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Immunostimulant Compounds Identification in Indonesian Underutilized *Zingiberaceae* Spices

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ABSTRACT. Spices are known for its health beneficial effect beside its function as flavouring and condiment. This study aimed to screen immunostimulant activities of 12 underutilized species of *Zingiberaceae* grown in Indonesia. The compound associated with immunostimulant activity in the most active sample was identified by linking the chromatogram profile of hexane, chloroform, ethyl acetate, methanol, and water fractions of the selected spices to its activity using orthogonal projection to the least square (OPLS) analysis, followed by LC-MS characterization. The result showed that *Alpinia malaccensis* and *Zingiber ottensii* had the highest lymphocyte cell proliferation index (SI) at concentrations of 400 ppm. *A. malaccensis* was chosen for the next step. The results of OPLS analysis showed that the retention time interval which strongly correlated with the immunostimulant activity was 27.0 – 28.5 minutes, which were abundant in methanol fraction. The mass spectra analysis of methanol fraction using UHPLC-HRMS showed that the respective retention time was attributed to flavokawain B. This compound was reported to increase rat splenocyte proliferation in vivo.

Keywords: *Alpinia malaccensis,* flavokawain B, immunostimulant, lymphocytes proliferation

INTRODUCTION

The immune system's role is to prevent numerous diseases by defending the body against pathogens or other substances, including viruses, bacteria, parasites, foreign proteins, and fungi (Catanzaro et al., 2018). Since the World Health Organization (WHO) declared the Corona virus (COVID-19) a global pandemic on March 12, 2020, the immune system needs to be boosted to prevent infection (Ciotti et al., 2020). Immunostimulants are molecules or compounds that strengthen the immune system. It can be synthesized (Bascones-Martinez et al., 2014) or extracted from natural substances like spices. Studies have shown that spices can provide immunostimulants because they contain various bioactive substances (Safriani et al., 2021).

Spices are used in cooking for various purposes, such as flavoring, preservatives, coloring, and aromatic elements. Scientific evidence of spices' health benefits is essential because they have been used medicinally for centuries (Viuda-Martos et al., 2011). One of the spice families that have the potential to stimulate the immune system is *Zingiberaceae*. Indonesians use the rhizome of many *Zingiberaceae* species as a spice and traditional medicine to treat many illnesses such as headaches, coughs, fever, itching, sore throats, and other problems (Demita et al., 2021).

Many of *Zingiberaceae* spices have medicinal and commercial value due to bioactive compounds such as tannins, flavonoids, alkaloids, and terpenoids (Anjusha & Gangaprasad, 2014). Around 50 genera and about 1500 species belong to this family, and they can be found worldwide, including in Indonesia (John Kress, Prince, & Williams, 2002). Even though the genera are distinct, the morphology of each species is comparable, with mainly functional differences. These spices are aromatic, pungent, and spicy (Ghosh et al., 2011; Rangsuji et al., 2000). Several *Zingiberaceae* species exhibit immunostimulating effects, namely ginger (*Zingiber officinale* Roscoe) (Rungkat et al., 2003) galangal (*Alpinia galanga* Linn) (Bendjeddou et al., 2003), temu mangga (*Curcuma mangga* Val), aromatic ginger (*Kaempferia galanga* L) (Safriani et al., 2021) and turmeric (*Curcuma longa* Linn) (Yue et al., 2010).

The phytochemical agents associated with immunostimulant activity in ginger are gingerol and

shogaol (Rungkat et al., 2003) α-turmerone and ar-turmerone in turmeric (Yue et al., 2010), quercetin in galangal and temu mangga (Jain et al., 2012; Yuandani et al., 2018), and methoxycinnamate in aromatic ginger (Dash et al., 2018). The spices mentioned above are commonly used as seasonings, condiments, and herbal drinks (jamu). However, several *Zingiberaceae* spices remain unexplored, underutilized (limited use as seasoning), and unfamiliar to the Indonesians. Thus, these spices are referred to as underutilized or minor spices. These minor spices are *Zingiber purpureum* Roscoe, *Z. ottensii* Val, *Amomum compactum* Sol. ex Maton, *A. malaccensis* (Burm. f), *Zingiber aromaticum* Val, *Zingiber zerumbet* L. Roscoe ex Sm, *Curcuma zedoria* Roscoe, *Curcuma aeruginosa* Roxb, *Curcuma heyneana* Val. & Zijp, *Boesenbergia rotunda* L. Mansf, *Curcuma petiolata* Roxb and *Curcuma purpurascens* Blume. Research on the immunostimulant effects and identification of bioactive compounds in these minor spices is limited.

Identifying the active components of plant extracts such as spices as immunostimulants is challenging because it requires repeated purification and is time-consuming. Therefore, Yuliana et al. (2020) who was identified the anti-diabetic component of cashew extract by correlating the extract's chromatogram profile with an anti-diabetic and antioxidant activity using orthogonal projection to least squares analysis (OPLS). It allows for rapid identification of the active component. Thus, this study aimed to screen 12 Indonesian underutilized *Zingiberaceae* species that have the potential as immunostimulants based on the lymphocyte cell proliferation test, where the ability to stimulate lymphocyte cell proliferation is expressed as the Stimulation Index (SI) value. Then, the selected spice with the highest stimulation index will be fractionated. The fractions were tested for lymphocyte proliferation and chemical profile analysis using High-Performance Liquid Chromatography (HPLC). Furthermore, chemical profile data in retention time intervals from HPLC and activity data in lymphocyte stimulation index were correlated using analysis OPLS. It aims to find retention time intervals positively correlated with immunostimulant activity. The peaks of compounds in the retention time of OPLS, which are potential immunostimulants, were identified using Ultra High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-HRMS).

EXPERIMENTAL SECTION

Sample Preparation

The twelve spices were collected from the Indonesian Spice and Medicine Research Institute (BALITRO) experimental garden in Bogor, Indonesia. Dedi Rosadi from Indonesian Spice and Medicine Research Institute identified the spices. **Table 1** shows both the local and scientific names of these spices. Samples were collected fresh, sorted, washed, and thinly sliced, then immediately stored in the freezer at a temperature of -20 °C. For the bioactivity screening, samples were dried in a freeze-dryer for 72 hours and, while for bioactive compound identification, the chosen spice was dried using a fluidized bed dryer for 4 hours at 45 °C. All spices were ground into a fine powder, and stored in the freezer.

Sample Extraction for Bioactivity Test

Sample extraction process followed the method described by Yuliana et al. (2020) twenty grams of spice powder were added with 80% methanol at twice of the powder's volume. It was sonicated using an Ultrasonic Bransonic Cleaner 8510E MTH USA for 20 minutes at room temperature. The extract was filtered, and the residue left on the filter was re-sonicated with 80% methanol. The filtrate was collected, and the solvent was evaporated until a dry extract was obtained using a rotary evaporator (Buchi Rotavapor R-300 Buchi Labortechnik Switzerland) at 40 °C.

Table 1. Names and used parts of 12 minor Indonesian spices of the *Zingiberaceae* family

No.	Scientific names	Local names	Used parts
1	*Zingiber purpureum* Roscoe	Bangle	Rhizome
2	*Zingiber ottensii* Val	Bangle hitam	Rhizome
3	*Alpinia malaccensis* (Burm.f.)	Laja gowah	Rhizome
4	*Zingiber zerumbet* L. Roscoe ex Sm	Lempuyang	Rhizome
5	*Zingiber aromaticum* Val	Lempuyang wangi	Rhizome
6	*Curcuma zedoria* Roscoe	Temu putih	Rhizome
7	*Curcuma aeruginosa* Roxb	Temu hitam	Rhizome
8	*Boesenbergia rotunda* L. Mansf	Temu kunci	Rhizome
9	*Curcuma petiolata* Roxb	Temu putri	Rhizome
10	*Curcuma heyneana* Val. & Zijp	Temu giring	Rhizome
11	*Curcuma purpurascens* Blume	Temu tis	Rhizome
12	*Amomum compactum* Sol. ex Maton	Kapulaga	Fruit

Sample Fractionation for Bioactivity Testing and Chemical Profiling using HPLC

The screening-selected sample with the highest immunostimulant activity was subjected to multi-solvent fractionation using Soxhlet method. Five solvents are used in fractionation, i.e. *n*-hexane, chloroform, ethyl acetate, methanol, and water. The fractionation process begins with 20 grams of spice powder wrapped in filter paper and placed in a Soxhlet apparatus. The boiling flask was filled with 175 mL of hexane solvent and extracted for about 2 hours. The *n*-hexane solvent that was mixed with the extract was separated first using the soxhlet system (distilled the solvent was in the soxhlet flask and collected; furthermore, the soxhlet flask containing the extract was put in an oven at 105 °C for 5 minutes). Then the sample extract was dried using nitrogen gas. After that, the spice powder of hexane fractions was reused to obtain chloroform, ethyl acetate, and methanol fractions. The process of extraction used hexane, methanol, chloroform, ethyl acetate, and methanol solvents for 2 hours using the Soxhlet method. The water fraction was extracted using a sonicator. The spice powder from the methanol fraction was put in an erlenmeyer and mixed with distilled water. Then it was sonicated for 20 minutes using an ultrasonic bath at room temperature. The filtrate was then collected. The solvent was evaporated using a rotary evaporator at 40–50 °C for 2 hours. Then, the sample extract was dried using nitrogen gas. All the extraction processes were performed six times for each fraction.

Isolation of Lymphocyte Cells

The IPB University's Research Ethics Commission approved this study for ethical reasons with number 489/IT3. KEPMSM-IPB/SK/2021. Lymphocytes were isolated using the method described by Erniati et al. (2020) with the number of blood samples modified. The peripheral blood was aseptically collected up to 20 mL from a healthy adult human female who signed a consent form and put into a vacutainer containing ethylenediaminetetraacetic acid. The blood was immediately transferred to a sterile tube and centrifuged at 1500 rpm for 10 minutes. The obtained buffy coat was diluted with phosphate-buffered saline (PBS) (Sigma-Aldrich), then put into a centrifuge tube containing a ficoll-hypaque solution (Sigma-Aldrich) carefully to form a layer, then centrifuged at 2700 rpm for 30 minutes. The upper suspension was diluted with PBS and centrifuged at 2000 rpm for 15 minutes to obtain cell pellets. These were washed with PBS, and the cell viability was calculated by adding 10 μL of trypan blue to 10 μL of lymphocyte cell suspension, followed by counting with a hemacytometer under a microscope. The cells were then diluted with Roswell Park Memorial Institute (RPMI-1640) (Sigma-Aldrich) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin to achieve a density of 1 x 10^5 cells/mL. The cell suspension was used to determine the stimulation index (SI), which had a viability ≥ 95%.

Lymphocyte Proliferative Activity Analysis

The lymphocyte proliferative activity test is based on the procedure of Erniati et al. (2020) with several modifications. A total of 80 μL of lymphocyte cell suspension (1 x 10^5 cells/mL per well) was added to 96-well plates and incubated for 24 hours at 37°C and 5% CO_2. After 24 hours of incubation, 20 μL of the spices extract with concentrations of 50 ppm, 100 ppm, 200 ppm, and 400 ppm was added to each well. In the other wells, lipopolysaccharide (LPS) and RPMI media were added as positive and negative controls, respectively. All preparations were incubated for 48 hours at 5% CO_2 and 37 °C. Four hours before incubation ended, 10 μl (5 mg/ml) MTT was added to each culture. After incubation, 100 μL of ethanol (p.a) was added to each well, and absorbance was measured using an Elisa Reader (Biogen) at 595 nm. The obtained optical density (OD) value is used to calculate the Stimulation Index (SI) value, which indicates proliferative activity, using the following equation:

$$\text{Stimulation Index (SI)} = \frac{\text{OD treatment cell}}{\text{OD negative control cell}}$$

HPLC Analysis

HPLC conditions followed Ma et al. (2012) with wavelength and gradient elution optimized. All fractions of selected spice (n-hexane, chloroform, ethyl acetate, methanol, and water) were dissolved and filtered through a 0.22 mm PTFE syringe filter. The HPLC-VWD (Variable Wavelength Detector) Agilent 1200 Series instrument was used, and the column was C18 with specifications (5μm, 4.6 mm x 150 mm), with a solvent flow rate of 1 ml/minute. The gradient elution times for mobile phase A (water and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid) were as follows: 0 - 1 minute (95% A: 5% B), 1 - 25 minutes (5% A: 95% B), 25 - 28 minutes (5% A: 95% B), and 28 - 30 minutes (95% A: 5% B). The injection volume was 2.5 μL and the wavelength was 254 nm. HPLC output is a chromatogram with peaks of compounds at a specific retention time. Each peak had relative area% data. It was converted into retention time intervals and tabulated in excel.

UHPLC-HRMS Analysis

The active fraction with the highest lymphocyte proliferative activity was tested with UHPLC-HRMS. The UHPLC-HRMS conditions followed the method of Barbosa et al. (2020) with some modifications. UHPLC instrument combined with Q Exactive Plus Orbitrap HRMS ThermoScientific. The sample was filtered using a 0.22 mm PTFE syringe filter. The column was a C18 accucore (1.5 μm, 2.1 mm x 100 mm) with a 0.2 mL/min solvent flow rate. Mobile phase A (water and 0.1% formic acid) and mobile phase B (acetonitrile and 0.1% formic acid) with

gradient elution for 30 minutes with the following composition: 0 - 1 minute (95% A: 5% B), 1 - 25 minutes (5% A: 95% B), 25 - 28 minutes (5% A: 95% B), and 28 - 30 minutes (95% A: 5% B). The mass spectral range was 100 - 1500 m/z, the ionization mode was positive and negative, and the injection volume was 2.5 μL.

Statistical Analysis

The immunostimulant activity of spices extract was analyzed using two-way variance analysis (ANOVA), followed by the DMRT (Duncan's Multiple Range Test) at a level of 5% using SPSS® version 25. Chemical profile data and immunostimulant activity were correlated for compounds identified using multivariate data analysis (PCA and OPLS with SIMCA version 16). The peak of interest was chosen based on variable to the projection (VIP) and coefficient correlation. The quality of multivariate data analysis models was assessed by the value of R^2X, R^2Y, Q^2, CV-ANOVA and permutation test.

RESULTS AND DISCUSSION

Lymphocyte Proliferation Activity of 12 Minor Spices *Zingiberaceae*

The comparison of SI values of 12 *Zingiberaceae* minor spice extracts of various concentrations normalized in control and mitogen wells (LPS) is shown in **Figure 1**. At 400 ppm, extracts of *A. malaccensis* and *Z. ottensii* had the potential as immunostimulants because they had the highest SI values of 2.42 ± 0,17 and 2.37 ± 0.97, respectively, both of which were not statistically significant, as indicated by the small letters in **Figure 1**. Both extracts at 400 ppm had SI values significantly different from those at 200 ppm, 100 ppm, and 50 ppm due to the observed p value being 0.000 less than the significance level of 0.05, as indicated by the capital letters (**Figure 1**). SI values of both spices increased with concentration. They were higher than LPS (the positive control) and RPMI (the negative control), indicating this concentration has high proliferative activity and is not toxic to cells. This result aligns with Jain et al. (2012) study, which found galangal (*Alpinia galanga*) extract concentrations of 100 ppm and 200 ppm also increased rat splenocyte stimulation index to 1.90 and 2.12, respectively. Moreover, this trend fits with the LPS trend of increasing SI values. LPS is an endotoxin isolated from gram-negative bacterial cell walls that can stimulate the proliferation of human T and B lymphocytes and the production of cytokines (Ulmer et al., 2000).

The crude extract effect of *A. malaccensis* and *Z. ottensii* on lymphocyte cell proliferation visually seen with a microscope with 400 times magnification is shown in **Figure 2**. In **Figure 2A**, it can be seen that cells cultured without the addition of extract (negative control) have lower cell density lymphocytes compared to those cultured with *A. malaccensis* and *Z. ottensii* extracts (**Figure 2A** and **Figure 2B**). It shows that the added extracts of *A. malaccensis* and *Z. ottensii* can increase lymphocyte cell proliferation.

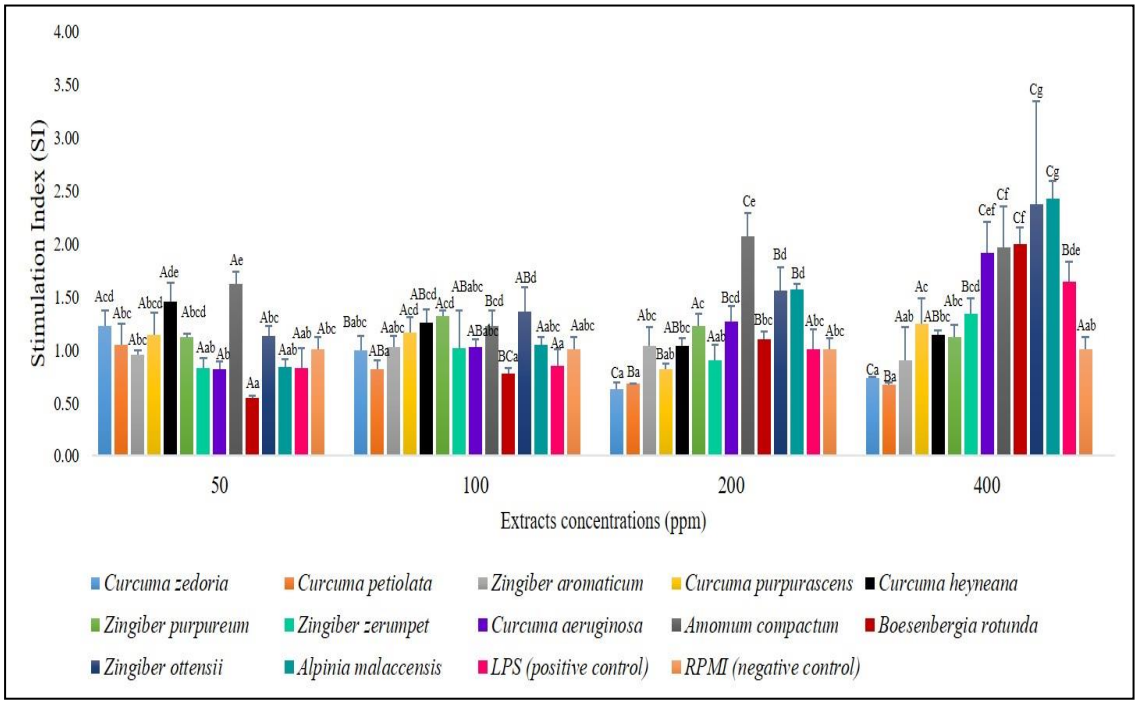
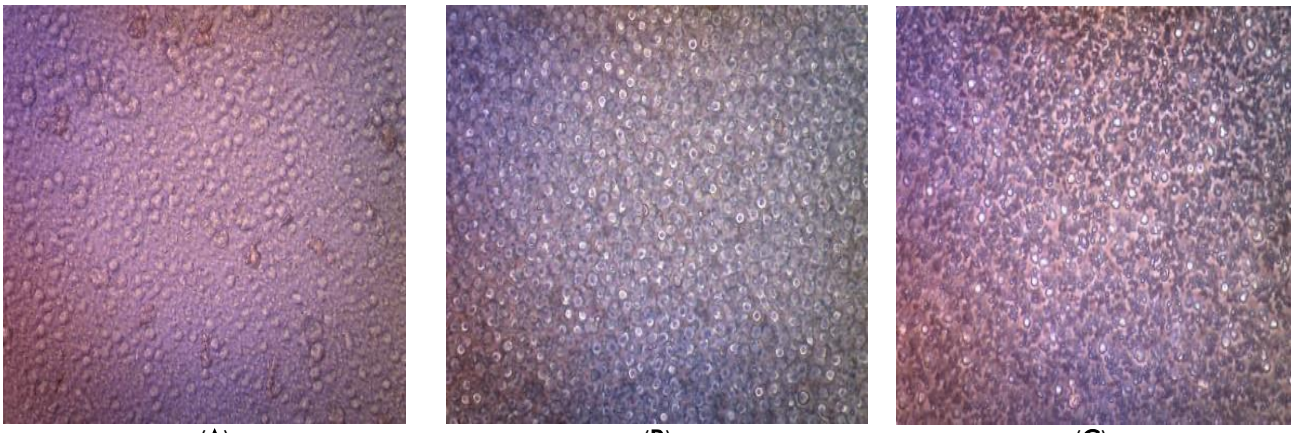


Figure 1. The effect of 12 Indonesian minor spice of Zingiberaceae crude extracts and concentrations on the value of the human lymphocyte stimulation index. *value = mean ± SD, n =3. The difference in capital letters on the graph represents the effect of concentrations significantly different at a significance level of 0.05. The difference in small letters on the graph indicates that the type of crude extract differs significantly at the 0.05 significance level.



(A) (B) (C)

Figure 2. Lymphocyte density in (A) cell culture with no extract added (negative control), (B) cell culture with 400 ppm of *A.malaccensis* crude extract, and (C) cell culture with 400 ppm of *Z.ottensii* crude extract.

The effect of lymphocyte cell proliferation after treatment with spices extracts is assumed to be influenced by various phytochemicals present in extracts, including carotenoids (curcumin, lutein) (Rajput et al., 2013) phenolics (p-coumaric acid, ferulic acid) (Chiang et al., 2003) and flavonoids (quercetin) (Cherng et al., 2008). The UHPLC-HRMS results (**Tabel 2**) of *A. malaccensis* and *Z. ottensii* crude extracts showed the presence of various phytochemical components from the flavonoids, carotenoids, amino acids, terpenoids, phenolic groups and so on. These compounds possibly played a role in the activity of lymphocyte cell proliferation. The mitogen attachment, such as LPS, to the surface of lymphocyte cells, was proposed as a mechanism of lymphocyte activation by bioactive components in spices. The bioactive components might function as mitogen ligands for T and B cell surface receptors (Gomez-Flores et al., 2008). Furthermore, the process activates signal transduction through second messengers such as inositol triphosphate and continues the release of Ca2+ into the cytoplasm, causing the Ca2+ concentration to rise. Increasing Ca2+ stimulates protein kinase C to induce cellular gene expression, such as interleukin-2 (IL-2) gene expression. The expression of these genes stimulates IL-2 production. IL-2 promotes T and B cell proliferation (Oh-hora & Rao, 2008).

Most spices showed increased activity with increasing concentration, except for some such as C. *zedoria* and C. *petiolate* (**Figure 1**). The stimulation index of both spices decreased by an increase in spice concentration. It indicated that it could suppress immune cell activity (immunosuppressive). This finding is supported by Yadav et al. (2005) who reported that curcumin from C. *longa* inhibited phytohemagglutin (PHA) induced lymphocyte cell proliferation and has immunosuppressive potential (suppressing immune cell activity). Curcumin might suppress cell proliferation by inhibiting ribonucleotide reductase and activating DNA polymerase, a key enzyme in DNA synthesis.

Table 2. Compounds of *A. malaccensis* and *Z. ottensii* crude extracts using 80% methanol with UHPLC-HRMS

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
					A.malaccensis					
1	Sakuranetin	$C_{16}H_{14}O_5$	10.18	[M+H]+	286.08	287.09	287.10	-2.13	167, 147	Flavonoid
2	Betaine	$C_5H_{11}NO_2$	1.08	[M+H]+	117.08	118.08	118.08	0.68	59, 58, 56	Amino acid
3	Methyl cinnamate	$C_{10}H_{10}O_2$	11.57	[M+H]+	162.07	163.07	163.07	-2.03	131, 103	Cinnamic acid esters
4	Choline	$C_5H_{13}NO$	1.04	[M+H]+	103.10	104.10	104.00	1.59	60	Vitamin
5	Arginine	$C_6H_{14}N_4O_2$	1.05	[M+H]+	174.11	175.11	175.12	-1.55	130, 116, 70 , 60	Amino acid
6	Zerumbone	$C_{15}H_{22}O$	14.32	[M+H]+	218.17	219.17	219.17	-2.42	151, 123, 109, 81, 69	Sesquiterpenoid
7	Isoleucine	$C_6H_{13}NO_2$	1.29	[M+H]+	131.09	132.10	132.10	-1.1	86	Amino acid
8	Guanidinobutyric acid	$C_5H_{11}N_3O_2$	1.09	[M+H]+	145.08	146.09	146.00	-1.44	128, 104, 87, 86	Amino acid
9	Desmethoxyyangonin	$C_{14}H_{12}O_3$	11.01	[M+H]+	228.08	229.08	229.08	-3.47	201, 141, 131, 103	Terpenoid
10	Benzocaine	$C_9H_{11}NO_2$	1.45	[M+H]+	165.08	166.08	166.08	-0.72	167, 147	Para-amino benzoic acid
11	Carvone	$C_{10}H_{14}O$	14.32	[M+H]+	150.10	151.11	151.11	-1.33	123, 109	Monoterpenoid
12	Pipecolic acid	$C_6H_{11}NO_2$	1.10	[M+H]+	129.08	130.09	130.09	-0.21	84	Amino acid

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
					A.malaccensis					
13	Flavokawain B	$C_{17}H_{16}O_4$	14.62	[M+H]+	284.10	285.11	285.00	-3.38	181, 131 103	Flavonoid
14	Phloretin	$C_{15}H_{14}O_5$	9.58	[M+H]+	274.08	275.09	275.09	-3.23	169, 149, 107	Flavonoid
15	Abietatriene	$C_{20}H_{30}$	14.03	[M+H]+	270.23	271.40	270.46	-2.12	229, 215, 201, 147	Diterpenoid
16	Mesuagin	$C_{24}H_{22}O_5$	13.12	[M+H]+	390.15	391.15	391.54	-2.6	191, 187, 155	Flavonoid
17	Gamma-Aminobutyric acid	$C_4H_9NO_2$	1.06	[M+H]+	103.06	104.10	104.00	2.12	60	Amino acid
18	Cinnamic acid	$C_9H_8O_2$	8.95	[M+H]+	148.05	149.05	149.05	-1.23	131, 121, 103	Phenolic acid
19	Alpha-Farnesene	$C_{15}H_{24}$	14.80	[M+H]+	204.19	205.19	205.19	-1.46	149, 121, 109, 95	Sesquiterpenoid
20	Norethynodrel	$C_{20}H_{26}O_2$	17.21	[M+H]+	298.19	299.19	299.20	-2.44	256, 239, 157, 143	Steroid
21	DL-Malic acid	$C_4H_6O_5$	1.11	[M-H]-	134.02	133.01	133.22	-8.05	115, 89, 71	Phenolic acid
22	Methylmalonic acid	$C_4H_6O_4$	1.29	[M-H]-	118.03	117.02	117	-9.3	73	Phenolic acid
23	9,12,13-Trihydroxy-10-octadecenoic acid	$C_{18}H_{34}O_5$	9.55	[M-H]-	330.24	329.23	329.24	-0.68	211, 171	Fatty acid

24	2',4'-Dihydroxy-4-methoxychalcone	$C_{16}H_{14}O_4$	12.45	[M-H]-	270.09	269.08	269	-1.78	254, 227, 226, 225	Flavonoid

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
					Z.ottensii					
25	Kaempferol	$C_{15}H_{10}O_6$	7.71	[M+H]+	286.05	287.05	287.05	-2.85	287, 153, 121	Flavonoid
26	Pipecolic acid	$C_6H_{11}NO_2$	1.12	[M+H]+	129.08	130.08	130.08	-0.33	84	Amino acid
27	Isoleucine	$C_6H_{13}NO_2$	1.29	[M+H]+	131.09	132.1	132.1	-1.45	86, 69	Amino acid
28	Mesuagin	$C_{24}H_{22}O_5$	13.12	[M+H]+	390.15	391.15	391.54	-2.6	191, 187, 155	Flavonoid
29	Gamma-Aminobutyric acid	$C_4H_9NO_2$	1.06	[M+H]+	103.06	104.10	104.00	2.12	60	Asam amino
30	Pipecolic acid	$C_6H_{11}NO_2$	1.12	[M+H]+	129.08	130.08	130.08	-0.33	84	Amino acid
31	Isoleucine	$C_6H_{13}NO_2$	1.29	[M+H]+	131.09	132.1	132.1	-1.45	86, 69	Amino acid
32	Proline	$C_5H_9NO_2$	1.09	[M+H]+	115.06	116.07	116.07	0.71	70	Amino acid
33	Valine	$C_5H_{11}NO_2$	1.12	[M+H]+	117.08	118.08	118.08	0.41	72	Amino acid
34	Choline	$C_5H_{13}NO$	1.05	[M+H]+	103.10	104.1	104	1.52	60	Vitamin
35	L-Tyrosine	$C_9H_{11}NO_3$	1.28	[M+H]+	181.07	182.08	182.08	-0.55	165, 136, 123, 147	Amino acid
36	L-Phenylalanine	$C_9H_{11}NO_2$	1.48	[M+H]+	165.08	166.08	166.08	-1.18	120	Amino acid
37	Zerumbone	$C_{15}H_{22}O$	14.38	[M+H]+	218.17	219.17	219.17	-2.7	201, 163, 151, 123	Sesquiterpenoid
38	Afzelin	$C_{21}H_{20}O_{10}$	7.71	[M+H]+	432.10	433.11	433.11	-2.1	287, 129	Flavonoid

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
					Z.ottensii					
39	Isokaempferide	$C_{16}H_{12}O_6$	9.80	[M+H]+	300.06	301.07	301.07	-2.09	286	Flavonoid
40	DL-Tryptophan	$C_{11}H_{12}N_2O_2$	4.18	[M+H]+	204.09	205.09	205.1	-1.13	188, 159, 146, 149	Amino acid
41	9-Oxo-ODE	$C_{18}H_{30}O_3$	14.53	[M+H]+	294.22	295.22	295.22	-2.06	277, 151, 95, 81	Fatty acid
42	4-Guanidinobutyric acid	$C_5H_{11}N_3O_2$	1.11	[M+H]+	145.09	146.09	146	-0.71	146, 86, 87	Amino acid
43	Hypoxanthine	$C_5H_4N_4O$	1.25	[M+H]+	136.04	137.04	137.04	-1.54	136	Nucleotide
44	Caryophyllene oxide	$C_{15}H_{24}O$	15.09	[M+H]+	220.18	221.18	221.19	-2.1	203, 147, 123, 109,	Sesquiterpenoid
45	Pulegone	$C_{10}H_{16}O$	5.32	[M+H]+	152.12	153.12	153.12	-1.48	135, 113, 107, 93, 81	Monoterpenoid
46	Trans-3-Indoleacrylic acid	$C_{11}H_9NO_2$	4.17	[M+H]+	187.06	188.07	188.08	-0.83	146, 133, 118, 105	Indol

47	Proline	$C_5H_9NO_2$	1.09	[M+H]+	115.06	116.07	116.07	0.71	70	Amino acid
48	4"-O-Acetylafzelin	$C_{23}H_{22}O_{11}$	8.55	[M+H]+	474.11	475.12	475.12	-2.57	287, 189, 171, 129	Flavonoid
49	Thymol	$C_{10}H_{14}O$	14.32	[M+H]+	150.10	151.11	151.63	-1.74	149, 121, 107, 93, 81	Monoterpenoid

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
						Z.ottensii				
50	Hexadecanoylpyrrolidine	$C_{20}H_{39}NO$	19.35	[M+H]+	309.30	310.3	310.31	-3.24	275, 149, 135, 111, 83	N-acylpyrrolidines.
52	Curcumene	$C_{15}H_{22}$	14.02	[M+H]+	202.17	203.17	203.17	-1.88	163, 147, 133, 119,	Sesquiterpenoid
53	Demethylphylloquinone	$C_{30}H_{44}O_2$	14.32	[M+H]+	436.33	437.34	437.34	-1.62	219, 201, 163, 151, 123	Vitamin k
54	Alpha-Farnesene	$C_{15}H_{24}$	10.44	[M+H]+	204.19	205.19	205.19	-1.39	149, 135, 123, 109	Sesquiterpenoid
55	Cynarine	$C_{25}H_{24}O_{12}$	9.89	[M+H]+	516.13	517.13	517.13	-2.06	153 ; 111 ; 83	Quinic acids
56	3,4-Dihydrocadalene	$C_{15}H_{20}$	14.32	[M+H]+	200.16	201.16	201.16	-0.79	173, 159, 145, 131	Sesquiterpenoid
57	Valerophenone	$C_{11}H_{14}O$	14.32	[M+H]+	162.10	163.11	163.11	-1.23	145, 121, 105, 107	Ketones
58	P-cymene	$C_{10}H_{14}$	6.59	[M+H]+	134.11	135.11	135.11	-0.95	107, 93, 91, 79	Monoterpenoid
59	Alpha-Farnesene	$C_{15}H_{24}$	15.05	[M+H]+	204.19	205.19	205.19	-1.39	149, 135, 123, 109, 95	Sesquiterpenoid
60	P-Cresol	C_7H_8O	14.32	[M+H]+	108.06	109.06	109	1.32	109, 91, 81, 79, 67	Phenol

No	Compounds	Molecular Formula	RT (min)	Ionization Mode (+/-)	Molecular weight	Observed (m/z)	Theoretical (m/z)	Mass error (ppm)	MS/MS Product Ions	Group
						Z.ottensii				
61	Alpha-Curcumene	$C_{15}H_{22}$	15.55	[M+H]+	202.17	203.17	203.17	-1.88	147, 133, 121, 107, 95	Sesquiterpenoid
62	Secoisolariciresinol	$C_{20}H_{26}O_6$	7.81	[M+H]+	362.17	363.17	363.17	-2.55	163, 149, 137, 123	Lignans
63	Citric acid	$C_6H_8O_7$	1.20	[M-H]-	192.03	191.01	191	-3.79	111, 87, 85	Phenolic acid
64	Malic acid	$C_4H_6O_5$	1.11	[M-H]-	134.02	133.01	133	-8.27	115, 71	Phenolic acid
65	Fumaric acid	$C_4H_4O_4$	1.13	[M-H]-	116.01	115	115	-9.25	71	Phenolic acid
66	Methylmalonic acid	$C_4H_6O_4$	1.18	[M-H]-	118.03	117.01	117	-9.04	99, 73	Phenolic acid
67	D-Glucose	$C_6H_{12}O_6$	1.07	[M-H]-	180.06	179.05	179.05	-5.03	101, 89, 71, 59	Monosaccharide

Effects of *A. malaccensis* fractions on Lymphocyte Proliferation

Based on the screening results, *A. malaccensis* and *Z. ottensii* extracts had the highest SI values at 400 ppm, which were not significantly different based on the DMRT test (**Figure 1**). However, *A. malaccensis* was chosen to proceed to the fractionation stage because it is more readily available and has a more fragrant aroma than *Z. ottensii*. The spice was fractionated using the soxhlet extraction method. This method has been widely used for various solid samples, particularly spice samples. Boothapandi and Ramanibai (2016) extracted the *Fabaceae* family's tarum leaves (*Indigofera tinctoria*) using ethanol as a solvent and the soxhlet method. Lymphocyte proliferation activity was higher in the Soxhlet ethanol extract than in the maceration method.

Fractionation of *A. malaccensis* using n-hexane, chloroform, ethyl acetate, methanol, and water-separated spices extracts based on the polarity. **Figure 3** showed the methanol fraction had the highest SI value of 1.20 ± 0.15 and was significantly different from the other fractions ($p<0.05$). Consequently, the hexane, chloroform, and ethyl acetate fractions were inactive because the SI value was lower than the RPMI (negative control). Meanwhile, the water and methanol fractions were active because the SI value was higher than the RPMI and close to the SI of the LPS value.

Principal Component Analysis (PCA)

PCA aims to identify class differences multivariate data sets, such as differences in a sample's chemical profile spectra (Worley & Powers, 2016). PCA was used to observe separation patterns and assess modeling on multivariate data. The PCA score plot (**Figure 4**) separated each fraction based on chemical profile polarity (retention time interval data). The polar water fractions (A) were clustered (on the right corner of the plot) and well separated from the non-polar hexane (H) fraction (on the left). The hexane (H), chloroform (K), and ethyl acetate (EA) fractions were also well separated. Consequently, all fractions were separated and grouped based on their polarity. The PCA model validity is determined by the value of R^2X (model validity) and the value of Q^2 (the ability to predict the model with actual data) (Eriksson et al., 2013). The R^2X and Q^2 value obtained is 0.74 and 0.53, respectively. Both values are above 0.4, indicating that models perform well in presenting data (Eriksson et al., 2005). The results of PCA indicate that the model can be analyzed to the OPLS, another type of multivariate data.

Orthogonal Projection to Least Square Analysis (OPLS)

The OPLS correlates both data metabolite chemical profiles and bioactivity data (Yuliana et al., 2011). The chemical profile consisted of retention time intervals obtained from HPLC analysis and bioactivity data consisted of immunostimulant activity (lymphocyte stimulation index value, SI). The OPLS score plot (**Figure 5A**) showed the grouping of fractions based on immunostimulant activity. Active fractions are methanol (M) and water (A), represented by red circles in the right quadrant, while inactive fractions are hexane (H), chloroform (K), and ethyl acetate (EA), represented by green circles in the left quadrant. The dominant retention time interval in the active and inactive fractions is represented in plot S (**Figure 5B**). The position of the retention time interval corresponds to the plot score. The further to the right, the more active the fraction. The retention time interval was positively correlated with immunostimulant activity in the right quadrant (methanol fraction) and negatively correlated in the left quadrant (inactive fractions). In contrast, there was no correlation between immunostimulant activity and retention time intervals nearing the quadrant's center.

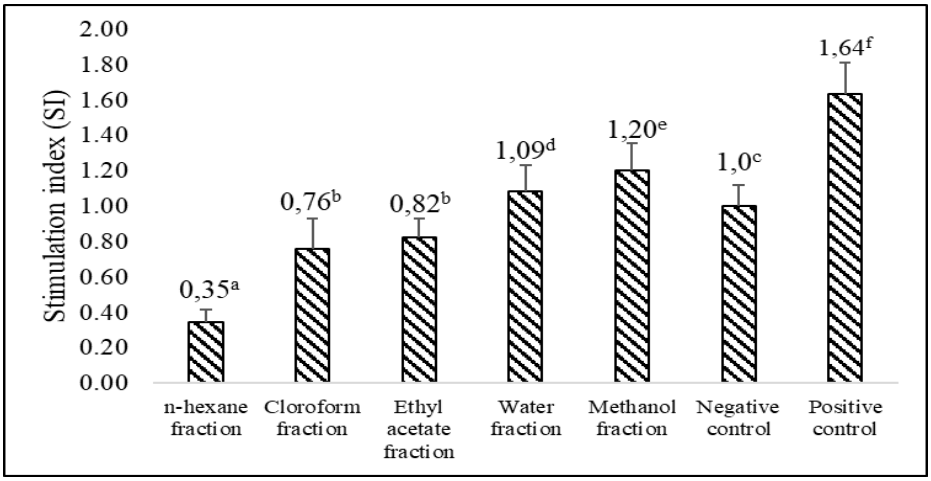


Figure 3. Lymphocyte proliferation of *A. malaccensis* fractions. *According to the DMRT test, the letter differences were statistically significant ($p < 0.05$)

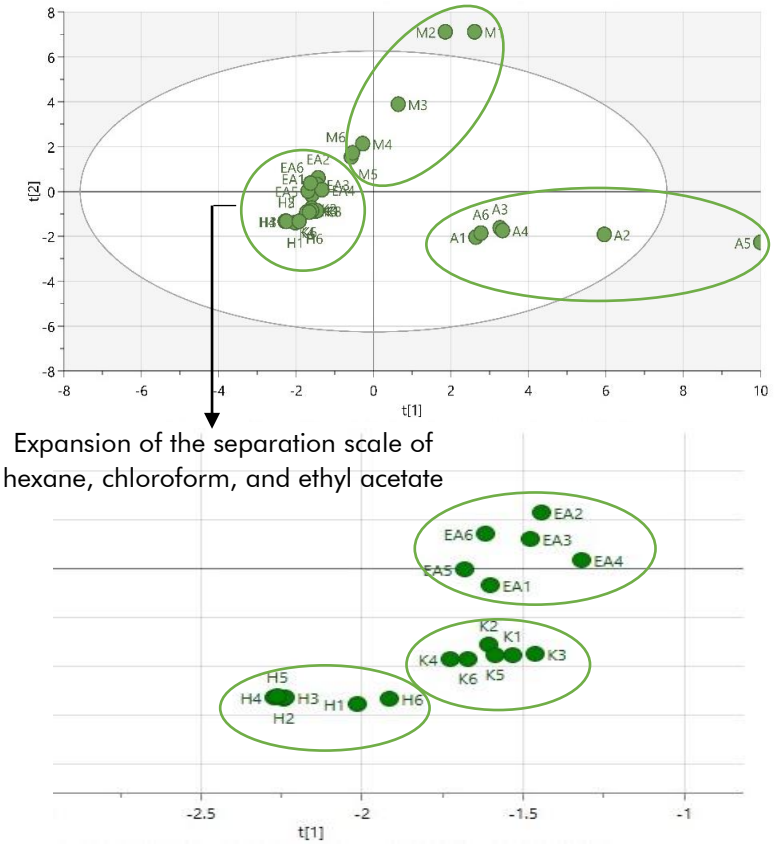


Figure 4. PCA Score Plot (A: water fraction, M: methanol fraction, EA: ethyl acetate fraction, K: chloroform fraction, H: hexane fraction). The number after the letter representing replication

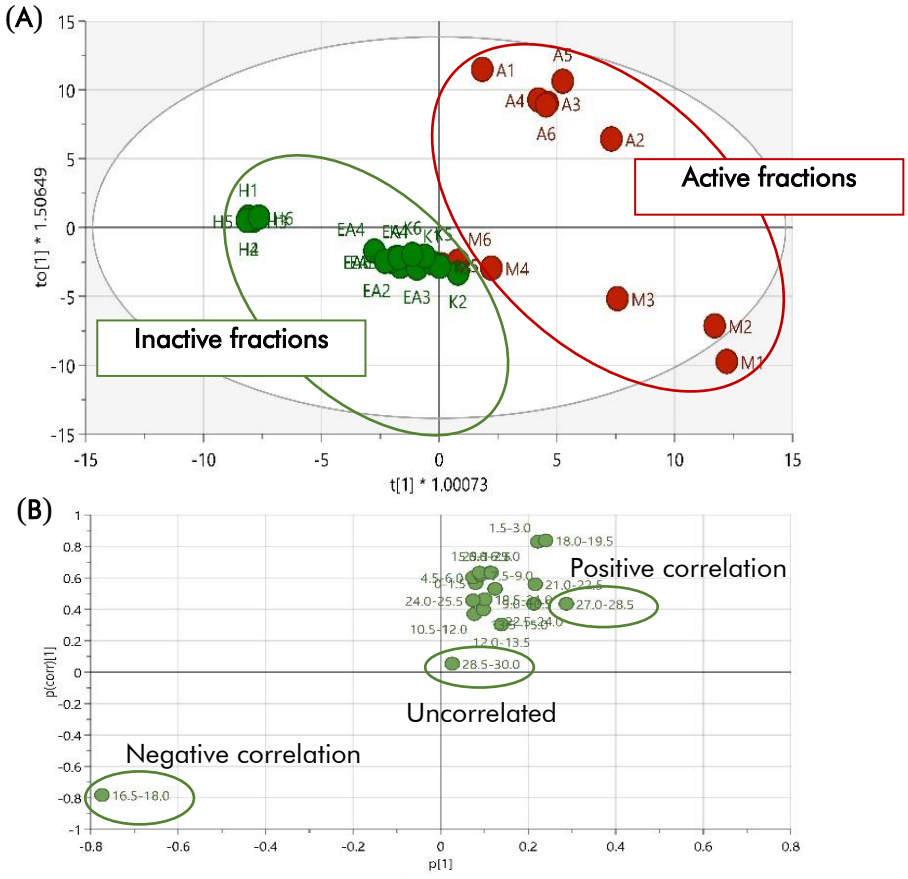


Figure 5. Output of OPLS (**A**) Score plot; (**B**) S-plot

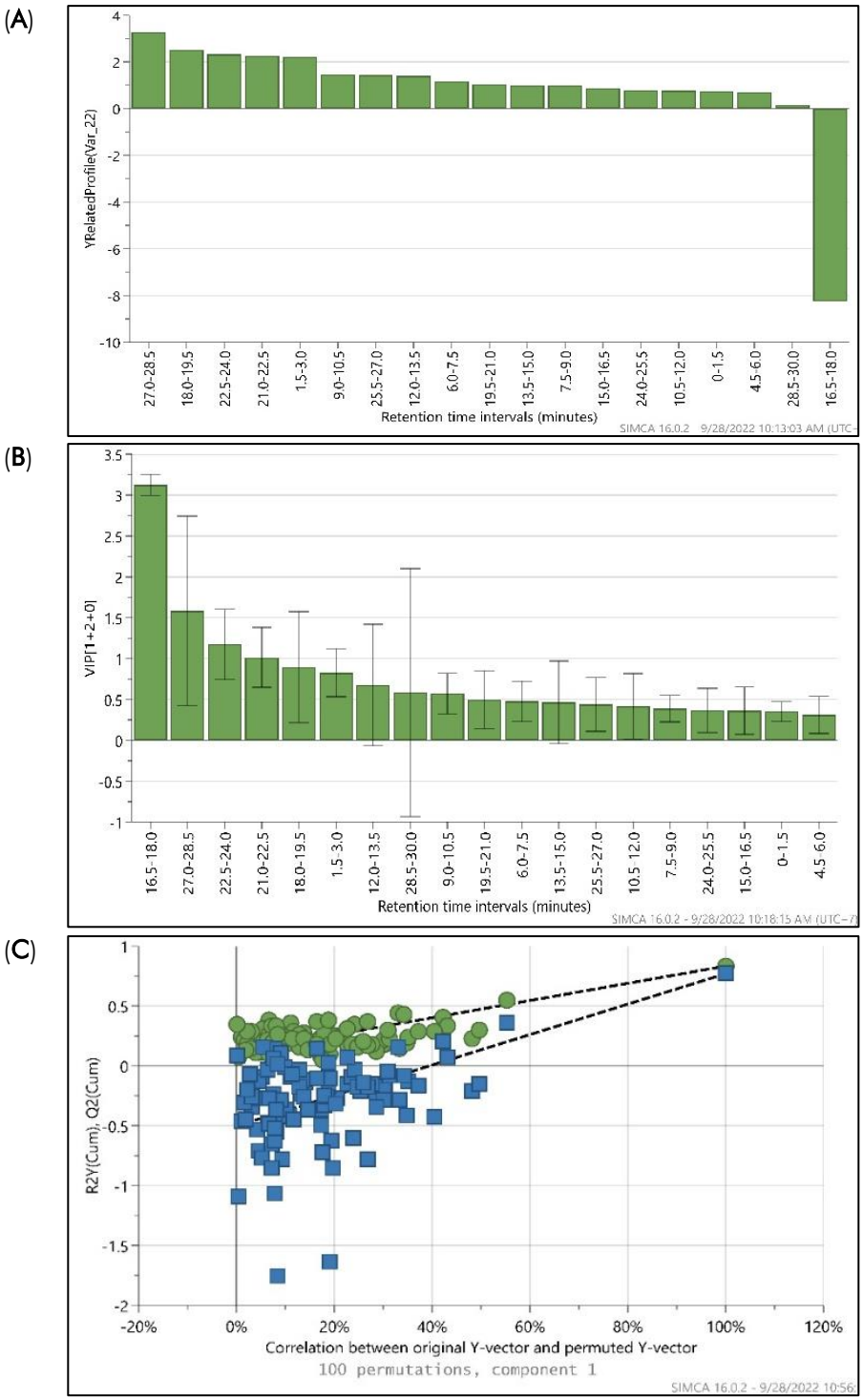


Figure 6. Output of OPLS (**A**) The Y-related coefficient plot; (**B**) VIP Plot; (**C**) Permutation plot

The Y-related coefficient plot (**Figure 6A**) indicated that the retention time interval and immunostimulant activity were positively correlated. The highest value, the strongest correlation with the activity. This data analysis used a positive value because of increasing immunostimulant activity, so the intensity of the compound is also high. Both data have a close relationship. The retention time range of 27.0 to 28.5 minutes has the highest Y-related coefficient, which is 3.26. It has the strongest positive correlation with immunostimulant activity. In addition to the Y-related coefficient plot, the VIP plot (**Figure 6B**) was used to obtain valid data. The VIP plot examines the effect of retention time intervals in grouping samples based on activity. The VIP value chosen is ≥ 1, and the error bar does not touch the X-axis (Worley & Powers, 2016). The highest VIP value (**Figure 6B**) was also obtained from the retention time interval of 27.0–28.5 minutes, which was 1.58. The VIP plot's retention time interval of 16.5–18.0 was disregarded

due to the low Y-correlated coefficient value. Retention intervals positively correlated with activity should have high Y-coefficients and VIP values.

The accuracy of the OPLS model was assessed by examining the R^2Y and Q^2Y values. The value of R^2Y is the number of Y variables explained by the model and the model's fit. Meanwhile, the value of Q^2Y is the result of cross-validation and quantitative measurements of both the predicted results and the actual data. The values of R^2Y and Q^2Y are 0.83 and 0.77, respectively. Because it is close to 1, the model obtained is quite good at presenting the data (Eriksson et al., 2013). Permutation plots and the cross-validation analysis of variance (CV-ANOVA) can be used to validate models. The permutation plot (**Figure 6C**) exhibits good model performance because the permutation model's R^2Y and Q^2Y values (the green circle and blue squares in the lower left of the plot) are smaller than the actual model's R^2Y and Q^2Y values (green circles and blue squares in the top right of the plot). Moreover, the CV-ANOVA validation value obtained in this study was 2.405 x 10^{-6}, less than the maximum value ($p < 0.05$). Thus, the OPLS model has good reliability (Eriksson et al., 2008).

Identification of bioactive compounds with immunostimulant potential

Based on OPLS results, the peak retention times of compounds associated with immunostimulant potential ranged between 27.0 and 28.5 minutes. Most of this interval was found abundant in the methanol fraction of *A.malaccensis*, which was the most active fraction (**Figure 3**). The compounds were identified by pattern recognition of HPLC (**Figure 7A**) and UHPLC-HRMS (**Figure 7B**) chromatograms of *A. malaccensis* methanol fraction. The HPLC system of methanol fraction used a longer column (150 mm x 4.6 mm, 5μm) than UHPLC-HRMS (100 mm x 2.1 mm, 1.5 μm), so compounds were retained longer, resulting in a shift in retention time between compounds in both chromatograms. The HPLC peak associated with immunostimulant activity was at a retention time of 27.71 minutes (marked with arrows in (**Figure 7A**). In UHPLC-HRMS, this peak can be attributed to 21.15 minutes retention times (marked with arrows in (**Figure 7B**). The mass spectrum of this peak was shown in **Figure 8**. Based on the fragmentation pattern, the peak was identified as flavokawain B (**Table 3**).

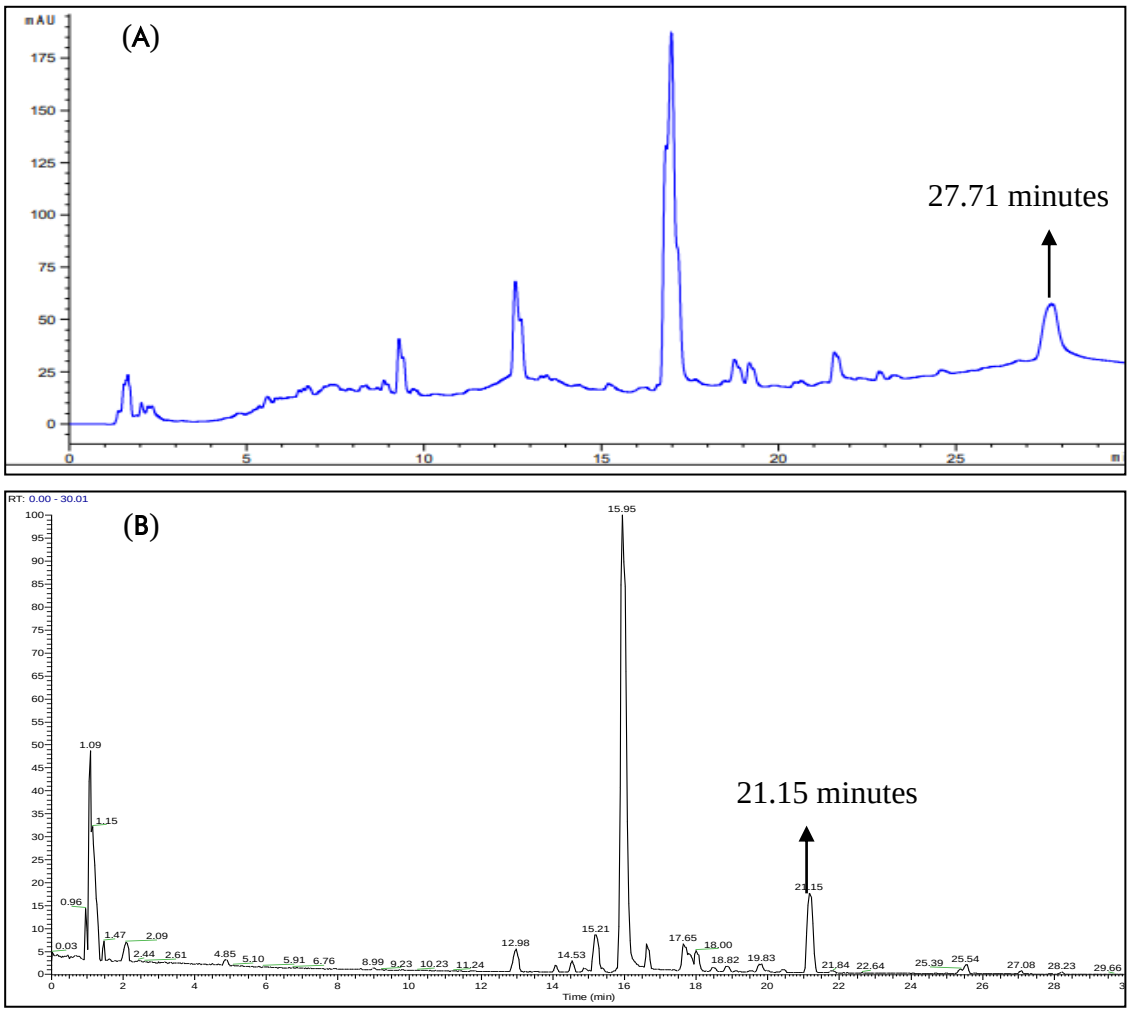


Figure 7. The chromatogram of *A. malaccensis* methanol fractions (**A**) HPLC; (**B**) UHPLC-HRMS

Table 3. Predictive compounds in the methanol fraction and correlated with an immunostimulant activity using UHPLC-HMRS positive ion mode [M+H]+

Retention time (minutes)	Compound	Molecular formula	Molecular weight	Molecular ions	Ions fragments observed (m/z)	Ions fragments databased (m/z)
21.15	Flavokawain B	$C_{17}H_{16}O_4$	284	285	181, 166, 131, 103	181, 166, 131, 103

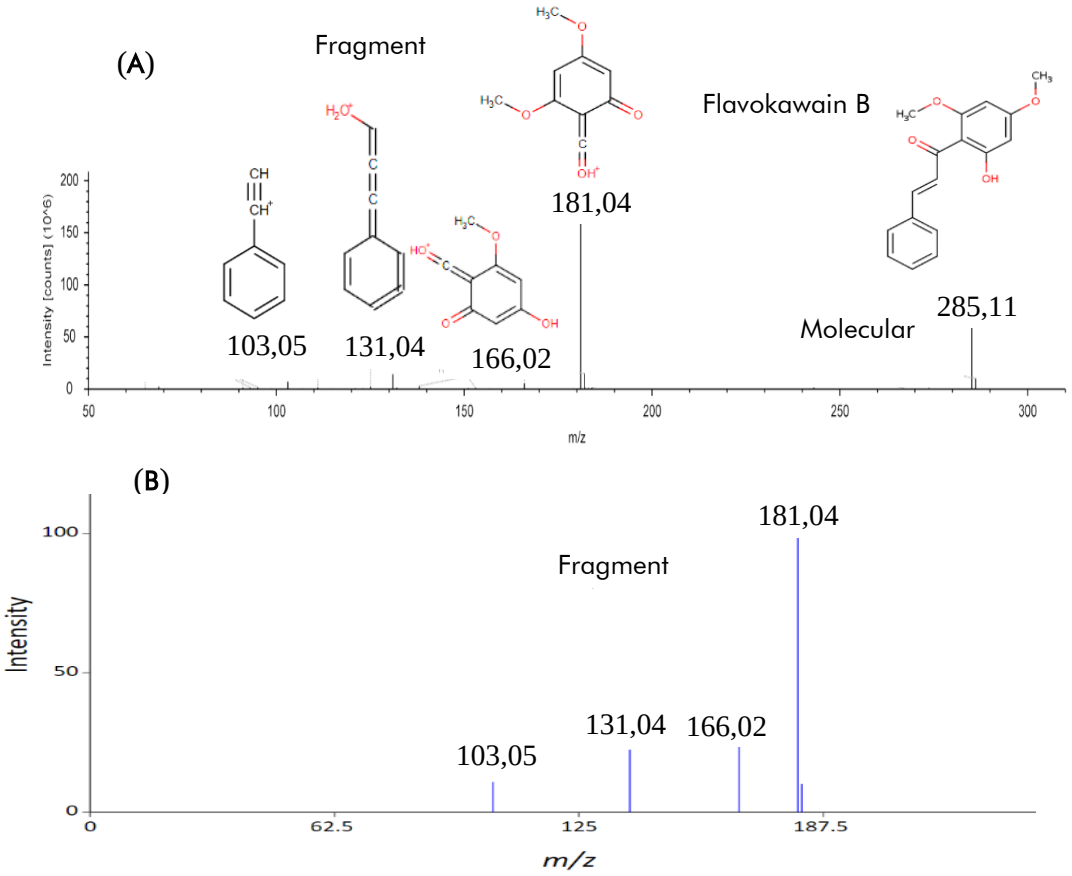


Figure 8. Fragmentation pattern (m/z) of flavokawain B (**A**) observed fragment; (**B**) databased fragment (https://pubchem.-ncbi.nlm.nih.gov/)

Flavokawain B is a compound in the chalcone class (Dharmaratne et al., 2002). Chalcone is a flavonoid derivative also known as benzalacetophenone or benzylidene acetophenone. Its basic molecular structure consists of two aromatic rings linked by a bridge of three unsaturated carbon chains (Szliszka et al., 2010). Flavokawain B consists of trans-chalcone substituted by a hydroxy group at position 2' and methoxy groups at positions 4' and 6' (**Figure 8A**) and has been found in the *Zingiberaceae* family plant, *Alpinia pricei* Hayata (Lin et al., 2009), and is capable of increasing the proliferation of rat splenocytes in vivo (Abu et al., 2015). This compound has a molecular ion [M+H]+ with m/z 285 and fragment ions (m/z) of 181, 166, 131, and 103 (**Figure 8A**). The ion fragment in the PubChem Human Metabolome Database (HMBD) (https://pubchem.-ncbi.nlm.nih.gov/) mass spectra backs this up (**Figure 8B**). Flavokawain B were also found in 80% crude methanol extract of *A.malaccensis* using UHPLC-HRMS (**Table 2**).

CONCLUSIONS

Screening the immunostimulant activity of 12 *Zingiberaceae* minor spices showed that *A. malaccensis* and *Z. ottensii* had high lymphocyte proliferation at 400 ppm. *A. malaccensis* rhizome was selected and fractionated, so the methanol fraction had the highest lymphocyte proliferation activity. According to the OPLS results, the retention time interval correlated with immunostimulant activity was 27.0-28.5 minutes, which was abundant in methanol fractions. The immunostimulant compound was preserved at 21.15 minutes based on UHPLC-HRMS and HPLC chromatograms. Flavokawain B was identified as a compound that could be attributed to the peak. Further research into the in vivo immunostimulant activity of *A. malaccensis*

extract is required. Testing the effect of this compound on other immune cells like natural killer (NK) cells, T-helper (Th) cells, T-cytotoxic (Tc) cells, B cells, and cytokines are also recommended.

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