A and C respectively) and *P. igniarius* (B and D respectively) in D_2O at 30ºC.

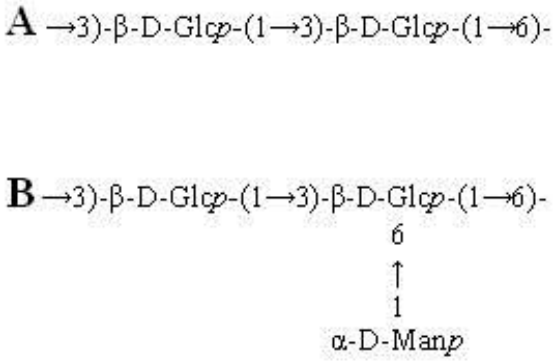

Fig. 27. Structure of β-polysaccharides from *P. linteus* (**A**) and *P. igniarius* (**B**)

CHAPTER 4

The immunomodulatory effects of polysaccharides from *Phellinus linteus* and *Phellinus igniarius*

Immunomodulatory activity has been found in many mushroom polysaccharides that have been described as (1→3; 1→6)-β-D-polysaccharides [85]. We extracted total RNA from the treated cell lines with the RNeasy mini kit. RT-PCR was performed for IL-6, IL-10, and TNF-α; G3PDH was used as control as described in Experimental. The mRNA profiles of IL-6 and IL-10 on RAW 264.7 cell lines are shown in figure 28. The results showed that polysaccharides from *P. linteus* and *P. igniarius* had no effect on the RAW 264.7 cells, as shown by the G3PDH mRNA profile. However, these polysaccharides significantly suppressed IL-6 after proinflammation-induction by 1 μg/ml LPS for 6 and 24 h (figure 28A). Furthermore, mRNA expression of IL-10 strongly increased after 6 h, and then mRNA expression of IL-10 was slightly decreased again after 24 h (figure 28B). In addition, both polysaccharides significantly suppressed TNF-α for 6 h (Fig. 29).

The culture medium of the treated cells was measured using sandwich ELISA kits for IL-6, IL-10 and TNF-α as described in Experimental. The results shown in figure 30 clearly indicated that at 6 h both polysaccharides significantly decreased TNF-α as compared to the inflammation stimulation control, i.e. LPS, (Fig. 30C), and this seemed related with mRNA suppression of TNF-α.

TNF-α is involved in systemic inflammation where it stimulates the acute phase reaction by activated macrophages [86]. The transcription of both TNF-α and of IL-6 slowed within 6 hours after exposing the cells to the suppressive activity of the *Phellinus* polysaccharides. Contrary to the reduced secretion of TNF-α in the culture medium (Fig. 30C), IL-6 secretion seemed not to be affected in the first 24 hours after addition of the polysaccharides (Fig. 30A). Furthermore, at 6 h the polysaccharides induced a very high positive IL-10 response compared to the LPS control. The secretion of IL-10 slightly decreased after 24 h which seemed related with the mRNA expression of IL-10 (Fig. 30B). LPS treatment caused no strong change of IL-10 production by the macrophages; the concentration remained almost identical independent of the extract they were treated with. This was different for the transcription values. LPS induced transcription of IL-6 was strongly reduced by both *Phellinus* polysaccharides after both 6 and 24 hours. For IL-10 also *Phellinus* polysaccharides caused reduction of transcription was observed but to a much lower degree than for IL-6. The difference between the PCR and ELISA observations could be explained by the relatively long time needed for processing and release of (cytokines) proteins compared to the process of transcription.

IL-6 is a pleiotropic cytokine which plays an important pathological role in inflammatory and autoimmune diseases. Because of its pro-inflammatory character it also has a significant function in the defense against pathogens and cancers. The anti-inflammatory cytokine IL-10 inhibits the production of a number of proinflammatory cytokines such as TNF-α and IL-6 and its role has been demonstrated in models of multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), diabetes, inflammatory bowel disease (IBD) and other

autoimmune diseases [87]. Clinically, a balanced IL-6/IL-10 ratio decreases the risk of chronic inflammation and resulting metabolic disorders, and of the mentioned autoimmune diseases [50]. Reduced IL-10, a higher IL-6/IL-10 ratio and the absence of a counter-balancing, immunoregulatory increase in IL-10 in responses to elevated IL-6 concentrations contribute to the pro-inflammatory physiological milieu that is known to be associated with major depression [88], a mental health condition in which feelings of sadness, anger, loss and frustration determine daily life for a longer period of time. Interestingly, IL-6 has been found to attenuate serotonin 2A receptor signalling [89] and is considered an interesting target for treatment of depression [90].

We calculated the IL-6/IL-10 ratio as well as the TNF-α/IL-10 ratio for the different treatments from our ELISA and PCR data. The TNF-α/IL-10 ratio is considered a parameter of chronic inflammation. Table 6 shows that the TNF-α/IL-10 ratios obtained from both the ELISA and PCR values are considerably decreased due to the influence of the *Phellinus* polysaccharides. For the IL-6/IL-10 ratio, the PCR data are analogous to the TNF-α/IL-10 ratio. The ELISA data we obtained for IL-6/IL-10 ratio decreased after 24 h. These observations suggest *Phellinus* polysaccharides could suppress chronic inflammation, and may reduce inflammation by balancing of IL-6/IL-10 ration in immune system. Although, polysaccharides from *P. linteus* and *P. igniarius* have different composition and structure, their immunomodulatory activity seems very similar. This may be because these polysaccharides do have the same main chain and a side chain that is recognized by the same receptor for immune recognition.

This study is a novel for polysaccharide from *P. linteus* and *P. igniarius* to reduce inflammation by ratio in immune system that could provide to investigate the immune system as a target for immunoregulatory substances of "medicinal" mushrooms in vivo for the further.

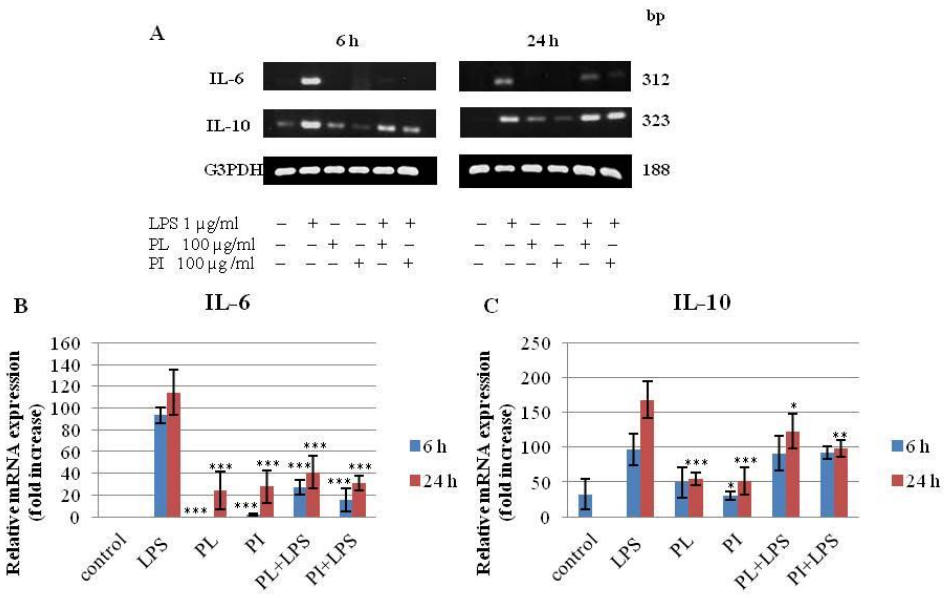

Fig. 28. The results of RT-PCR analysis (A) on the mRNA expression of IL-6 (B), IL-10 (C) and G3PDH in RAW 264.7 cell line. Cells were treated with LPS 1 µg/ml or with samples, for 6 and 24

h. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Turkey's test for selected pairs. The results represent the mean ± SE of duplicate cultures of two representative experiments. *p<0.05; **p<0.01; ***p<0.001 versus LPS positive control.

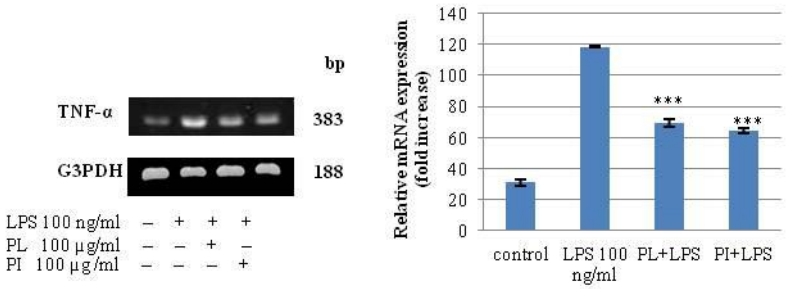

Fig. 29. The results of PCR analysis on the mRNA expression of TNF-α and G3PDH in RAW 264.7 cell line. Cells were treated with LPS 100 ng/ml or samples for 6 h. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Turkey's test, for selected pairs. The results represent the mean ± SE of duplicate cultures of two representative experiments. *p<0.05; **p<0.01; ***p<0.001 versus LPS positive control.

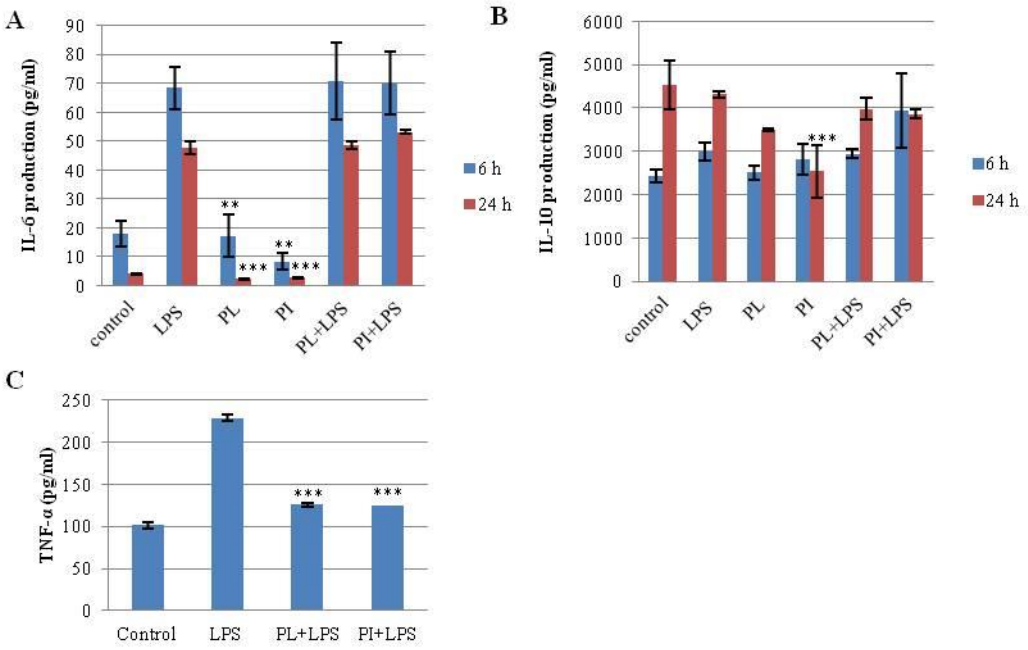

Fig. 30. Production of IL-6 (A) and IL-10 (B) by the RAW 264.7 cell lines in response to polysaccharides from *P. linteus* and *P. igniarius*. Cells were treated with LPS 1 µg/ml or samples for 6 and 24 h as described in Experimental (C) Production of TNF-α by the RAW 264.7 cell line treated for 6 h with LPS 100 ng/ml or samples as described in Experimental. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Turkey's test,

selected pairs. The results represent the mean ± SE (n=3). *p<0.05; **p<0.01; ***p<0.001 versus LPS positive control.

Table 10. The ratio of cytokines in RAW 264.7 cells

	mRNA expression ratio			cytokines production ratio		
	TNF-α/IL-10	IL-6/IL10		TNF-α/IL-10	IL-6/IL10	
	6 h	6 h	24 h	6 h	6 h	24 h
Control	0.713±0.272	n.d.	n.d.	0.043±0.003	0.003±0.002	0.001±0.000
LPS	1.022±0.047	0.996±0.178	0.708±0.259	0.074±0.007	0.023±0.004	0.011±0.001
PL+LPS	0.622±0.002*	0.333±0.182***	0.331±0.095*	0.042±0.003	0.024±0.005	0.012±0.001
PI+LPS	0.681±0.017**	0.172±0.113***	0.313±0.050**	0.031±0.008	0.018±0.005	0.014±0.000

Values are mean ± SE (n = 4/mRNA expression), (n=3/cytokines production), * p\0.05, ** p\0.01 and *** p\0.001 versus LPS group

n.d. not determined

CONCLUSION

The simple LC-MS method that we described was used to identify the molecular mass of the DMSO soluble ethanol extract of *P. linteus* and *P. igniarius* fruit bodies in negative mode. The polyphenols extract from *P. linteus* consisted mainly of 9 compounds that were 3,4-dihydroxybenzaldehyde, 4-(3,4-dihydroxyphenyl-3-buten-2one, Interfungin A, Inonoblin C, Phelligridin D, Interfungin B, Inoscavin E, Inoscavin C and Methylinoscavin D in figure 7. The characterization of the different components was done by assignment of their proton resonances that found strong signals of 3,4-dihydroxybenzaldehyde, Interfungin A, Inonoblin C, Phelligridin D, Inoscavin E and Inoscavin C. Furthermore, polyphenol extract from *P. igniarius* mainly contains 3,4-dihydroxybenzaldehyde, 4-(3,4-dihydroxyphenyl-3-buten-2one, Inonoblin C, Phelligridin D, Inoscavin C, Phelligridin C and Interfungin B in figure 32. In the addition, ^{1}H NMR resonances of polyphenols were assigned, identifying Inonoblin C, Phelligridin D, Inoscavin C and Phelligridin C. We suggest that the difference in the upmentioned biological effects of *P. linteus* and *P. igniarius* might be caused by the absence respiratory presence of Inoscavin C and Inoscavin D in the extracts.

In *in vitro* experiments, we observed that 5 µg/ml and lower concentration PI EtOH extract prevented the effects of 2 and 5 µM acrolein, and also could prevent 100 µM H_2O_2. The results showed that PI EtOH extract strongly prevented the effects acrolein and to a higher extent than the effects H_2O_2. The polyphenol extract from *P. igniarius* greatly reduced the growth inhibition of Neuro-2a cells induced by acrolein. The effects of polyphenols from *P. igniarius* on artificially induced brain infarction showed that the average volume of infarction was decreased significantly. Furthermore, the concentration of PC-Acro at the locus of brain infarction and in plasma was decreased. Our observations suggest that the polyphenols of *P. igniarius* could serve as a substance for new neuroprotective treatment of artificially induced ischemic stroke by scavenging acrolein.

We determined the composition and structure of polysaccharides isolated from *P. linteus* and *P. igniarius* by HPLC, GCMS and NMR (1-H, 13-C, COSY, NOESY and TOCSY). The results showed that polysaccharides from *P. linteus* and *P. igniarius* contain mainly glucose, i.e. 78.88% and 57.58% respectively, with minor proportions of mannose, galactose, xylose, arabinose and rhamnose. Methylation analyses showed that the majority of glycosidic linkages are 1→3, 1→6 or 1→ 3,6 from glucose. The two-dimensional COSY, NOESY and TOCSY confirmed these polysaccharides to have a main chain of →3,6)-β-D-Glc*p*-(1→. In addition, polysaccharide from *P. igniarius* showed a side chain of α-D-Man*p*-(1→. Our observations indicated that these complex polysaccharides from *P. linteus* and *P. igniarius* are (1→3; 1→6)-β-D-polysaccharides containing some neutral sugar as galactose, rhamnose, arabinose, and xylose that have molecular weight (20,708 Da and 18,518 Da, respectively).

In vitro measurements of immunomodulatory effects by RT-PCR and ELISA showed that polysaccharides from *P. linteus* and *P. igniarius* significantly decreased TNF-α suggesting immune-

suppressive activity. Furthermore, these polysaccharides stimulated a highly positive IL-10 response and strongly suppressed IL-6 and TNF-α mRNA transcription early after LPS treatment. In addition, these polysaccharides reduced TNFα/IL-10 and IL-6/IL-10 ratios of the messenger RNA's. These in vitro results suggest that polysaccharides from *P. linteus* and *P. igniarius* could possibly use in the development of mushroom derivatives that have in vivo anti-inflammatory effects.

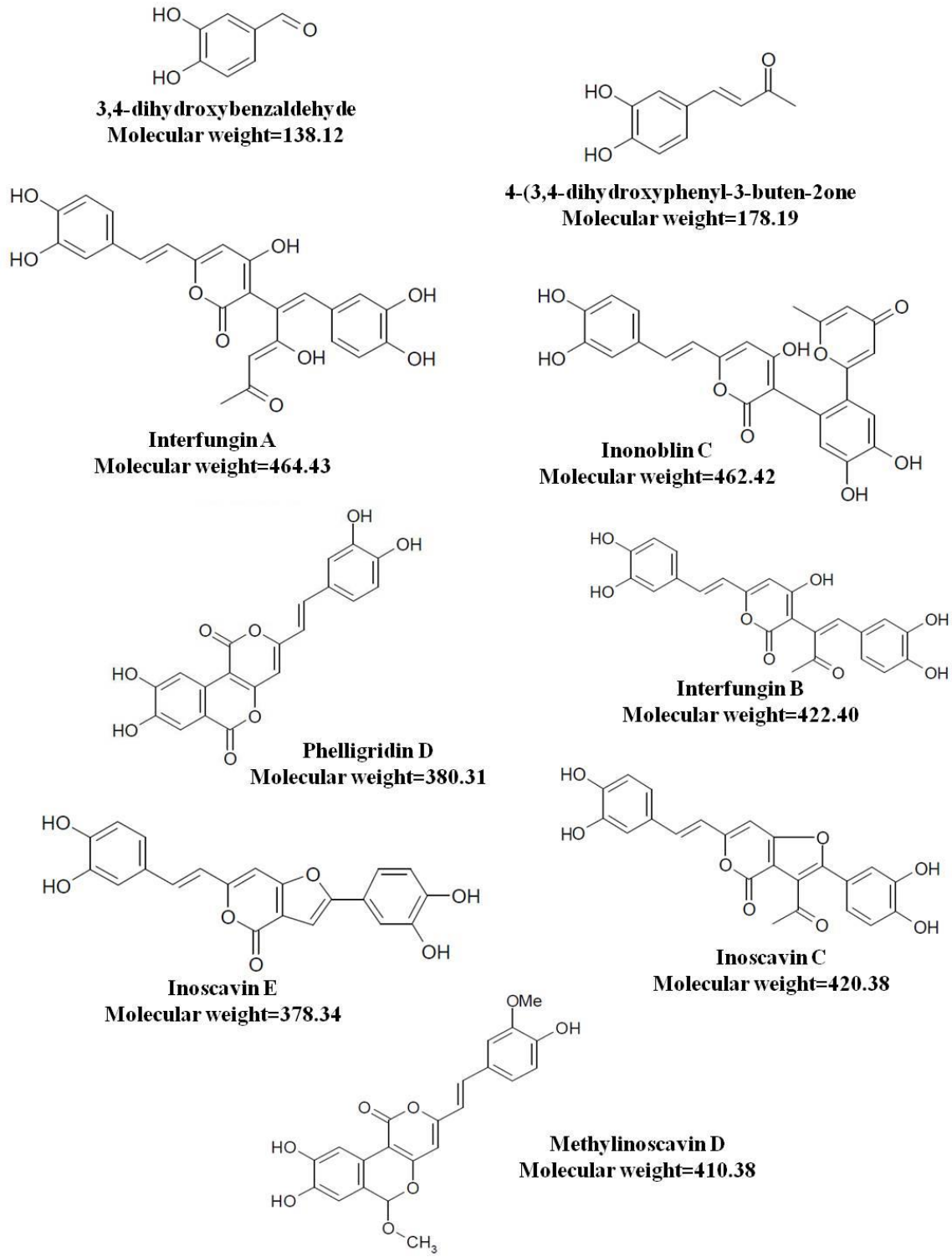

Fig. 31. Structure of polyphenols from *P. linteus*

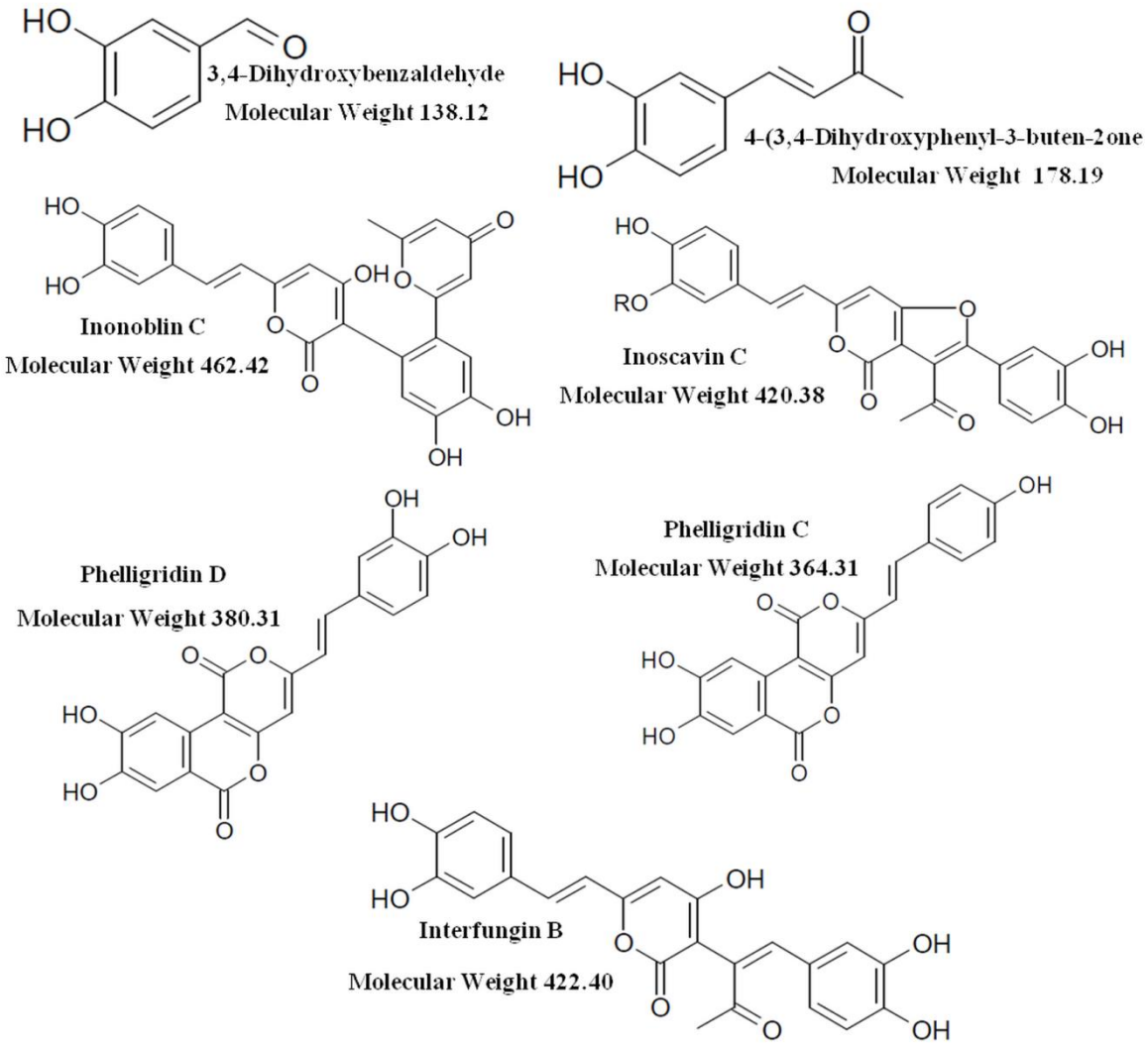

Fig. 32. Structure of polyphenols from *P. igniarius*

EXPERIMENTAL

Materials and chemicals

Extraction reagents were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan) that are 95% ethanol and Dimethyl sulfoxide (DMSO). The hydrophilic 0.22 µm filters were purchased from Merck Ltd. (Tokyo, Japan). Total Phenolics Determination, Phenol reagent solution (Acid 1.8 N) was purchased from *Nacalai Tesque*, Inc. (Kyoto, *Japan*), and Gallic acid standard was purchased from Sigma–Aldrich Co. (Tokyo,Japan).

Analysis reagents and standard were mostly purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan), which are Ethanol HPLC grade, Formic acid solution, Acetonitrile HPLC grade, Esculetin and 3,4 Dihydroxybenzaldehyde.

Chromatographic separation was performed on a Hitachi LaChrom Elite HPLC ultra-high-pressure liquid chromatography (U-HPLC) system from Hitachi High-Technologies Corporation (Tokyo, Japan). The chromatographic separation was performed on an Inertsil column (ODS-3 150 mm × 4.6 mm, 5 µm from GL Sciences Inc., (Tokyo, Japan). Mass spectrum analysis was performed using a Bruker amaZon SL Ion Trap Mass Spectrometer from *Bruker Japan* Co., LTD. (Yokohama, Japan) LC-MS Data were collected with Bruker Compass Data Analysis 4.2 from *Bruker Japan* Co., LTD. (Yokohama, Japan).

NMR experiments were performed by Jeol 600 MHz instrument from JEOL Ltd. (Tokyo, Japan). Dimethyl sulfoxide-d6, 99.9% containing 0.05 v/v TMS was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

Deionized water used for all the solutions and dilutions was prepared by Milli-Q® Integral Water Purification System of Merck Ltd. (Tokyo, Japan).

Extraction of P. linteus and P. igniarius

Dried fruiting body powder of wild-type *P. linteus* and *P. igniarius* were kindly provided by Amazing Grace Health Products Limited Partnership (Bangkok, Thailand). Identification of the wild types had been done by Dr. Usa Klinhom (Mahasarakham University, Thailand) and representative samples of both species were stored in the collection of the Mushroom Museum of said university as published before (Sittiwet & Puangpronpitag, 2008).

Mushrooms powders from *P. linteus* and *P. igniarius* were extracted with 70% ethanol (10% w/v) at 70ºC for 16 hrs. The supernatant was removed by centrifugation at 10,000 g for 20 min. Crude *P. linteus* (PL EtOH) and *P. igniarius* (PI EtOH) ethanol extracts were filtered and stored at -20ºC for further use. Crude aqueous extracts were prepared by hot water extraction [91] for 16 hrs. The *P. linteus* (PL DDW) and *P. igniarius* (PI DDW) aqueous extracts were then filtered and stored at -20ºC for further use. The *P. linteus* (PL EtOH) and *P. igniarius* (PI EtOH) ethanol extracts were lyophilized and stored at -20ºC for further use. PI EtOH (PIO) and PL EtOH (PLO) powders were dissolved in water, filtered with a hydrophilic 0.22 µm filter, and stored at -20ºC for MTT assays. PI EtOH (PIDMSO) and PL EtOH (PLDMSO) powders were dissolved in DMSO,

filtered with a hydrophilic 0.22 µm filter, and stored at -20ºC for MTT assays, mouse stroke model and analysis.

After ethanol extraction the tissue was further extracted with hot water [91] for 16 hrs. Crude polysaccharides were precipitated by addition of 2.5 volumes of cold 99% ethanol, after which the suspension was kept at -20ºC for 16 to 24 hrs. The suspension was then centrifuged at 15,000 g for 20 minutes. The precipitate was dissolved in a small volume of water and the polysaccharides were reprecipitated with alcohol as before for 4-5 times. Ethanol-soluble phenolic compounds were removed by a Sep-Pak C18 Plus Light Cartridge. The polysaccharides of *P. linteus* (PLP) and *P. igniarius* (PIP) were lyophilized and stored at -20ºC for further use.

Total Phenolics Determination

The concentration of total phenols was estimated by the Folin-Ciocalteu assay (Ainsworth et al., 2007). The procedure consisted of diluting 50 µl polyphenol extract in 400 µl water followed by the addition 50µl of the Phenol reagent solution. The mixture was incubated at room temperature for 5 minutes. 500 µl of 7% Na_2CO_3 Solution was added to the mixture, and then the mixture was incubated at room temperature for 90 minutes. Absorbance was measured at 750 nm. Gallic acid was used as a standard in concentrations ranging from 0 to 500 µg/ml.

Analysis of polyphenols by liquid chromatography mass spectrometry (LC-MS)

Chromatographic separation was performed on a Hitachi LaChrom Elite HPLC ultra-high-pressure liquid chromatography (U-HPLC) system fitted with a Bruker amaZon SL Ion Trap Mass Spectrometer. The chromatographic separation was performed on an Inertsil column (ODS-3 150 mm × 4.6 mm, 5 µm). The mobile phase was composed of water (A) with formic acid solution (pH=3.0) and 100% acetonitrile (B) with formic acid solution (pH=3.0) under gradient elution conditions at 0–60 min, 0–50% B; 60-90 min, 50-100% B and 90-100 min, 100% B. The flow rate of the mobile phase was 0.4 ml/min, and the column temperature was maintained at 30ºC. Mass spectrum analysis was performed using a Bruker amaZon SL Ion Trap Mass Spectrometer fitted with an ESI source and operated in negative ion mode. The key optimized ESI parameters were as follows: spray voltage: -3.5 KV; capillary temp: 220ºC; Ultra scan. Data were collected with Bruker Compass Data Analysis 4.2 and analyzed by Mass++ software (Tanaka et al., 2014) with the MassBank mass spectral library.

Analysis of polyphenols by nuclear magnetic resonance spectroscopy (NMR)

One-dimensional spectra of 10 mg of dry sample exchanged in dimethyl sulfoxide-d6, 99.9% containing 0.05 v/v TMS was acquired using a Jeol 600 MHz instrument. The operation condition for ^{1}H NMR was as follows: spin, 15 Hz; relaxation delay, 5 s; temperature, 25ºC.

Cell culture for acrolein and H_2O_2 cytotoxicity

Mouse mammary carcinoma FM3A cells [92] (5×10^4 cells/ml) were cultured in low glucose DMEM (Dulbecco's modified Eagle's medium; Nacalai Tesque, Inc., Japan) under 6 conditions: control (standard conditions); samples alone at 50, 5 and 0.5 µg/ml; samples with acrolein at 10 µM, 5 and 2 µM; only acrolein; samples with H_2O_2 at 200, 150 and 100 µM; and only H_2O_2 for 24 and 48 hrs.

Mouse neuroblastoma cells (Neuro-2a cells) [93] were grown in DMEM culture medium (Nacalai Tesque, Inc., Japan) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Inc., Japan) at 37°C in 5% CO_2 in a humidified incubator. Neuro-2a cells (3×10^4 cells/ml) were plated in 96-well microtiter plates in DMEM medium under the standard conditions for 12 hrs. Then, the Neuro-2a cells were treated with samples and medium for a control. The activity of the Neuro-2a cells was then measured by MTT assay.

Trypan blue assay

Viability of cells was tested in culture fluid with 0.25% trypan blue. After 1 minute the cells were counted under a microscope and unstained cells were considered alive. Cell counts were done in triplicate.

MTT assay

Cells were plated in 96-well microtiter plates and treated as described above. Thereafter, the medium was removed and 50 µl/ml of MTT solution in DMEM and antibiotics (penicillin and streptomycin) were added. After a 1 hr incubation, the MTT solution was replaced with 100 µl of DMSO to dissolve the tetrazolium crystals. Finally, the absorption was read at a test wavelength of 540 nm and a reference wavelength of 650 nm with a Multiskan JX microplate reader (Thermo Labsystems, UK). Cell viability (%) was calculated as [optical density (OD) of the treated wells]/[OD of the control wells] x 100 [94].

Photochemically Induced Thrombosis Model Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chiba University and carried out according to the Guidelines for Animal Research of Chiba University. Male C57BL/6NCrSlc mice (8-week-old) were purchased from Japan SLC Inc. (Japan). Nine-week-old mice weighing 22 to 27 g were anesthetized with inhalation of 3% isoflurane (Abbott, Japan). Anesthesia was continued with 2% isoflurane during the operation. A thrombotic occlusion of the middle cerebral artery was induced by a photochemical reaction [95]: an incision was made between the left orbit and the external auditory canal, and the temporalis muscle was detached from the dura mater to expose the proximal section of the middle cerebral

artery. Immediately after intravenous injection of a photosensitizer, Rose Bengal (20 mg/kg), through a jugular vein, green light (wavelength: 540 nm) emitted from a xenon lamp (Hamamatsu Photonics, Japan) illuminated the middle cerebral artery for 10 min. After middle cerebral artery occlusion, the incised skin was restored. At 24 hrs after the induction of photochemically induced thrombosis (PIT) stroke, we took blood from the anesthetized mice via the cardiac puncture method. After that, the brain was removed and sectioned into 2-mm thick coronal slices. Each slice was incubated with 5% triphenyltetrazolium chloride solution at 37°C for 10 min. The volume of the infarction was analyzed using ImageJ software [96]. Where indicated, *P. igniarius* ethanol extract dissolved in DMSO (PIDMSO) at 200 ng to 20 mg/kg in phosphate-buffered saline was injected intraperitoneally at 0 hr after infarction induction. The experiments were performed using 6 mice in each group [97].

Measurement of PC-Acro

Brain tissues at the locus of infarction in the PIT model mice and at the same locus in control mice were homogenized using an Ultra-Turrax homogenizer in 0.5 ml of buffer A containing 10 mmol/L Tris/HCl (pH 7.5), 1 mmol/L dithiothreitol, 10% glycerol, 0.2 mmol/L EDTA, and 0.02 mmol/L FUT-175 (6-amino-2-naphthyl-4-guanidinobenzoate), a protease inhibitor [98]. Total proteins (5 µg) were stained with Coomassie Brilliant Blue R-250 after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the level of PC-Acro was measured by Western blotting [99] using 5 µg of tissue homogenate protein and a polyclonal antibody against bovine serum albumin-conjugated acrolein (MoBiTec, Germany). The level of albumin was measured similarly using an antibody against bovine serum albumin-conjugated acrolein mouse albumin (Bethyl, USA). Relative levels of PC-Acro and albumin were estimated by measuring the density of bands of 6 mice with a LAS-1000 imaging analyzer (Fuji film, Japan). Cardiac blood containing 3 U/ml heparin was centrifuged at 3,000 g for 10 minutes at 4°C. The supernatant (plasma) was carefully collected to avoid contamination by erythrocytes. PC-Acro, in which acrolein is converted to N^{ε}-(3-formyl-3,4-dehydropiperidino-lysine) (FDP-lys) in plasma, was measured by the ACR-LYSINE ADDUCT ELISA system (NOF Corporation) using 0.05 ml of plasma [100]. After the reaction was terminated, absorbance at 450 nm was measured by a Bio-Rad Model 550 microplate reader. Protein concentrations were determined by the method of Bradford et al [101].

Analysis of monosaccharide and Amino sugar composition by HPLC

Polysaccharides (100 µg) were hydrolyzed with 2.5 M trifluoroacetic acid (TFA) at 100ºC for 6 h. The aqueous solutions were evaporated to dryness under a N_2-stream at 40ºC. The residues were dissolved in 100 µl water and analyzed for monosaccharide composition on a HPLC post-column system with TSK gel Sugar AXI (150×4.6 mm i.d.; Tosoh, Tokyo, Japan). The column temperature was kept at 70ºC, equilibrated with 0.5 M Borate buffer (pH 8.5), and flow rate was 0.4 ml/min. The eluent from the column was combined with 0.5% (w/v) 2-cyanoacetoamide and 1.0 M NaOH, both of which were delivered at 0.25 ml/min and heated at 120°C within the reaction loop.

Rhamnose, Mannose, Galactose, Glucose, Xylose and Arabinose at 100 ppm were used as standards [102].

The samples were analyzed for amino sugar composition on a HPLC post-column system with TSK gel SCX (150×4.6 mm i.d.; Tosoh, Tokyo, Japan). The column temperature was kept at 70°C, equilibrated with 0.35 M Borate buffer (pH 7.6), and flow rate was 0.6 ml/min. The eluent from the column was combined with 0.5% (w/v) 2-cyanoacetoamide and 1.0 M NaOH, both of which were delivered at 2.5 ml/min and heated at 120ºC within the reaction loop. The monosaccharide and amino sugars were detected by fluorescence detector with emission at 383 nm and excitation at 331 nm. D-glucosamine, D-galactosamine and D-mannosamine at 100 ppm were used as standards for amino sugar composition analysis.

Sulfate groups determination

The samples were hydrolyzed as described in analysis of monosaccharide and Amino sugar composition by HPLC. The samples were analyzed for Sulfate group determination by HPLC with TSK gel IC-Anion-PW (10 µm, 50×4.6 mm i.d.; Tosoh, Tokyo, Japan). The column temperature was kept at 45°C, equilibrated with the mixture of 1.42 mM Sodium hydrogen carbonate ($NaHCO_3$) and 1.5 mM Sodium carbonate (Na_2CO_3). The sulfate groups were detected by refractive index detector. The flow rate was 0.8 ml/min. Sodium sulfate at 1000, 500, 300, 100, 50 and 10 ppm were used as standards.

Analysis of molecular weight by HPLC

The samples were analyzed for molecular weight on HPLC with OHpak SB-806M HQ (300×8 mm i.d.; Shodex, New York, America) and refractive index detector. The eluent was 10 mM Ammonium bicarbonate (NH_4HCO_3) with flow rate at 0.5 ml/min. The sulfate groups were detected by refractive index detector. The 5,900 to 2,350,000 molecular weights of pullulans were used as standards.

Analysis of monosaccharide composition by GC-MS

Polysaccharides were lyophilized and dried in vacuum over P_2O_5 for 16 h. Polysaccharides were hydrolyzed with 1.0 M HCl in MeOH at 80ºC for 24 hours, and 3 times washed by n- hexan. Anhydrous pyridine and acetic anhydride were added at room temperature to the lower phases for 30 minutes and evaporated to dryness under a N_2-stream at 40ºC. The methanolyzed polysaccharides were trimethylsilylated with 30 ml of a 2:1 (v:v) mixture of *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA)–pyridine at 80°C for 30 minutes [103] in figure 33. The trimethylsilylated polysaccharides were then analyzed by GC-MS, and identified by their typical retention times and electron impact profiles. Gas liquid chromatography mass spectrometry (GC-MS) was performed using a Hewlett–Packard model 6890 series II gas chromatograph with Helium

as carrier gas. A capillary column (25 m × 0.24 mm i.d.) of Silicone OV-101 held at 280ºC during injection and then programmed at 160ºC for 5 min and 160ºC to 260ºC (2ºC/min).

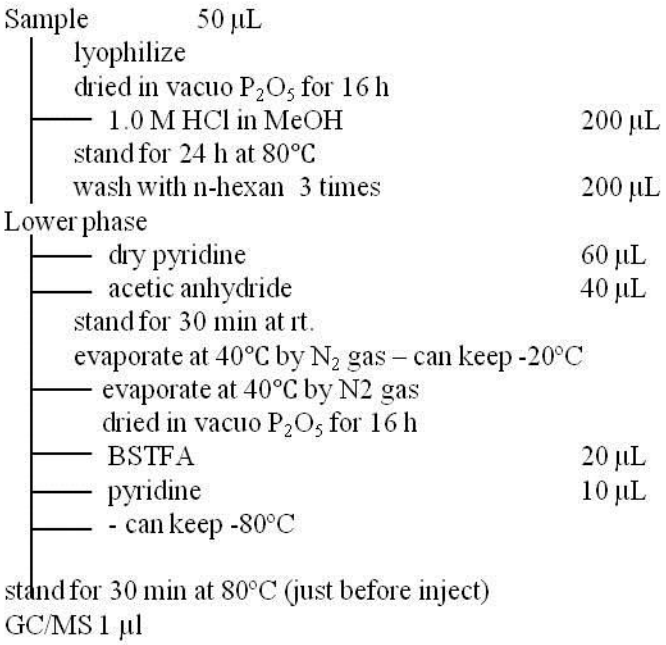

Fig. 33. Procedure for composition analysis of polysaccharide by GC-MS

GC-MS Analysis of Partially Methylated Alditol Acetates (PMAAs)

The PMAA strategy involves methylation of all free (non-linkage involved) OH-groups in poly- or oligosaccharides using methyl iodide (CH_3I) in DMSO at high pH, also known as "permethylation". Polysaccharides 100 µg were dissolved in 200 µl dehydrated DMSO, NaOH-DMSO (0.2 ml of 50% (w/v) NaOH with 5 ml dehydrated DMSO) and iodomethane 0.1 ml. After 3 times of sonication for 5 minutes, methylated polysaccharides were extracted by chloroform and washed by water. The residues were evaporated to dryness under a N_2-stream at 40°C. The dry methylated polysaccharides were hydrolyzed with 2.5 M trifluoroacetic acid (TFA) and heated at 100°C for 4 h. The aqueous solutions were evaporated to dryness under a N_2-stream at 40°C. The partially methylated sugars were reduced by $NaBH_4$ (5 mg/ml in 30% methanol containing 0.03 M NaOH). After removal of borate by evaporation as methyl borate, partially O-methylated alditols were acetylated by acetic anhydride [104] in figure 34. The partially methylated alditol acetates were analyzed by a Hewlett–Packard model 6890 series II gas chromatograph with a Silicone OV-101 capillary column (25 m × 0.24 mm i.d.). The analysis was performed in the electron impact ionization mode, and an ionizing voltage of 70 eV was used. Several temperature programs were investigated from which the best results were obtained at the following gradient: 3 min 100°C, 4°C/min to 160°C, 0.5ºC/min to 180ºC then 20°C/min to 260°C.

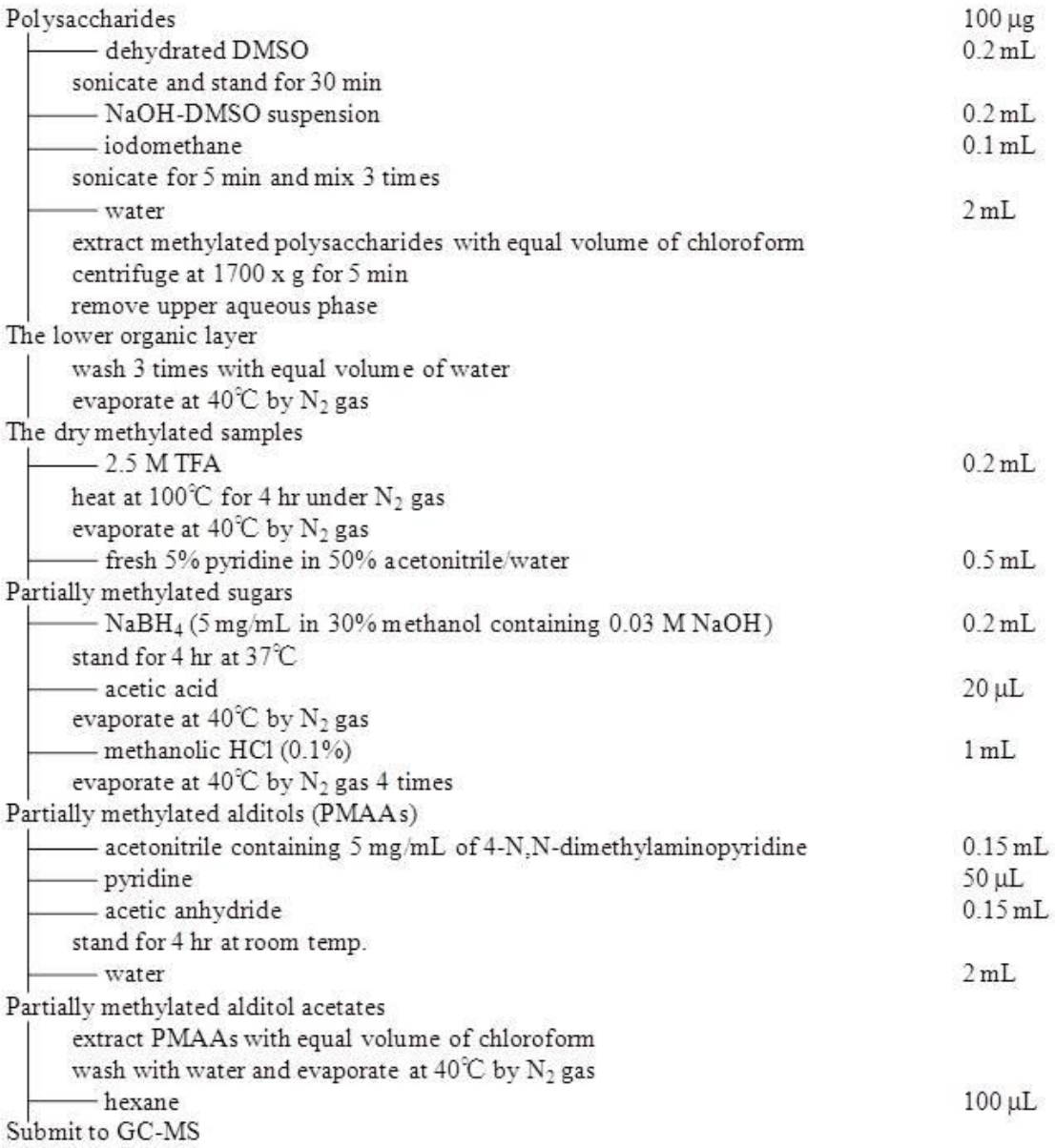

Fig. 34. Procedure for methylation analysis of polysaccharides.

Nuclear magnetic resonance spectroscopy (NMR)

The one-dimensional spectra, on 4-50 mg of dry, exchanged samples in deuterium oxide (0.6 ml, 99.9 atom % with 3-(trimethylsilyl) propionic acid-d4 sodium salt (internal standard), were performed using a Jeol 600 MHz instrument. The operation conditions for one-dimensional spectra were as follows: 1-H NMR; spin, 15 Hz; relaxation delay, 2 s; acquisition, ,1000 scans; temperature,

30ºC: 13-C NMR; spin, 15 Hz; relaxation delay, 2 s; acquisition, 33,000 scans; temperature, 30ºC. The water resonance was suppressed by selective irradiation during the relaxation delay.

The two-dimensional spectra were obtained by using COSY, NOESY and TOCSY function. The operation conditions for two-dimensional spectra were as follows: COSY; spin, 15 Hz; acquisition, 16 Proton scans; acquisition, 128 COSY scans; temperature, 30ºC: NOESY;mixing time, 150 ms; acquisition, 40 Proton scans; acquisition, 40 NOESY scans; temperature, 30ºC: TOCSY; spin, 15 Hz; acquisition, 16 Proton scans; acquisition, 64 TOCSY scans; spin locking, MLEV17; temperature, 30ºC.

Cell culture for immunomodulatory study

The mouse Abelson leukemia virus transformed monocyte macrophage cell line (RAW 264.7 cell lines) was obtained from Dr. Atsushi Ichikawa. RAW 264.7 cell lines were grown in DMEM culture medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% Fetal Bovine Serum (Life Technology, Tokyo, Japan) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Inc., Kyoto, Japan), at 37°C in 5% CO_2 in a humidified incubator. RAW 264.7 cells 5×10^5 cells/ml) were treated in 6-wells polystyrene tissue culture plates with 2 ml cell suspension in each well for 24 h. The medium was then removed and replaced by fresh medium containing polysaccharides at 100 μg/ml or LPS (100 ng/ml or 1 μg/ml) as proinflammatory controls. After 6 or 24 h, cells and medium were harvested for further experiments.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the treated cell lines with the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized in a 20 μl reaction mixture with 4 μg of total RNA by using the Blend taq with 10× Buffer for Blend Taq (Toyobo Co Ltd., Osaka, Japan) PCR was performed with the PCR Thermal Cycler (Takara Bio Inc. Otsu, Japan) and each 2 mM dNTPs (Toyobo Co Ltd., Osaka, Japan) Primer sequences are listed in Table 7. The PCR conditions were as follows; G3PDH; 5 min at 95˚C, (30 s at 95°C, 30 s at 60°C and 60 s at 72°C) × 30 cycles, and 5 min at, 72°C: IL-6; 5 min at 94˚C, (60 s at 94°C, 120 s at 61.6°C and 120 s at 72°C) × 30 cycles, and 5 min at 72°C: TNF-α; 5 min at 94°C, (60 s at 94°C, 130 s at 60°C and 140 s at 72°C) × 30 cycles, and 5 min at 72°C [105]: IL-10; 5 min at 94˚C, (30 s at 94˚C, 30 s at 55˚C and 60 s at 72˚C) × 30 cycles, and 5 min at 72˚C [106]. All data were normalized to the internal standard G3PDH.

Table 11. Primer sequences for PCR.

Gene	NCBI reference	Primer sequences (5'-3')	Product size (bp)
IL-6	NM_031168	F: GTGACAACCACGGCCTTCCCTACT R: GGTAGCTATGGTACTCCA	312
TNF-α	NM_013693	F:GCGACGTGGAACTGGCAGAAG R: GGTACAACCCATCGGCTGGCA	383
IL-10	NM_010548	F: CCAGTTTTACCTGGTAGAAGTGATG R: TGTCTAGGTCCTGGAGTCCAGCAGACTC	323
G3PDH		F: GGTATCGTGGAAGGACTCATGAC R: ATGCCAGTGAGCTTCCCGTTCAGC	188

F, forward primer; R, reverse primer.

Quantitative determination of cytokines by ELISA

Reactions were carried out by enzyme linked immunosorbent assay (ELISA), using eBioscience ELISA Ready-SET-GO (Affymetrix, Tokyo, Japan) for IL-6, IL-10 and TNF-α. Coat Corning Costar 9018 ELISA plate with 100 µl/well of capture antibody in Coating buffer was sealed and incubated overnight at 4ºC. After incubation, ELISA plates were aspirated well and washed 3-5 times with 250 µl/well wash buffer. Then ELISA plates were blocked with 200 µl/well of 1×Assay Diluent and incubated at room temperature for 1 h. After that ELISA plates were aspirated and washed same as before. After that, 100 µl/well of cell culture medium or standards were added and incubated at room temperature for 2 h. After incubation for 2 h, plates were aspirated and washed again. Then, 100 µl/well of Avidin-HPR were added and incubated at room temperature for 30 minutes. ELISA plates were aspirated and washed 5-14 times. Then, 100 µl/well of substrate solution were added at room temperature for 15 minutes and 50 µl of stop solution. Absorbance was read at 450 nm in tecan sunrise plate reader A-5082 (Tecan, Tokyo, Japan). Values were converted to weight in pg/ml.

Statistics

Statistical analyses were carried out using GraphPad Prism version 4.0 for Windows 7 (GraphPad,USA). ANOVA with Tukey's multiple comparison test was applied for multiple comparisons. Values are indicated as means ± SE, and significant differences are shown as probability values.

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LIST OF PUBLICATION

1. Suabjakyong, P., Saiki, R., Van Griensven, L. J., Higashi, K., Nishimura, K., Igarashi, K., Toida, T.: Polyphenol Extract from *Phellinus igniarius* Protects against Acrolein Toxicity In Vitro and Provides Protection in a Mouse Stroke Model. ***PLoS One***, 10(3), e0122733 (2015)

2. Suabyakyong, P., Nishimura, K., Toida, T., Van Griensven, L. J.: Structural characterization and immunomodulatory effects of polysaccharides from *Phellinus linteus* and *Phellinus igniarius* on the IL-6/IL-10 cytokine balance of the mouse macrophage cell line RAW 264.7. ***Food & Function***, 6, 2834-2844 (2015)

DISSERTATION COMMITTEE

This dissertation was evaluated by the following committee authorized by the Graduate School of Pharmaceutical science, Chiba University.

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