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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi: 10.1016/S2221-1691(15)30170-2

Immunomodulatory effects of selected Malaysian plants on the CD18/11a expression and phagocytosis activities of leukocytes

Nurul Hikmah Harun[1*], Abdi Wira Septama[2], Ibrahim Jantan[3]

[1]*School of Biomedicine and Diagnostics, Faculty of Medicine and Health Sciences, Universiti Sultan Zainal Abidin, 21300 Kuala Terengganu, Terengganu, Malaysia*

[2]*Faculty of Pharmacy, University of Sumatera Utara, Jalan Tri Dharma No. 3, Medan, Sumatera Utara, Indonesia*

[3]*Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

PEER REVIEW

Peer reviewer

Dr. Nilesh Prakash Nirmal, Department of Biopharmacy, Faculty of Pharmaceutical Sciences, Burapha University, 169, Bangsaen Road, Saensuk, Muang, Chonburi-20131, Thailand.
E-mail: nirmalnp21@yahoo.co.in

Comments

This is a valuable research work in which authors have demonstrated the immunemodulatory effect of 20 Malaysian medicinal plant extracts using CD18/11a expression and phagocytosis activity of leukocytes. Methanolic extracts of *A. galangal*, *O. aristatus* and *A. muricata* are able to modulate innate immune system and give the prospect for discovery the therapeutic agent for modulating immune system.
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ABSTRACT

Objective: To investigate the effects of 20 methanolic extracts from Malaysian selected plants on CD18/11a expression and phagocytosis activity of leukocytes using flow cytometry analysis.
Methods: The effects of methanolic extracts on CD18/11a expression and phagocytosis of leukocytes were measured by labelling the cells with CD18-fluorescein isothiocyanate and ingestion labelled with *Escherichia coli*-fluorescein isothiocyanate and then analyzed using flow cytometer.
Results: About 12 out of 20 methanolic extracts of selected Malaysian medicinal plants significantly ($P \leqslant 0.05$) inhibited the CD18/11a expression of leukocytes at both concentrations of 6.25 μg/mL and 100 μg/mL in dose dependent manner. The most active inhibitory was shown in *Citrus aurantifolia* (Christm.) Swingle and *Alpinia galangal* (L.) Willd. at dosage 100 μg/mL. Moreover, the *Orthosiphon aristatus* (Blume) Miq (*O. aristatus*). showed the highest stimulatory activity at the concentration of 100 μg/mL. Other than that, four plant extracts significantly ($P \leqslant 0.05$) rose the phagocytosis activities of leukocytes in dose dependent manner. However, *Annona muricata* L. and *O. aristatus* showed the highest stimulated activities at the 100 μg/mL concentration.
Conclusions: The results suggest that methanolic extracts of *Citrus aurantifolia*, *Alpinia galangal*, *O. aristatus* and *Annona muricata* are able to modulate innate immune system and can potentially be recognized as therapeutic agents for modulating immune system.

KEYWORDS
Selected Malaysian medicinal plants, Immunomodulatory effects, CD18/11a expression, Phagocytosis activity, Flow cytometry analysis

1. Introduction

Innate immune system is our first line of defense against invading pathogen. It consists of an accumulation of phagocyte cells that will form immediately at the site of infection[1]. Polymorphonuclear neutrophil (PMN) cells are the most abundant and the first responder of phagocyte cells to migrate towards the site of infection. These cells play an important role to destroy the pathogens[2]. The migration of phagocyte cells occurs in multistep processes that involve rolling, adhesion of the cells to the vascular endothelial cells, diapedesis, followed by migration of these cells to the site of infection, phagocytosis and finally degradation of pathogen[3].

*Corresponding author: Nurul Hikmah Harun, School of Biomedicine and Diagnostics, Faculty of Medicine and Health Sciences, Universiti Sultan Zainal Abidin, 21300 Kuala Terengganu, Terengganu, Malaysia.
Tel: +60149137502
Fax: +609 627 5771/5772
E-mail: nurulhikmah@unisza.edu.my, heekmah_85@yahoo.com
Foundation Project: Supported by Ministry of Higher Education, Malaysia (Grant No. GUP-SK-07-23-042).

Article history:
Received 20 Sep 2014
Received in revised form 8 Oct, 2nd revised form 3 Nov 2014
Accepted 5 Nov 2014
Available online 7 Nov 2014

Adhesion of phagocyte cells to the endothelium during innate immunity involves specific membrane receptor called beta 2 integrin (CD18/11a,b,c) that originates from a family of proteins[4]. This receptor mediates adherence of phagocyte cells to endothelium cell receptors which are known as intercellular adhesion molecule-1 and intercellular adhesion molecule-2. The phagocyte-endothelial interaction will promote phagocyte cells recruitment into infected tissue and later would result in phagocytosis of pathogen[5,6]. Phagocytosis is a process that phagocyte cells engulf and ingest solid particles including microbial pathogens. Through this process, the microorganisms can be destroyed, processed for antigen presentation and further introduced them to later immune responses towards this pathogen[7]. Phagocyte cells contain surface receptors such as Fc gamma receptor and complement fragment C3b receptor that are involved in phagocytosis process[8,9]. Pathogen that enters our body will be firstly opsonized by C3b which is the result of complement activation. Then, the phagocytosis process will be initiated after C3b binds into a complement fragment C3b receptor[10].

Several plant extracts such as *Panax ginseng*, *Pelargonium graveolens*, *Ganoderma tsugae, Codonopsis pilosula, Angelica sinensis*, *Emblica officinalis*, *Zingiber officinale, Alpinia galangal* (L.) Willd. (*A. galangal*) and *Averrhoa bilimbi* have been reported for their activities on the immune system[11-14]. Many bioactive compounds that have been isolated from plants such as cordifolioside A, syringing, curcumin, flavopiridol, combretastatin, lycopene and polysaccharides, exhibit potent immunomodulatory agents[15-17]. Several types of immunommodulatory agents that have been found from fungi and bacteria also exhibit immunomodulatory activities to immune system[18,19]. However, little information is available about activity of Malaysian medicinal plants that can induce or inhibit natural immune system, particularly expression of adhesion molecule and phagocytosis activity of leukocytes.

The present study aimed to investigate the effects of 20 methanolic extracts from Malaysian selected plants on CD18/11a expression and phagocytosis activity of leukocytes using flow cytometry analysis.

2. Materials and methods

2.1. Chemical

Fluorescein isothiocyanate-labeled CD18 (anti-LFA-1/CD 18/11a), goat anti-mouse polyclonal antibody and FACS lysing solution were purchased from BD Biosciences. Phosphate buffer saline (PBS) was purchased from Sigma, St. Louis, MO, USA. Phago test kit was purchased from Orpegen Pharma, Germany.

2.2. Plant materials

A total of 20 selected plants were collected between March and July 2008 from different places in the peninsular of Malaysia. The plants were identified by botanist of Universiti Kebangsaan Malaysia (UKM) and the voucher specimens were deposited at the herbarium of UKM, Bangi, Malaysia. The traditional usage of these plants and previous studies on their biological activities are presented in Table 1.

2.3. Preparation of methanolic plant extracts

A total of 100 g of each plant material was soaked into methanol for 3 d at room temperature. Supernatant was obtained by filtration and collected into a container. The collection of supernatant was removed from the organic solvent by rotary evaporator.

Table 1
Plant species and their bioactivities, traditional usage and percentage yields of methanol extracts based on dry/fresh weight.

Sample No.	Scientific names	Family	Traditional usage[a]	Part used	Voucher No.	Yield (%)
1	*Phyllanthus amarus* Schum & Thonn	Euphorbiaceae	Treatment for jaundice, diabetes, skin ulcer	Whole plant	B-29769	10.40
2	*O. aristatus*	Lamiaceae	Anti-hipertension, anti-diabetes, anti-inflammation	Whole plant	B-29770	10.20
3	*Andrographis paniculata* (Burn.f.) Ness	Acanthaceae	Anti-inflammation, analgesic	Whole plant	B-29771	19.73
4	*Tinospora crispa* L.	Menispermaceae	Anti-pyretic, analgesic	Stem	B-29772	30.85
5	*Labisia pumila* Var alata	Myrcinaceae	Phytoestrogens	Whole plant	B-29773	15.30
6	*Curcuma domestica* L.	Zingiberaceae	Anti-inflammation, anti-ulcer, jaundice, antiparasite	Rhizome	B-29774	35.24
7	*Curcuma mangga* (Valton & Vazsjip)	Zingiberaceae	Anti-allergic	Rhizome	B-29775	15.00
8	*Curcuma xanthorrhiza* Roxb.	Zingiberaceae	Anti-microbial	Rhizome	B-29776	17.00
9	*Curcuma aeruginosa* Roxb.	Zingiberaceae	Anti-microbial	Rhizome	B-29777	15.25
10	*Boesenbergia pandurata* (Roxb.) Schlecht	Zingiberaceae	Anti-inflammation, anti-fungus, treatment for rheumatism and dry cough	Rhizome	B-29778	10.12
11	*A. galangal*	Zingiberaceae	Anti-inflammation, treatment for asthma, cough, headache	Rhizome	B-29779	15.61
12	*Kaempferia galanga* L.	Zingiberaceae	Anti-microbial	Rhizome	B-29780	12.50
13	*Zingiber officinale* Rosc.	Zingiberaceae	Anti-inflammation, treatment for asthma and diabetes	Rhizome	B-29781	10.50
14	*Averrhoa bilimbi* Linn.	Oxalidaceae	Anti-inflammation, anti-diabetic, anti-hypertensive	Fruit	B-29782	19.15
15	*A. muricata*	Annonaceae	Antiseptic, treatment for dermatosis and malarial fever	Fruit peel	B-29783	22.57
16	*Garcinia mangostana* L.	Clusiaceae	Immunostimulation	Fruit peel	B-29784	26.53
17	*Garcinia atroviridis* Griff ex T. Anders	Clusiaceae	Immunostimulation	Fruit	B-29785	39.20
18	*Citrus hystrix* DC.	Rutaceae	Spice in food	Leave	B-29786	24.77
19	*C. aurantifolia*	Rutaceae	Anti-inflammation, anti-infection, treatment for asthma	Fruit	B-29787	16.32
20	*Piper nigrum* L.	Piperaceae	Anti-microbial	Seed	B-29788	25.86

O. aristatus: *Orthosiphon aristatus* (Blume) Miq.; *A. muricata*: *Annona muricata* L; *C. aurantifolia*: *Citrus aurantifolia* (Christm.) Swingle. [a]Source: Burkill[20].

2.4. Effect of methanolic plant extracts on CD18/11a expression

A total of 100 μL of heparinized blood was incubated with 20 μL plants extract in CO_2 incubator at 37 ˚C in 5% CO_2 for 90 min. After 90 min, tubes were placed on ice. Next, 10 μL of CD18 was added to each tube. Meanwhile, for negative control, 10 μL of goat anti-mouse was added. Tubes were incubated on ice for 60 min. After that, FACS lyses was added and incubated for 20 min to lyse erythrocytes. Samples were washed twice with PBS (1 630 r/min) at 50 ˚C for 5 min. Samples were analyzed by flow cytometer[21]. The use of human blood was approved by the Human Ethical Committee of UKM (Approval No. FF-220-2008).

2.5. Effect of methanolic plant extracts on phagocytosis activities

A total of 20 μL plant extracts were mixed with 100 μL heparinized blood and incubated in closed shaking water bath at 37 ˚C for 30 min (60 r/min). After that, tubes were put on ice to stop the reaction[21]. Modified protocol of Phago test kit was used to determine the phagocytic activities of plant extracts. Briefly, mixed samples were added with 20 μL of fluorescent-labeled *Escherichia coli* (*E. coli*) at 0 ˚C. Samples were incubated in shaking water bath at 37 ˚C for 10 min, while for negative control, the samples were put on ice. After incubation, the samples were put on ice to stop reaction, followed by addition of quenching solution, to suppress fluorescence of the bacteria to attach to the outside of the cell. The samples were then mixed.

Next, the samples were washed twice with 3 mL of PBS (washing solution). After washing, samples were added 2 mL of lysing solution to lyse erythrocytes and incubated at room temperature for 20 min. The samples were centrifuged at 50 ˚C for 5 min (1 630 r/min). Samples were then washed twice with 3 mL washing solution. Finally, samples were added with 200 μL DNA staining solution. Each sample was analyzed with flow cytometer.

2.6. Statistical analysis

All the data were analyzed by using SPSS version 15.0. Data were analyzed using independent group *t*-test to compare the value of samples with negative control. Data were presented as SEM and the differences were considered to be significant at $P \leqslant 0.05$ level.

3. Results

3.1. Effect of methanolic plant extracts on CD 18/11a expression

The effect of methanolic plant extracts on CD 18/11a expression was measured by flow cytometer. Figure 1 shows the CD 18/11a expression of leukocytes as mean fluorescence intensity (MFI). From the results, it was found that 12 plant extracts (Sample No. 3, 5, 7, 8, 10, 11, 12, 13, 14, 15, 17, 20) at the concentration of 6.25 μg/mL and 100 μg/mL showed significant ($P \leqslant 0.05$) inhibition on CD18/11a expression compared with negative control samples. Meanwhile, the plant extracts with the concentration of 6.25 μg/mL showed only moderate inhibition when compared with negative control samples. The methanolic extract of *C. aurantifolia* and *A. galangal* showed the most active inhibitory activity on CD18/11a expression activity with value of (310.69±3.80) and (327.85±3.20) respectively at 100 μg/mL compared to negative control samples.

Interestingly, methanolic extract of *O. aristatus* showed the significant ($P \leqslant 0.05$) stimulation effect only at the high concentration of 100 μg/mL (721.92±1.45). Meanwhile, for the low concentration of 6.25 μg/mL, the extract of this plant showed the

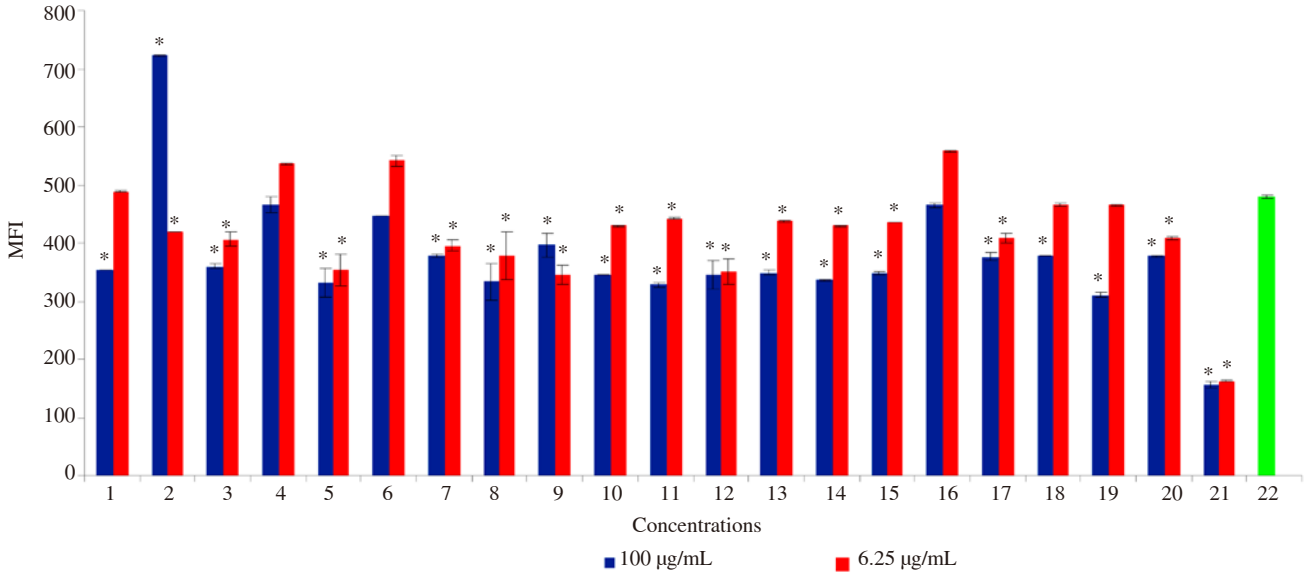

Figure 1. Effect of samples on the CD18/11a expression of leukocytes.
Code number of samples used as in Table 1. 21: Aspirin as positive control; 22: Dimethyl sulfoxide solution as negative control. Each bar: A mean triplicate reading. *: Sample shows significant effect ($P \leqslant 0.05$) at 6.25 μg/mL or 100 μg/mL dosages when compared to negative control using *t*-independent test analysis.

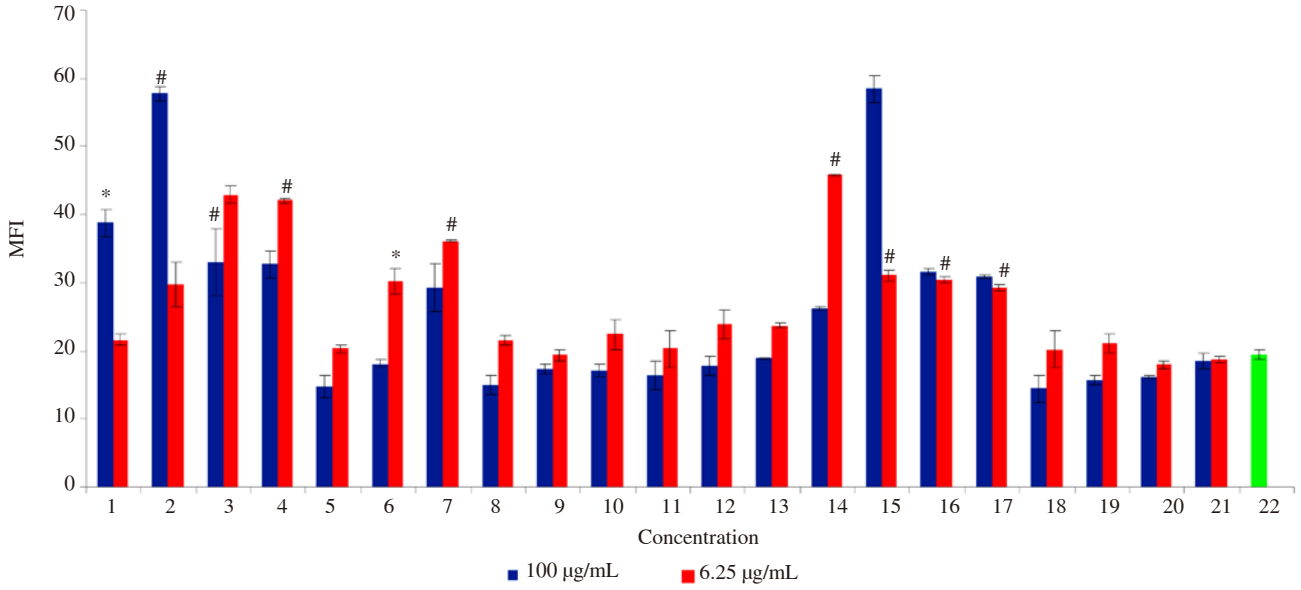

Figure 2. Effect of samples on the phagocytosis of leukocytes.
Code number of samples used as in Table 1. 21: Aspirin as positive control; 22: Dimethyl sulfoxide solution as negative control. Each bar: A mean triplicate reading. #: Sample shows significant effect ($P \leqslant 0.05$) for both concentrations when compared with negative control using *t*-independent test analysis; *: Sample shows significant effect ($P \leqslant 0.05$) at dosage 6.25 µg/mL or 100 µg/mL when compared to negative control using *t*-independent test analysis.

suppression for CD18/11a expression of leukocytes.

3.2. Effect of plant extracts on phagocytosis activity

Figure 2 shows the effect of plant samples on the phagocytosis of leukocyte cells. The phagocytosis activity was determined as the MFI of intracellular bacteria. From the analyzed results, four plant samples (Sample No. 2, 15, 16, 17) showed significant ($P \leqslant 0.05$) immunostimulation effect in dose dependent manner. However, the methanolic extract of *A. muricata* (58.43±1.93) at 100 µg/mL dosage showed the most active stimulatory activity on phagocytosis of lekocytes followed by *O. aristatus* (57.72±1.05) compared to negative control samples.

4. Discussion

The migration of leukocytes cell to the site of infection is an important process and it occurs in several stages. CD18/11a,b,c is the membrane glycoprotein complex that facilitates adhesion between leukocyte cells and endothelial cells[22]. This adhesion complex allows migration of leukocytes to the site of infection[23].

Inhibition on CD18/11a expression by *C. aurantifolia* and *A. galangal* extract might be due to the ability of bioactive compounds from the plant extracts to inhibit the intracellular and extracellular mechanisms such as blockade nuclear factor-kappa B activation and pro-inflammatory pathways. In traditional medicine, both of these plants are commonly used as anti-inflammatory and treatment for asthma. It has been reported that bioactive compound of citrus plant decreased COX-2 mRNA expression and also inhibited the gene expression of pro-inflammatory cytokines including interleukin-1α, interleukin-1β, TNF-α and interleukin-6 in mouse[24]. Besides, *A. galangal* extract has the anti-inflammation effect and is able to downregulate interleukin-1β-induced matrix metalloproteinases and COX-2 expression in human synovial fibroblast. This extract has also shown activity in reducing the expression of nuclear factor-kappa B signaling biomarker[25,26].

Phagocytic activity of leukocyte cells is the third step involved in the phagocytosis process[27]. This activity begins when membrane receptor of leukocytes binding to the microbes. The membrane then closes up and pinches off and the microbes are internalized in the membrane-bound vesicle called phagosome[27,28]. The phagocytosis activity is determined as the MFI of intracellular bacteria.

The immunostimulation of *A. muricata* and *O. aristatus* samples on phagocytic activities of leukocytes could be due to the active compound of these plants activating the complement pathway and generating C3b and C3bi which will bind to *E. coli*. Then, this bounded *E. coli* will be recognized by phagocyte receptor such as CR1 (CD35) and CR3 (CD11b) to start the phagocytosis process[29]. Furthermore, there is a probability that the stimulation effect is caused by the direct influence of active compounds from all of these plants which increase the expression of Fcγ receptor on the phagocyte cells and then promotes the binding of opsonized *E. coli* to the receptor. The expression of Fcγ receptor such as FcγRIII (CD16) and FcγRII (CD32) will enhance the phagocytosis process[30,31].

This finding supports the previous studies on bioactivities of the plants used in this study. The ethanolic extract of *A. muricata* demonstrated inhibition of acute and chronic inflammation by using animal model[32,33]. According to previous literature, the main bioactive compound of this plant is acetogenin. This compound is able to stimulate expression of Toll-like receptor 4 on cancer cell, which enhances activity of innate immune cell to clear the target cell[34]. This compound has also exhibited anti-inflammatory activities by decreasing level of pro-inflammatory cytokines, such as

tumor necrosis factor-α and interleukin-6[35].

O. aristatus is commonly used as folk medicine to treat several illnesses. The plant has also been used to enhance immune system. Several studies have reported bioactivities of this plant. The ethanolic extract of *O. aristatus* revealed anti-inflammatory activities by inhibiting nitric oxide production in LPS-stimulated RAW 264.7 cells. Moreover, its bioactive compound like ursolic acid suppresses LPS-induced nitric oxide and PGE2 production by inhibiting reactive oxygen species generation[36].

All the results obtained from this study provide the information about the potential use of several Malaysian medicinal plants for the treatment of immune-related diseases. Our findings demonstrate the activity of active plant extracts in modulating activities of immune cell, such as CD18/11a expression and phagocytosis activity. The active plant extracts possess high potential of being discovered as new therapeutic agents for modulating immune system.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by Ministry of Higher Education, Malaysia (Grant No. GUP-SK-07-23-042) and University Sultan Zainal Abidin (UniSZA) as fellowship provider.

Comments

Background

Natural immune system is the first line of defense mechanism against infection of the body. Plant extracts have been reported as the immune modulator agent owing to the presence of phytochemicals. There is small information on Malaysian medicinal plants with immune modulatory effect. Therefore, it is beneficial information to study the effect of Malaysian medicinal plants on immunomodulation.

Research frontiers

The present research work investigates the immune modulatory effect of various Malaysian medicinal plants methanolic extract. Study was conducted using CD18/11a expression and phagocytosis activity of leukocytes.

Related reports

Phagocytosis activity and CD18/11a expression are affected during infection, which facilitate defense mechanism. Phytochemical or plant extracts have been known for their immune modulation effects.

Innovations and breakthroughs

Innovative part of this paper belongs to screening of 20 Malaysian medicinal plant methanolic extracts for their immune-modulatory effect using CD18/11a expression and phagocytosis activity of leukocytes.

Applications

This scientific study will support the application of those 20 medicinal plant extracts as per their immune-modulatory effect for particular disease condition.

Peer review

This is a valuable research work in which authors have demonstrated the immune-modulatory effect of 20 Malaysian medicinal plant extracts using CD18/11a expression and phagocytosis activity of leukocytes. Methanolic extracts of *A. galangal*, *O. aristatus* and *A. muricata* are able to modulate innate immune system and give the prospect for discovery the therapeutic agent for modulating immune system.

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