CHAPTER III

RESULTS

3.1 Characterization of phytochemicals

3.1.1 Characterization of xanthone

To confirm that xanthone, used in this study is pure; the characterization performed by High Performance Liquid Chromatogrphy method was conducted. HPLC chromatogram is shown in Figure 18. The retention time of xanthone was 20.872, as same as, a standard α-mangostin from Sigma-Aldrich® (USA).

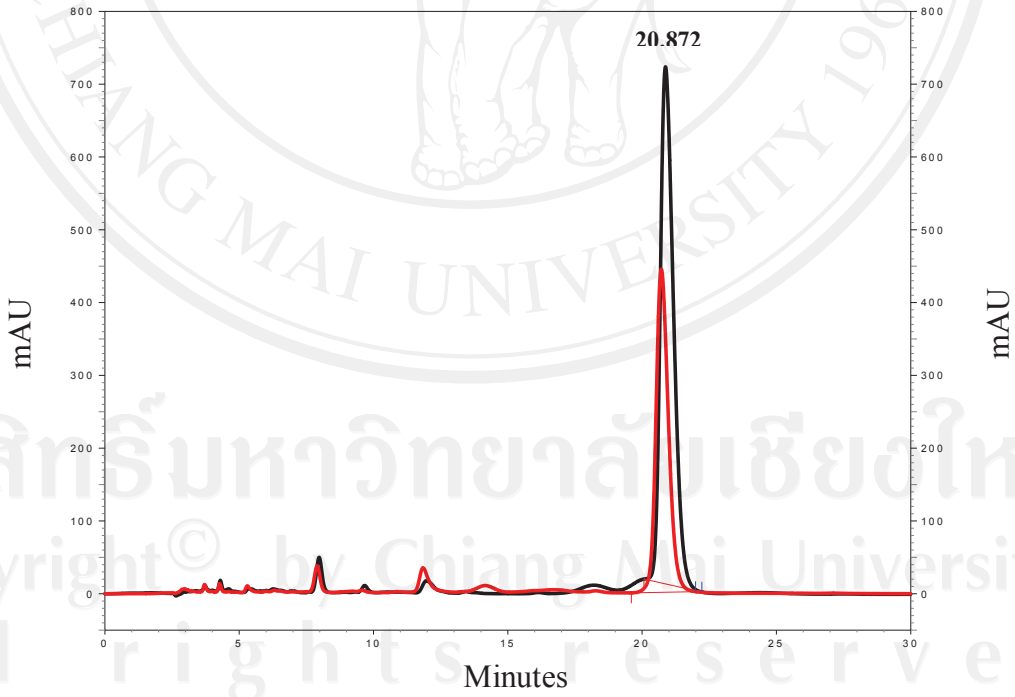


Figure 18. HPLC chromatogram is shown the retention time at 20.872, as same as, standard α-mangostin from Sigma-Aldrich® (USA). The *black line* was standard α-mangostin and the *red line* was xanthone, used in this study.

3.1.2 Characterization of sesamin

To confirm that sesamin, used in this study and extracted at Dr. Wilat's laboratory is pure; the characterization performed by High Performance Liquid Chromatogrphy method was conducted. HPLC chromatogram is shown in Figure 19. The retention time of sesamin was 8.055, as same as, a standard sesamin from Sigma-Aldrich® (USA).

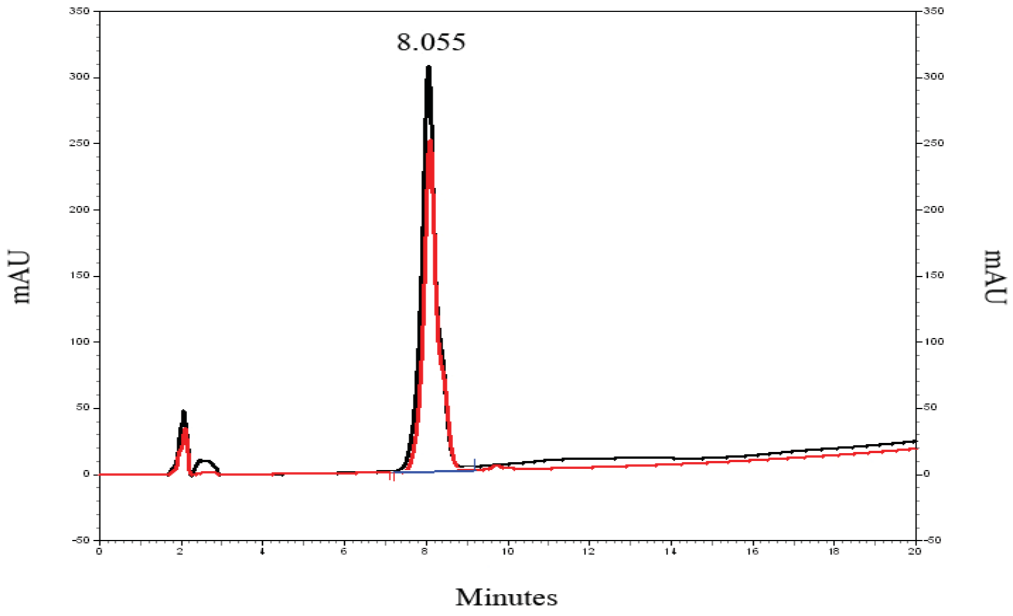


Figure 19. HPLC chromatogram is shown the retention time at 8.055. The *black line* was standard sesamin and the *red line* was sesamin, used in this study. The concentration of both sesamin was 100 μg/ml.

3.1.3 Characterization of *Andrographis paniculata*

HPLC fingerprint of methanol extract of dry powdered *Andrographis paniculata* was analyzed by HPLC system for isocratic elution, with water: acetonitrile: ethanol mobile phase in the ratio 60: 20: 20, Hypersil® ODS-2 C18 (5µm), 250×4.6 mm column and 1.0 ml/min flow rate as shown in Figure 20.

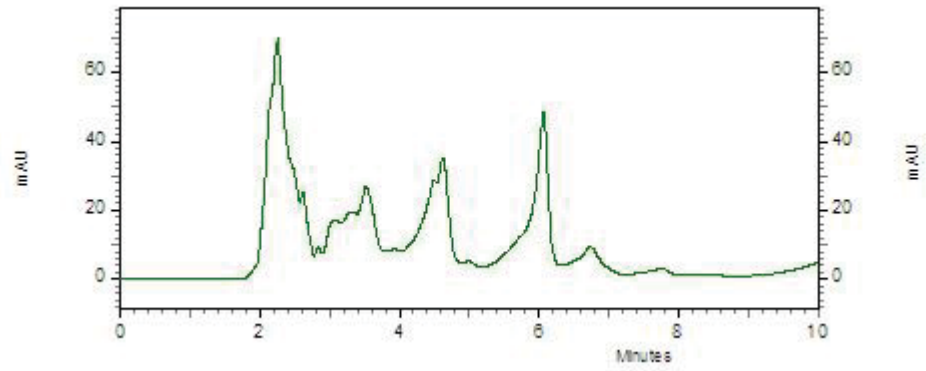


Figure 20. HPLC chromatogram is shown the fingerprint of ethanol extract of dry powdered *Andrographis paniculata*.

3.1.4 Characterization of *Moringa oleifera*

HPLC fingerprint of extract of dry powdered *Moringa oleifera* (water and ethanol extraction) and extract of fresh leaves of *Moringa oleifera* (water and ethanol extraction) were analyzed by HPLC system for isocratic elution, with water: acetonitrile: ethanol mobile phase in the ratio 60: 20: 20, Hypersil® ODS-2 C18 (5µm), 250×4.6 mm column and 1.0 ml/min flow rate as shown in Figure 21.

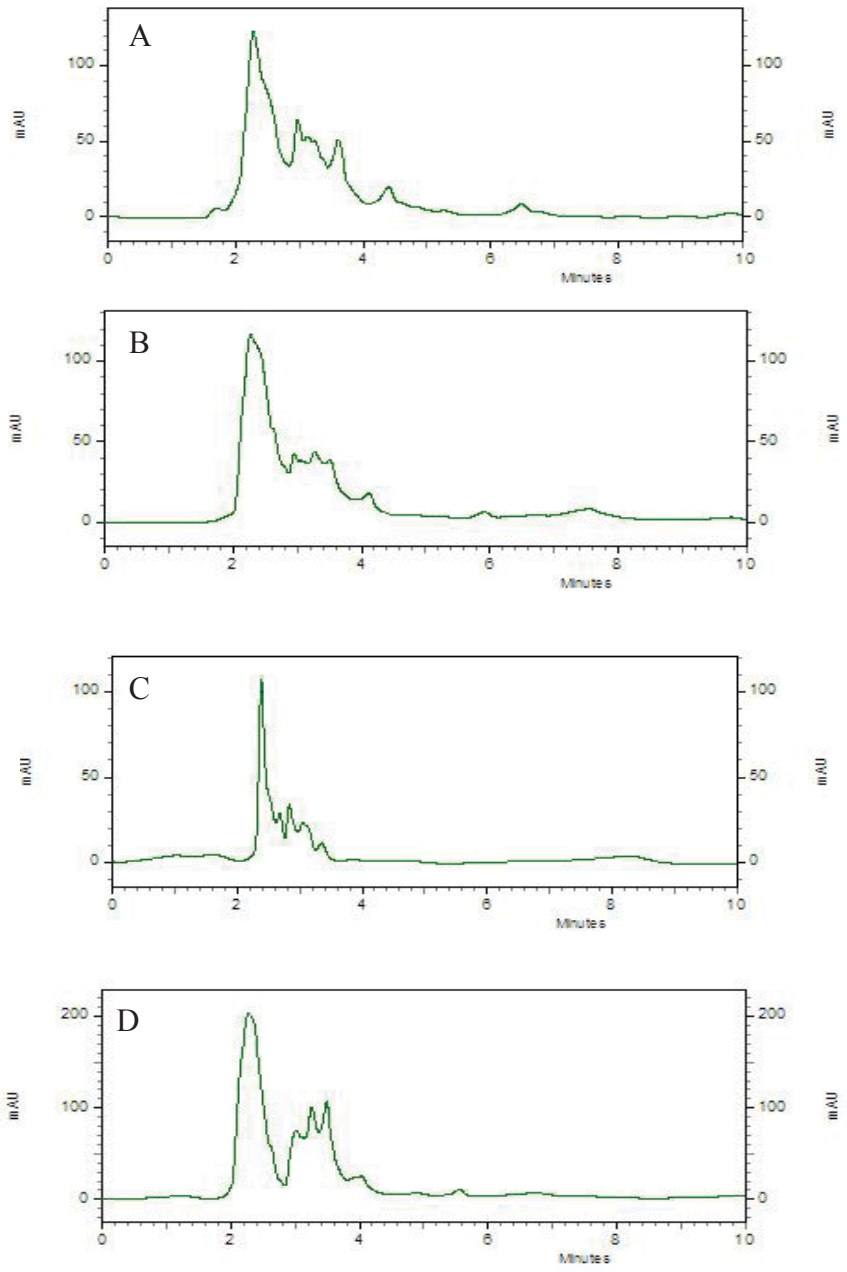


Figure 21. HPLC chromatogram is shown the fingerprint of *Moringa oleifera* extract.

(A) HPLC fingerprint of water extract of dry powdered *Moringa oleifera*,

(B) HPLC fingerprint of ethanol extract of dry powdered *Moringa oleifera*,

(C) HPLC fingerprint of water extract of fresh leaves of *Moringa oleifera*,

(D) HPLC fingerprint of ethanol extract of fresh leaves of *Moringa oleifera*

3.1.5 Characterization of *Houttuynia cordata*

HPLC fingerprint of *Houttuynia cordata* extract (water and ethanol extraction) and fermented *Houttuynia cordata* extract (water and ethanol extraction) were analyzed by HPLC system for isocratic elution, with water: acetonitrile: ethanol mobile phase in the ratio 60: 20: 20, Hypersil® ODS-2 C18 (5µm), 250×4.6 mm column and 1.0 ml/min flow rate as shown in Figure 22.

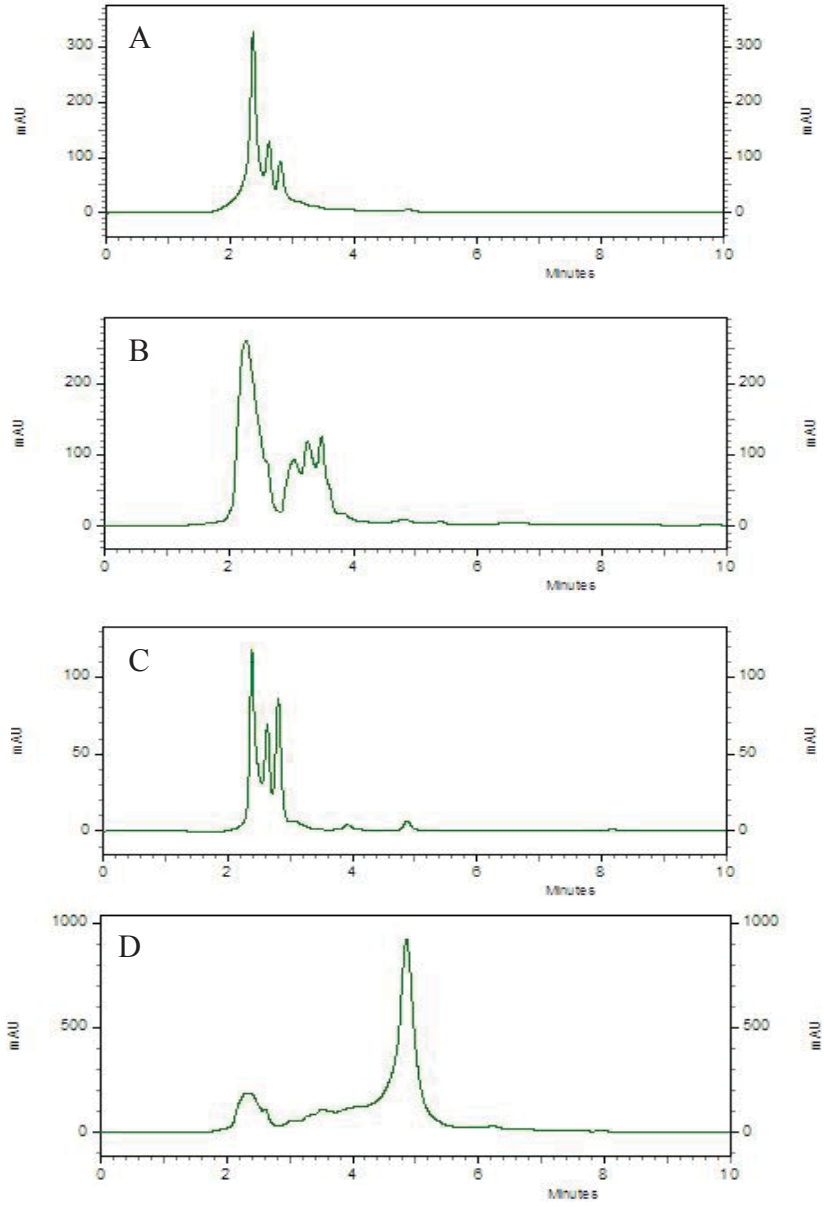


Figure 22. HPLC chromatogram is shown fingerprint of *Houttuynia cordata* extract.

(A) HPLC fingerprint of water extract of *Houttuynia cordata*,

(B) HPLC fingerprint of ethanol extract of *Houttuynia cordata*,

(C) HPLC fingerprint of water extract of fermented *Houttuynia cordata*,

and (D) HPLC fingerprint of ethanol extract of fermented *Houttuynia cordata*.

3.1.6 Characterization of *Hericium erinaceus*

HPLC fingerprint of *Hericium erinaceus* extract (water and ethanol extraction) was analyzed by HPLC system for isocratic elution, with water: acetonitrile: ethanol mobile phase in the ratio 60: 20: 20, Hypersil® ODS-2 C18 (5µm), 250×4.6 mm column and 1.0 ml/min flow rate as shown in Figure 23.

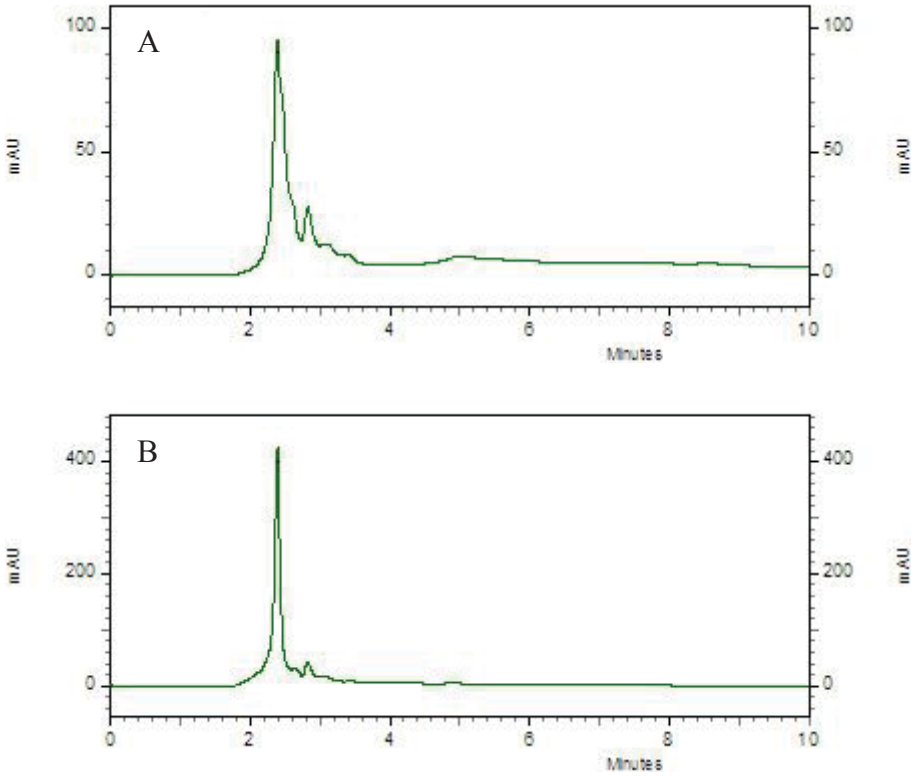


Figure 23. HPLC chromatogram is shown the fingerprint of *Hericium erinaceus* extract.

(A) HPLC fingerprint of water extract of *Hericium erinaceus*, and

(B) HPLC fingerprint of ethanol extract of *Hericium erinaceus*.

3.1.7 Characterization of acetone extract of *Alpinia galanga*

The acetone extract of *Alpinia galanga* used in this study that was extracted at Dr. Wilat's laboratory, the pure *p*-hydroxycinnamaldehyde was identified by MS/NMR. HPLC fingerprint of acetone extract of *Alpinia galanga* was analyzed by HPLC system for isocratic elution, with water: CH_3CN mobile phase in the ratio 80: 20, Shimadzu CLASS-VP V5.02 ODS C18 (5µm), 250×4.6 mm column and 1.0 ml/min flow rate as shown in Figure 24.

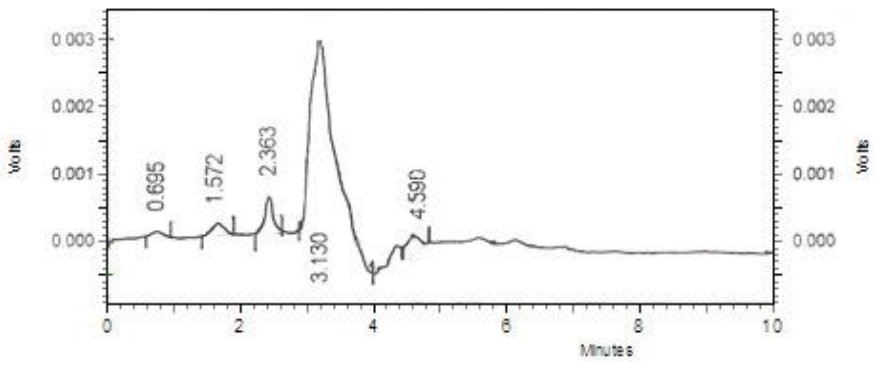


Figure 24. HPLC chromatogram is shown the fingerprint of acetone extract of *Alpinia galanga*.

3.2 Screening phase of the effects of phytochemicals on cytokine markers release from influenza type A H1N1 induced PBMC.

3.2.1 Study of the cytotoxicity of phytochemicals.

In this study, phytochemical extracts were divided into two groups. The first group; xanthone, sesamin, *Moringa oleifera* dry powder extract, *Moringa oleifera* ethanol extract, *Houttuynia cordata* ethanol extract, fermented *Houttuynia cordata* ethanol extract, and *p*-hydroxycinnamaldehyde were dissolved in dimethylsulfoxide (DMSO). The second group; *Andrographis paniculata* extract, *Moringa oleifera* water extract, *Herricium erinaceus* extract, *Houttuynia cordata* water extract, and fermented *Houttuynia cordata* water extract were dissolved in deionized water. To assess whether phytochemicals toxic to PBMC, we investigated the cytotoxicity effect of phytochemicals by alamarBlue® assay. The concentrations ranging from 0.5 to 10.0 μg/ml were not toxic to the cells (Figure 25) and these concentrations had been used in the next study which investigating the activity and gene expression of cytokine markers.

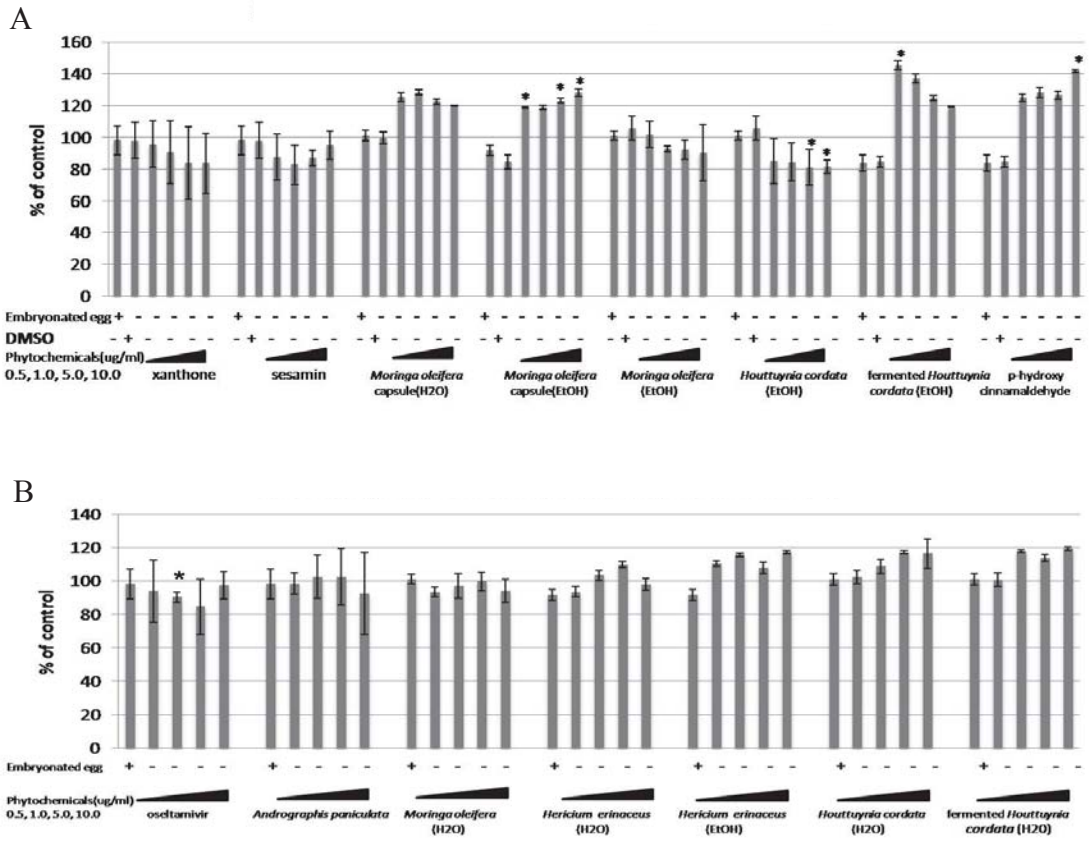


Figure 25. The cytotoxicity effect of phytochemicals on the growth of PBMC by the alamarBlue® assay. PBMC was cultured with various concentrations of phytochemicals or oseltamivir (positive control) or RPMI 1640 medium 1640 control or DMSO control for 24 h.

(A) The cytotoxicity effect of phytochemicals dissolved in DMSO.

(B) The cytotoxicity effect of phytochemicals dissolved in water.

Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from RPMI 1640 medium control, (p<0.05) respectively.

3.2.2 Study of activity of cytokine markers by ELISA kit

The alamarBlue® assay revealed the suitable concentrations of phytochemicals used in this part were 0.5-10.0 μg/ml.

The inhibitory effect of phytochemicals on cytokine storm was focus on IL-1β, TNF-α, and IL-2 release from influenza type A H1N1 induced peripheral blood mononuclear cells (PBMC) model. The phytochemical extracts were divided into two groups. The first group; xanthone, sesamin, *Moringa oleifera* dry powdered extract, *Moringa oleifera* ethanol extract, *Houttuynia cordata* ethanol extract, fermented *Houttuynia cordata* ethanol extract, and *p*-hydroxycinnamaldehyde were dissolved in dimethylsulfoxide (DMSO). The second group; *Andrographis paniculata* extract, *Moringa oleifera* water extract, *Herricium erinaceus* extract, *Houttuynia cordata* water extract, and fermented *Houttuynia cordata* water extract were dissolved in deionized water.

The screening for phytochemicals which reduced IL-1β, TNF-α, and increased IL-2 releasing from PBMCs showed that xanthone, sesamin and *Andrographis paniculata* extract could reduce IL-1β and TNF-α level and increase IL-2 level in dose dependent manner (Figure 26, 27).

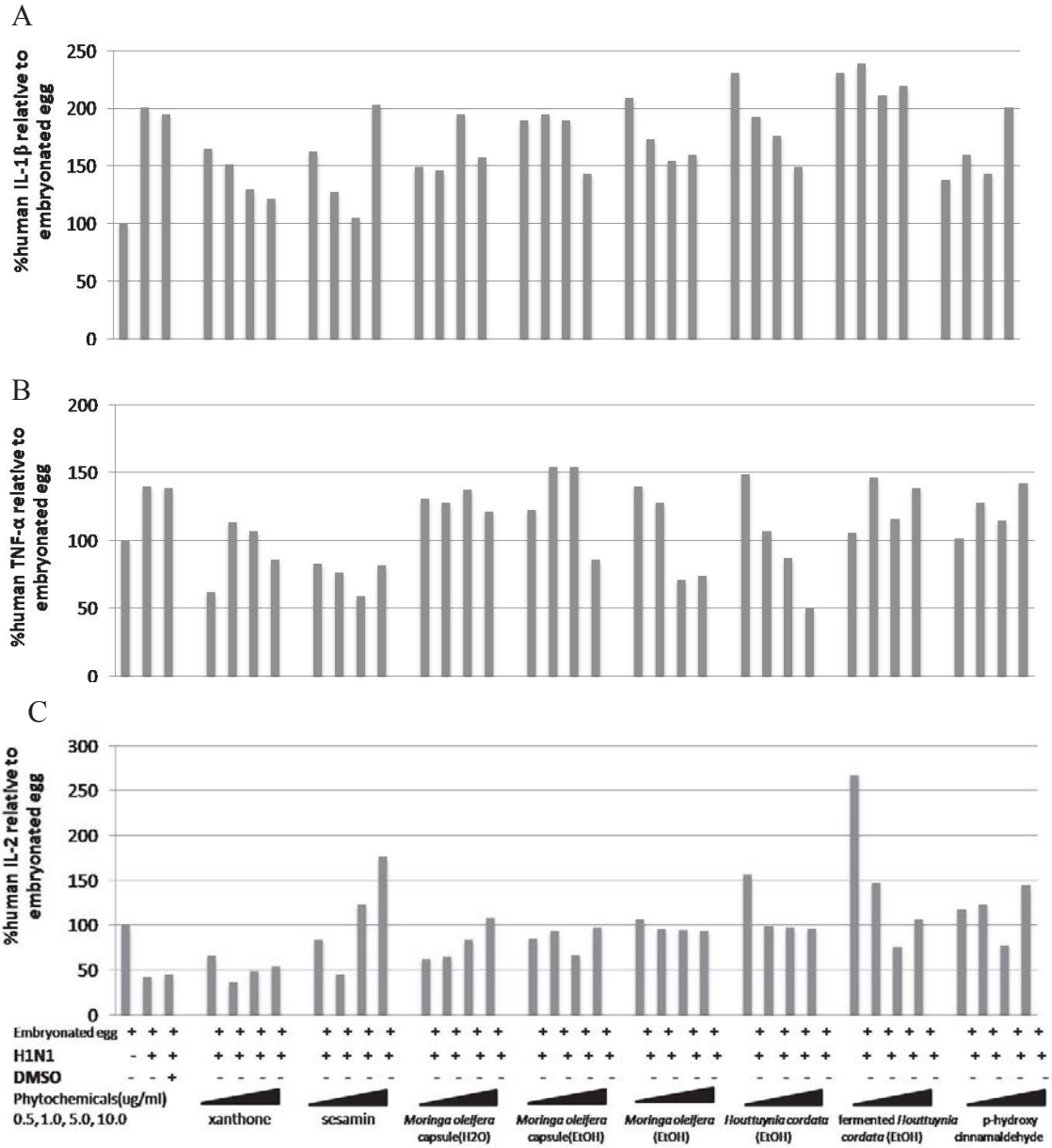


Figure 26. The effect of phytochemicals which is dissolved in DMSO on screening activity of IL-1β (A), TNF-α (B) and IL-2 (C) by ELISA. The data showed that xanthone and sesamin could reduce IL-1β and TNF-α level and increase IL-2 level in dose dependent manner.

Data shown are mean value of duplicate assay per treatment.

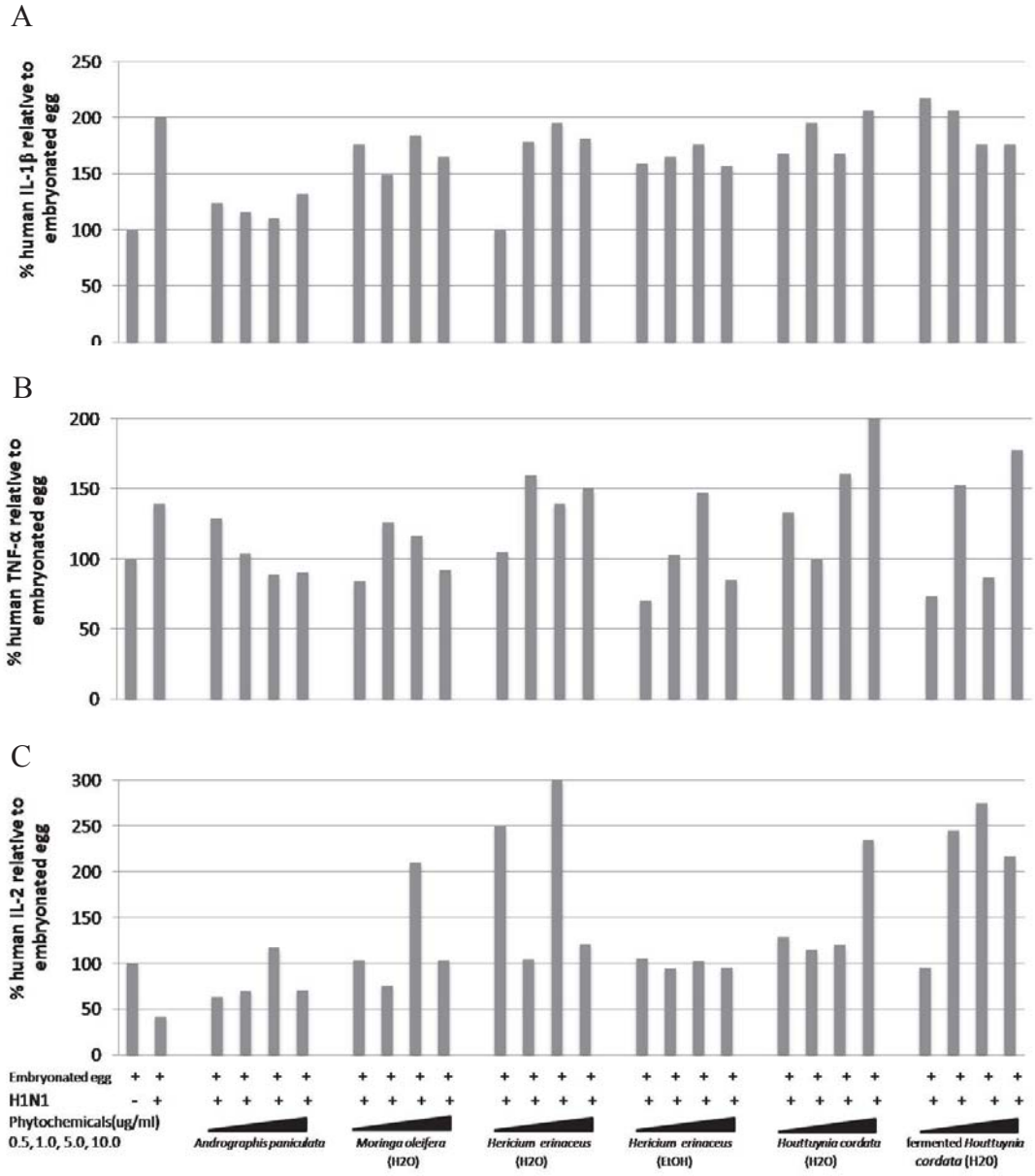


Figure 27. The effect of phytochemicals which is dissolved in deionized water on screening activity of IL-1β (A), TNF-α (B) and IL-2 (C) by ELISA. The data showed that *Andrographis paniculata* extract could reduce IL-1β and TNF-α level and increase IL-2 level in dose dependent manner. Data shown are mean value of duplicate assay per treatment.

3.3 Confirming phase of the effect of phytochemicals on cytokine markers release from influenza type A H1N1 induced PBMC.

3.3.1 Study of activity of cytokine markers by ELISA kit

The inhibition of cytokine storm that involved in the activity of cytokines was used as cytokine markers. As the results, the screening activity of IL-1β, TNF-α, and IL-2 showed that xanthone, sesamin and *Andrographis paniculata* extract could reduce IL-1β and TNF-α level and increase IL-2 in dose dependent manner.

In this study, we investigated further the effects of selected phytochemicals; xanthone, sesamin and *Andrographis paniculata* extract, in comparison with positive control oseltamivir (Tamiflu®) on IL-1β, TNF-α, and IL-2 level. To confirm the activity of cytokines markers, repeated ELISA method was used in this study. We found that xanthone, sesamin and *Andrographis paniculata* extract in contrast with oseltamivir (Tamiflu®) could reduce IL-1β and TNF-α level and increase IL-2 in dose dependent manner as shown in Figure 28.

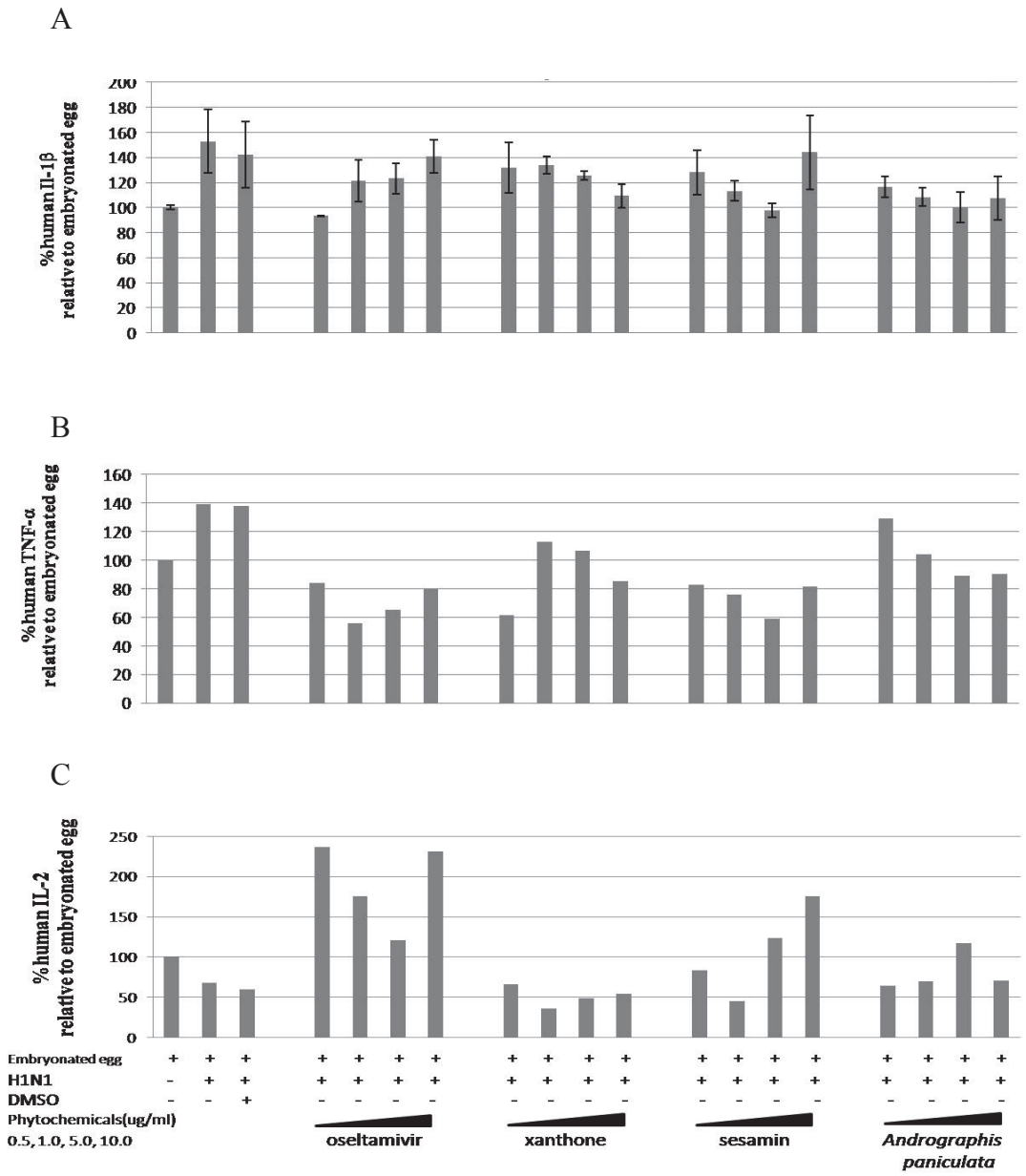


Figure 28. The effect of phytochemicals; xanthone, sesamin and *Andrographis paniculat* extract compare with oseltamivir (Tamiflu®) on IL-1β (A), TNF-α (B) and IL-2 (C) level.

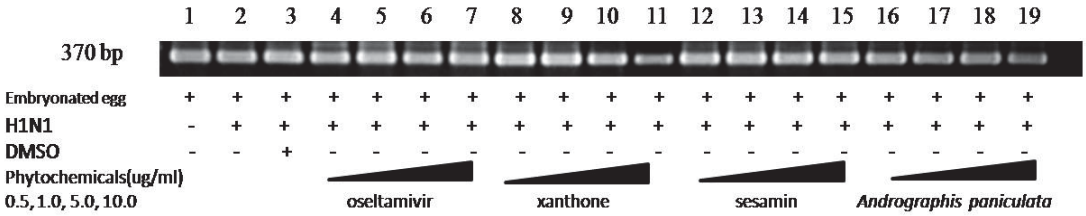
(A) Data shown are mean value ± standard deviation of triplicate assay per treatment.

(B, C) Data shown are mean value of duplicate assay per treatment.

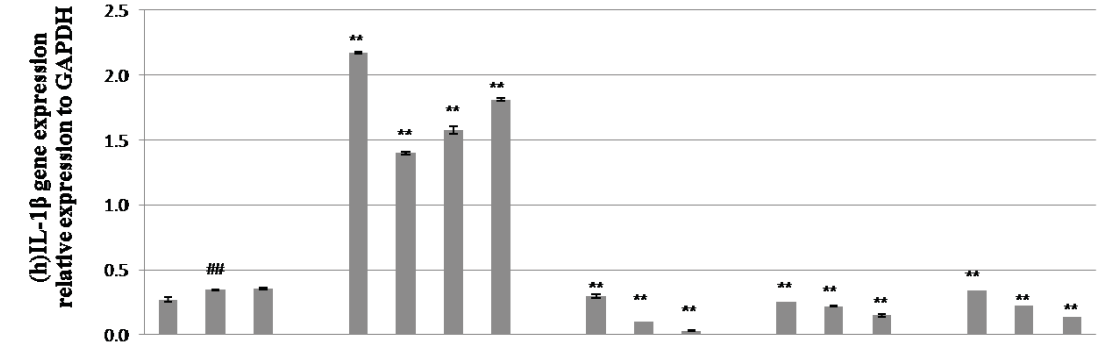
3.3.2 Study of cytokines markers gene expression by real-time qPCR

The inhibition of cytokine storm that involved in the expression of cytokines was used as cytokine markers. In this study, we investigated the effect of selected phytochemicals; xanthone, sesamin and *Andrographis paniculata* extract, compare with positive control oseltamivir (Tamiflu®) on IL-1β, TNF-α, and IL-2 mRNA level. After 24 h of phytochemicals or oseltamivir (Tamiflu®) treatments, PBMC were lysed and accompanied the GAPDH gene expression by RT-PCR. The band of GAPDH gene expression appeared on 1.5% agarose gel was shown in Figure 30, top.

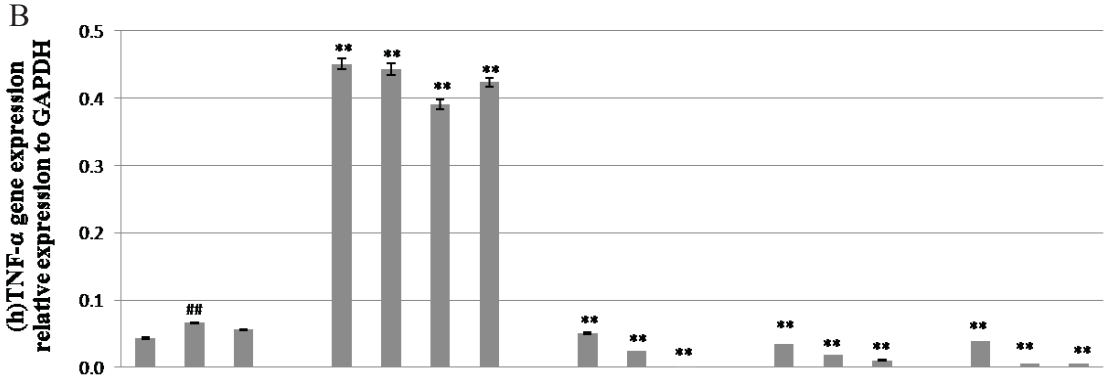
To confirm the gene expression of cytokine markers, real-time qPCR method was used in this study. The PBMC were treated with selected phytochemicals; xanthone, sesamin and *Andrographis paniculata* extract, and oseltamivir (Tamiflu®) in the range of concentration 0.5-10.0 μg/ml. Then, analysis of the gene expression was exhibited by real-time qPCR. We found that xanthone, sesamin and *Andrographis paniculata* extract could reduce IL-1β and TNF-α mRNA level ($p<0.01$) whereas increased IL-2 mRNA level ($p<0.01$) in dose dependent manner, in contrast with oseltamivir (Tamiflu®) as shown in Figure 29.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
370 bp
Embryonated egg + + + + + + + + + + + + + + + + + + +
H1N1 - + + + + + + + + + + + + + + + + + +
DMSO - - + - - - - - - - - - - - - - - - -
Phytochemicals(ug/ml)
0.5, 1.0, 5.0, 10.0
oseltamivir
xanthone
sesamin
Andrographis paniculata

A


(h)IL-1β gene expression
relative expression to GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
##
**

B


(h)TNF-α gene expression
relative expression to GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
##
**

C

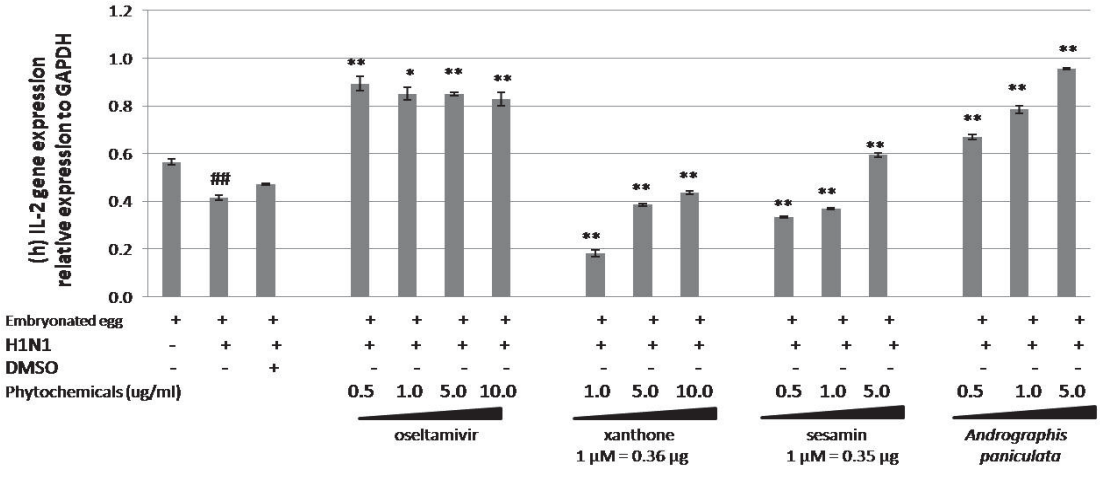

(h) IL-2 gene expression
relative expression to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
**
Embryonated egg + + + + + + + + + + + + + + + + + + +
H1N1 - + + + + + + + + + + + + + + + + + +
DMSO - - + - - - - - - - - - - - - - - - -
Phytochemicals (ug/ml)
0.5 1.0 5.0 10.0
1.0 5.0 10.0
0.5 1.0 5.0
0.5 1.0 5.0
oseltamivir
xanthone
1 μM = 0.36 μg
sesamin
1 μM = 0.35 μg
Andrographis paniculata

Figure 29. The effect of phytochemicals; xanthone, sesamin and *Andrographis paniculat* extract compared with oseltamivir (Tamiflu®) on IL-1β (A), TNF-α (B) and IL-2 (C) mRNA level. The band of GAPDH gene expression was appeared on 1.5% agarose gel was shown in the top.

Data shown are mean value ± standard deviation of triplicate assay per treatment.

Denote value that was significantly different from embryonated egg treated control ($p < 0.01$).

** Denote values that were significantly different from H1N1 treated control ($p < 0.01$).