

# Immunomodulatory activity and phytochemical analysis of *Hibiscus sabdariffa* L. flower fractions

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## ABSTRACT

Immunomodulators are compounds that are capable of enhancing the immune system. This research examined the immunomodulatory activity of *Hibiscus sabdariffa* L. flower fractions and identified the chemical compounds contained. A *H. sabdariffa* L. flower ethyl acetate extract was fractionated by the vacuum liquid chromatography method, yielding seven fractions: Fractions A, B, C, D, E, F, and G. Immunomodulatory activity testing was carried out by the phagocytosis method, and phytochemical analysis was conducted using liquid chromatography-mass spectroscopy. The test results showed that Fractions A, B, C, D, F, and G were significantly different from the negative control sodium carboxymethyl cellulose. Fraction A exhibited the best activity. The phytochemical analysis results showed that the *H. sabdariffa* L. flower fractions exhibited a number of compounds that have the potential to act as immunomodulatory active compounds.

## INTRODUCTION

The immune system is a system responsible for the protection of the body against foreign matters entering, thereby preventing the body function from being disturbed. The functions of the immune system include improving human DNA, preventing infections from fungi, bacteria, viruses, and other organisms, and producing antibodies (immunoglobulin) to combat the assault of foreign bacteria and viruses on the body. The non-specific immune system is the first-line protection against foreign microorganisms or matters that come into the body (Balekar *et al.*, 2014).

In general, diseases related to the immune system result from either deficient or excessive expression of the immune system. Deficiency in the immune system is called immunodeficiency. Such a condition makes the body vulnerable toward infectious disease attacks. It is, therefore, of utmost importance to preserve

the body's condition in order to maintain the immune system in the body. One effort that can be made for this purpose is to consume immunostimulants that are capable of promoting the immune system of the body (Balekar *et al.*, 2014).

Some *in vitro* and *in vivo* tests may be conducted to examine the immunomodulatory activity. Phagocytosis is one of the methods frequently used to examine the immune response activity. It is a primary defensive mechanism against foreign substances that penetrate the body which is triggered by neutrophils and macrophages. The process of phagocytosis consists of sequential stages such as motility, adhesion to microorganisms, ingestion of microorganisms, degranulation, and intracellular killing of microorganisms (Uribe-Querol *et al.*, 2017).

A previous study by Ulfah *et al.* (2013) has proven that the ethanol extract of the *Hibiscus sabdariffa* L. flower demonstrates immunomodulatory activity. The ethanol extract of the *H. sabdariffa* L. flower stimulates lymphocyte proliferation in Swiss Webster mice *in vitro* in the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay at concentrations of 50, 100, 200, and 400 µg/ml. Another study by Fakaye (2008)

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tested the ethyl acetate fraction of *H. sabdariffa* L. which showed immunomodulatory activity at 100 mg/kg BW.

This study was conducted to further assay the immunomodulatory activity of *H. sabdariffa* L. flower. The aims of this study were to fractionate the ethyl acetate fraction of *H. sabdariffa* L. using vacuum liquid chromatography (VLC), to test the immunomodulatory activity of the *H. sabdariffa* L. fraction, and to identify the phytochemical compounds of the *H. sabdariffa* L. fraction using liquid chromatography-mass spectroscopy (LC-MS).

## MATERIALS AND METHODS

### Chemicals

The chemicals used in this study are as follows: 96% ethanol, ethyl acetate, n-hexane, chloroform, silica gel GF 254 (Merck), nutrient agar media, Aquadest, Stimuno, 1% BaCl<sub>2</sub>, 1% H<sub>2</sub>SO<sub>4</sub>, 0.9% NaCl, 4% Giemsa stain, methanol, and immersion oil. All chemicals were purchased from a standard local source.

### Animals

Albino mice (20–30 g) were caged in a hygienic condition at the Pharmaceutical Biology Laboratory. They were caged at a standard temperature (25°C ± 5°C) in a 12:12 hour light and dark cycle, in which a standard pellet diet was applied to the mice. Animal testing was carried out with the approval of the Animal Ethics Committee with Letter No. 177a/UN29.20.1.2/PG/2021.

### Plant materials and fractionation

The locally sourced *H. sabdariffa* L. flower was subjected to determination at the Department of Biology of Universitas Haluoleo. The flower was dried at room temperature and then pulverized and macerated with an ethanol solvent. The ethanol extract was freed from glycosides by the liquid-liquid extraction method using ethyl acetate and water. The ethyl acetate fraction was fractionated by the VLC method with hexane, a combination of hexane and ethyl acetate, ethyl acetate, and methanol solvents. From this fractionation, 20 fractions were obtained to be analyzed by node analysis using thin-layer chromatography (TLC) with a chloroform : methanol mobile phase of 1:9. Fractions with the same TLC profiles were merged as single fractions.

### Phytochemical analysis

*Hibiscus sabdariffa* L. flower fractions were analyzed using LC-MS. LC-MS acted as an interfaced with a Quadrupole Time of Flight (Q-TOF) Mass Spectrometer fitted with an ESI source. A full-scan mode from *m/z* 500 to 1,200 was carried out. The solvents were methanol and water at 1:9. Solvents were delivered at a total flow rate of 0.3 ml/minute. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out in 25 minutes under the following conditions: gradients starting at 95% A and 5% B in 8 minutes, to 60% A and 40% B in 3 minutes, to 100% B in 2 minutes, and to 95% A in 2 minutes. Components were identified by matching their mass spectra with those of the Wiley library database.

## Immunomodulatory evaluation

The immunomodulatory activity testing involved 45 mice (*Mus musculus*). The experimental animals were assigned to nine treatment groups, with each group consisting of five mice (number of experimental animals per group based on Federer's formula).

The divisions are as follows:

Group I	Positive control, mice given Stimuno (herbal immunostimulant) at 0.13 mg/30 g body weight
Group II	Negative control, mice given 0.5% sodium carboxymethyl cellulose (Na-CMC)
Group III	Mice given Fraction A at the concentration 600 µg/ml
Group IV	Mice given Fraction B at the concentration 600 µg/ml
Group V	Mice given Fraction C at the concentration 600 µg/ml
Group VI	Mice given Fraction D at the concentration 600 µg/ml
Group VII	Mice given Fraction E at the concentration 600 µg/ml
Group VIII	Mice given Fraction F at the concentration 600 µg/ml
Group IX	Mice given Fraction G at the concentration 600 µg/ml

All the groups were given treatments with each sample orally every day for 1 week. On day 8, every mouse was intraperitoneally infected with a 0.5 ml (0.5 McFarland standard) *Staphylococcus aureus* bacterial suspension and left to stand for 1 hour. The mice were euthanized with ether to be dissected in their bellies using sterile surgical scissors and tweezers. The peritoneal liquid was extracted using a syringe. It was daubed on the object glass and fixated with methanol for 5 minutes. Then, it was stained with 4% Giemsa stain, left to stand for 20 minutes, and rinsed with flowing water. After the preparations were dried, they were then observed under a microscope using immersion oil at 10–100× and computed for macrophage phagocytosis (Fig. 1) (Parawansah *et al.*, 2018).

The phagocytosis values were computed using the following formula (Parawansah *et al.*, 2018):

$$\% \text{ Activity} = \frac{\text{Number of active macrophages}}{\text{Number of the overall macrophages}} \times 100$$

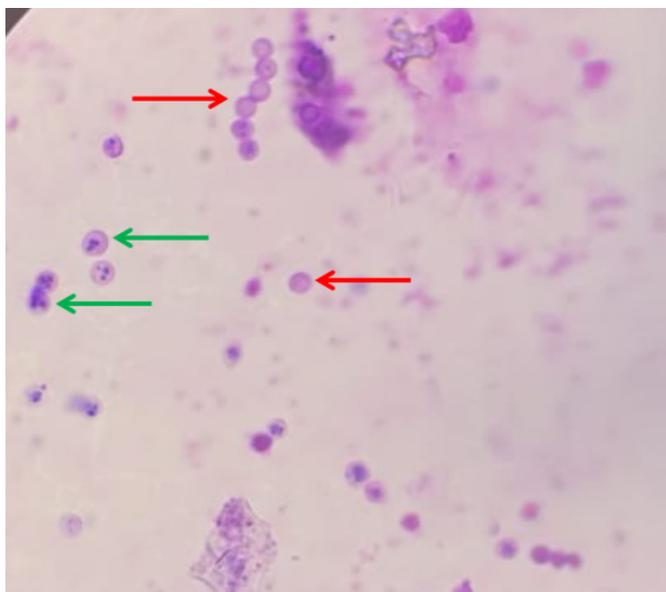
### Statistical analysis

Values are expressed as mean values ± standard error. One-way analysis of variance, followed by Tukey's test was applied for the statistical evaluation of the results obtained in the tests. *P*-values < 0.05 were regarded as significant.

## RESULTS AND DISCUSSION

### Extraction and fractionation

The extraction of the *H. sabdariffa* L. flower yielded 42.5 g of thick extract (8.2% yield). The liquid-liquid extraction yielded 20.75 g of ethyl acetate extract. The fractionation of the ethyl acetate extract by the VLC method yielded 20 fractions. Of the 20 fractions from VLC fractionation with a chloroform : methanol mobile phase of 1:9, some shared the same TLC profiles, necessitating some merging. Seven fractions were derived as the final results: Fractions A (0.19 g), B (0.37 g), C (1.76 g), D (0.87 g), E (3.81 g), F (2.24 g), and G (1.99 g).



**Figure 1.** Phagocytosis microscopic view (red arrows: inactivated phagocytes, green arrows: activated phagocytes).

### Phytochemical analysis

The results of the phytochemical analysis of the *H. sabdariffa* L. fractions using LC-MS are presented in Table 1 and Figure 2.

### Immunomodulatory evaluation

The results of the immunomodulatory evaluation can be seen in Table 2 and Figure 3. It was shown that all the treatments, except the treatment with Fraction E use, were significantly different from the negative control (Na-CMC). This shows that Stimuno and Fractions A, B, C, D, F, and G have immunomodulatory activity. The samples with Fractions A, B, F, and G were significantly different from the positive control (Stimuno), suggesting even better activity. Fraction A showed the best activity (95.83%).

### DISCUSSION

This research aimed at examining the immunomodulatory activity of *H. sabdariffa* L. flower fractions with the phagocytosis method using mice (*M. musculus*) as experimental animals and at identifying the chemical compounds with the LC-MS method.

The activity examination was conducted by administering fractions, Stimuno (positive control), and Na-CMC (negative control) to the experimental animals for 7 days in a row orally to give the samples time to improve their nonspecific immune response. On day 8, each mouse was infected with a *S. aureus* bacterial suspension intraperitoneally at 0.5 ml. *S. aureus* was used as it is categorized as a Gram-positive bacterium. Gram-positive bacteria are able to bind Giemsa stain clearly, thereby making it easy to compute under the microscope. Moreover, containing no protein A, a protein that is antiphagocytic in nature, *S. aureus* is unable to escape from peritoneal macrophage phagocytosis (Fristiody *et al.*, 2019).

After being injected with bacterial suspension, all the treatment groups were left to stand for 1 hour prior to dissection. This was to know to which extent the macrophage bacterial activation ability was. The macrophages were able to withstand infection for the first period of about 1 hour before another immunity mechanism could be mobilized. Therefore, macrophage extraction was carried out approximately for 1 hour after bacterial induction. In this manner, to which extent the macrophages were able to deal with bacterial invasion could be identified (Wahyuni *et al.*, 2019).

The peritoneal liquids obtained were then smeared thinly on prepartate slides to be observed under a light microscope at 1,000× with immersion oil applied. Active macrophages were marked as growing macrophages in size and shape, with extremely varied pseudopodia protrusions. The phagosomes grew with increasingly wavy membranes, the lysosomes grew in quantity, the Golgi apparatuses enlarged, and the rough endoplasmic reticulus developed. On the other hand, the inactive macrophages were smaller in size and shape relative to the active macrophages (Rougerie *et al.*, 2013).

The *S. aureus* bacterial infection involved in this research triggered infection in mice. During the infection process, T lymphocytes produce some lymphokines that will attract macrophages to places in need and activate them. Active macrophages release a number of important substances: enzymes, lysozymes, elastases, collagenases, complements, and cytokines. Cytokines that are secreted by macrophages include interleukin (IL)-1, IL-6, IL-8, IL-12, IL-15, and tumor necrosis factor (TNF- $\alpha$ ). Under a normal condition, this process runs slowly, so the active macrophages that undergo phagocytosis resulting are small in quantity (Wahyuni *et al.*, 2019).

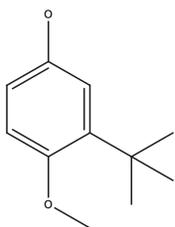
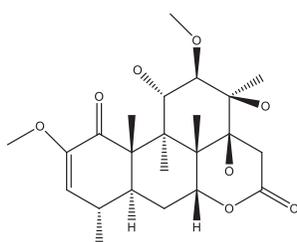
The test results showed that the percentages of phagocytosis with Fractions A, B, C, D, F, and G suggested immunomodulatory activity levels that statistically were significant in their differences from the negative control (Na-CMC). This research also conducted a comparison with a positive control that used Stimuno. Stimuno is an immunomodulatory traditional medication preparation used in Indonesia. Fractions C and D did not show any difference in their activity from Stimuno. Meanwhile, Fractions A, B, F, and G did show better activity than Stimuno.

The results of the compound identification of the *H. sabdariffa* L. flower fractions show several compound components in the fractions. Some of the previous studies have reported these compounds in the *H. sabdariffa* L. flower: coumarin (Adeyemi *et al.*, 2014), 3-hydroxy-7-methoxy baicalin (Alara *et al.*, 2020), 5,7,2',5'-tetrahydroxy-flavone (Da-Costa-Rocha *et al.*, 2014), undecanoic acid (Jabeur *et al.*, 2019), 9,12-octadecadienoic acid (*Z,Z*)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester (Hagr *et al.*, 2020), salvianolic acid A (Amin *et al.*, 2005), and kaempferol derivatives (Vargas-Álvarez *et al.*, 2018).

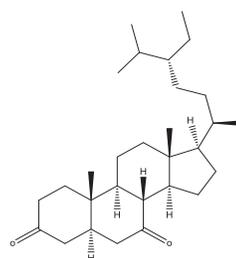
Some compounds have been reported to exhibit activity as immunomodulators. Stefanova *et al.* (2007) have reported that coumarin treatment enhanced the macrophage migration activity in the presence and absence of lipopolysaccharide (LPS) and increased nitric oxide release. *In vitro*, coumarins induced

**Table 1.** Results of LC-MS analysis of *H. sabdariffa* L. flower fractions.

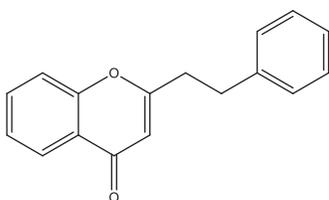
No	Component name	Observed <i>m/z</i>	Observed RT (minutes)
Fraction A			
1.	3-tert-Butyl-4-methoxyphenol	181.1221	7.82
2.	Nigakilactone H	425.2153	9.55
3.	Stigmastane-3,6-dione	429.3720	10.38
4.	Candidate mass C45H84O15	887.5691	10.85
5.	Candidate mass C45H84O16	903.5645	10.77
Fraction B			
1.	2-(2-Phenylethyl)chromone	251.1063	8.58
2.	Coumarin	147.0435	5.06
3.	3-tert-Butyl-4-methoxyphenol	181.1219	8.12
4.	5,7-Dihydroxy-3-(4'-hydroxybenzyl)chromone	285.0756	8.02
5.	Candidate mass C35H42O9	607.2914	9.64
Fraction C			
1.	Coumarin	147.0437	4.77
2.	3-Hydroxy-7-methoxy baicalein	301.0704	6.74
3.	5.7.2',5'-Tetrahydroxy-flavone	287.0550	6.09
4.	Digitopurpone	271.0599	6.64
5.	Undecanoic acid	209.1531	6.35
Fraction D			
1.	3-Hydroxy-7-methoxy baicalein	301.0702	6.75
2.	5.7.2',5'-Tetrahydroxy-flavone	287.0548	6.10
3.	Artemisinin I	207.1376	4.95
4.	Azedarachin C	609.2719	9.51
5.	Digitopurpone	271.0599	6.63
Fraction E			
1.	5.7.2',5'-Tetrahydroxy-flavone	287.0548	6.06
2.	5,7-Dihydroxychromone	179.0335	3.85
3.	9,12-Octadecadienoic acid (Z,Z)-(2,2-dimethyl-1,3-dioxolan-4-yl) methyl ester	417.2967	9.68
4.	Imperanene	331.1534	5.87
5.	Candidate mass C25H12O8	441.0602	7.53
Fraction F			
1.	5.7.2',5'-Tetrahydroxy-flavone	287.0549	6.05
2.	5,7-Dihydroxychromone	179.0337	3.86
3.	Kaempferol-3-o-β-D-glucopyranoside	449.1077	4.75
4.	Salvianolic acid A	495.1288	4.66
5.	Wedelolactone	337.0341	8.53
Fraction G			
1.	Kaempferide-3-O-α-L-rhamnosyl-7-O-α-L-rhamnoside	593.1862	5.86
2.	Kaempferol-3-O-rutinoside	595.1662	4.57
3.	Kaempferol-3-O-β-D-glucopyranoside	449.1075	4.75
4.	Kaempferol-7-O-α-L-rhamnoside	433.1124	5.20
5.	Methyl gallate	185.0441	3.65

**Fraction A**3-*tert*-Butyl-4-methoxyphenol

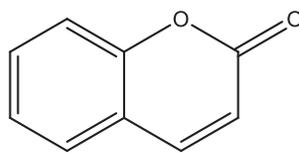
Nigakilactone H



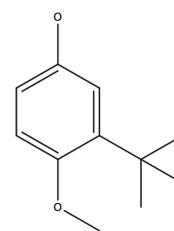
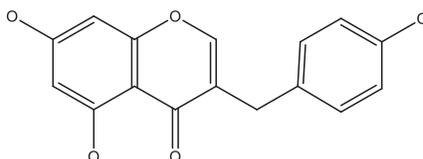
Stigmastan-3,6-dione

**Fraction B**

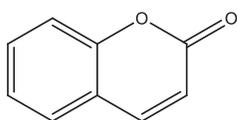
2-(2-Phenylethyl) chromone



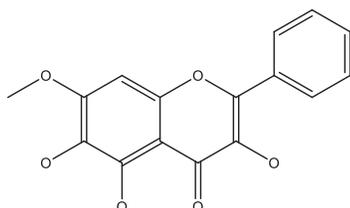
Coumarin

3-*tert*-Butyl-4-methoxyphenol

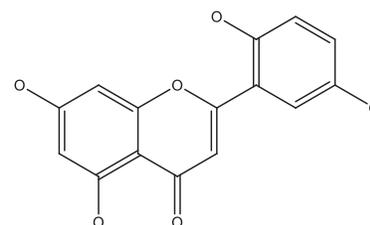
5,7-Dihydroxy-3-(4'-hydroxybenzyl) chromone

**Fraction C**

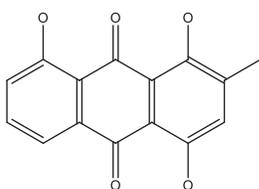
Coumarin



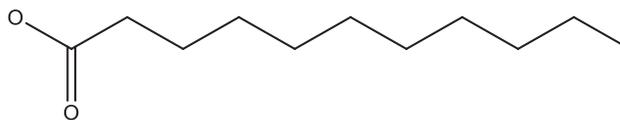
3-Hydroxy-7-methoxy baicalein



5,7,2',5'-Tetrahydroxy-flavone

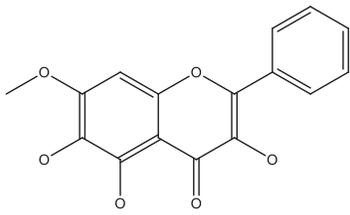


Digitopurpone

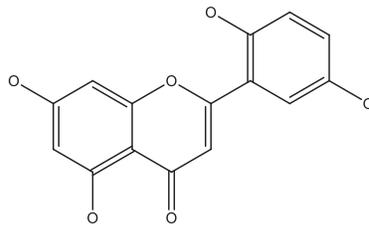


Undecanoic acid

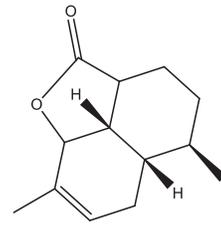
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**Fraction D**

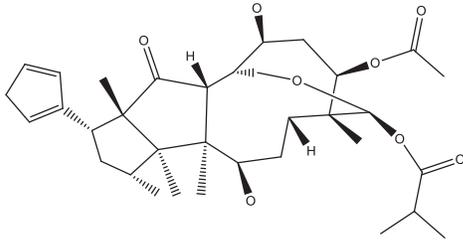
3-Hydroxy-7-methoxy baicalein



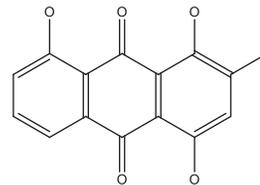
5,7,2',5'-Tetrahydroxy-flavone



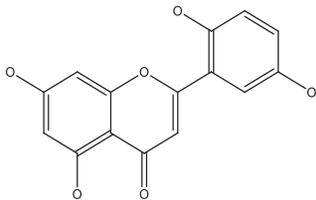
Artemisinin I



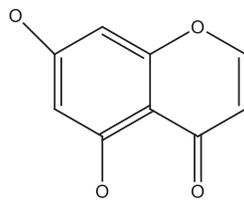
Azedarachin C



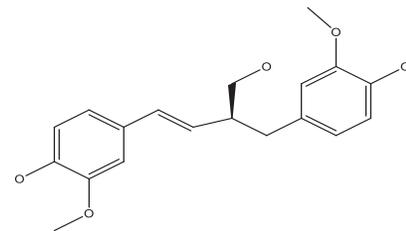
Digitopurpone

**Fraction E**

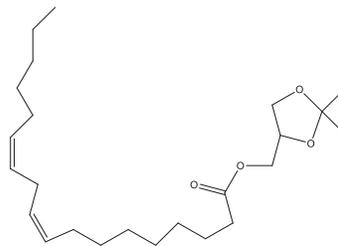
5,7,2',5'-Tetrahydroxy-flavone



5,7-Dihydroxychromone

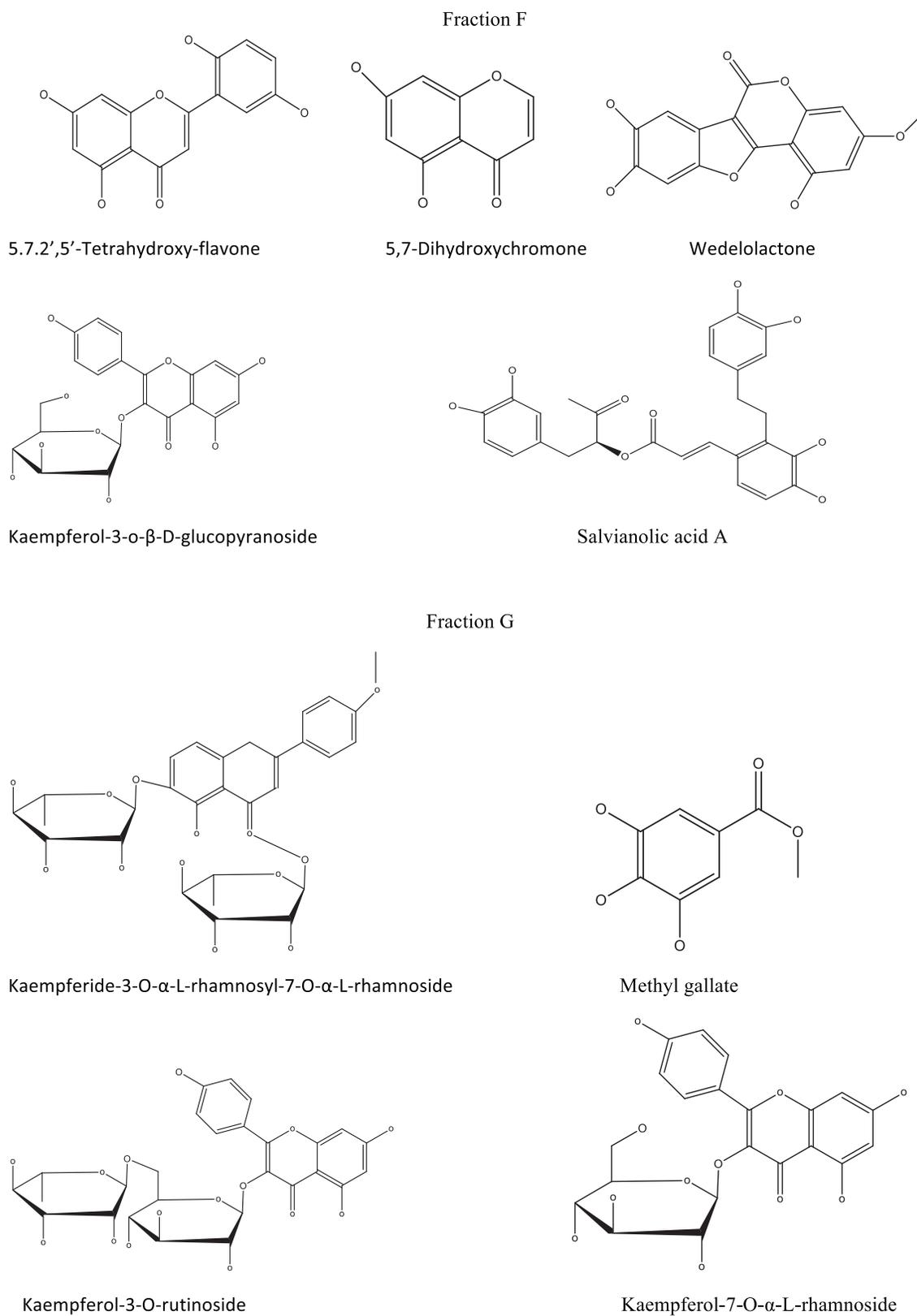


Imperanene



9,12-Octadecadienoic acid (Z,Z)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester

*Continued*

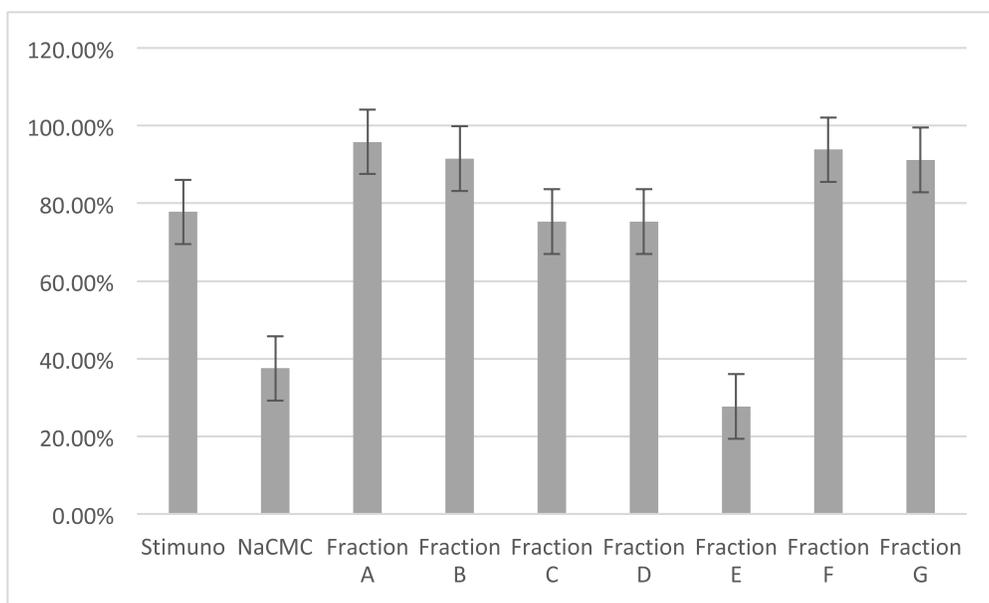


**Figure 2.** Identified compounds of *H. sabdariffa* L. flower fractions.

**Table 2.** Immunomodulatory evaluation of *H. sabdariffa* L. flower fraction.

Group	Phagocytosis activity
Stimuno (positive control)	77.83% ± 7,34*
Na-CMC (negative control)	37.50% ± 6,95
Fraction A	95.83% ± 4,89*
Fraction B	91.50% ± 4,89*
Fraction C	75.28% ± 4,15*
Fraction D	75.28% ± 7,70*
Fraction E	27.67% ± 6,19
Fraction F	93.83% ± 3,82*
Fraction G	91.17% ± 3,37*

Values are expressed as mean ± SEM ( $n = 5$ ). Values of  $p$  are considered statistically significant to the negative control group.  $p < 0.05$ .

**Figure 3.** Immunomodulatory evaluation of *H. sabdariffa* L. flower fraction.

IL-12 in murine macrophages and additively increased the LPS-induced IL-12 release. Orzechowska *et al.* (2014) reported that the *Scutellariae radix* extract, whose active compound is baicalin, was able to modulate the immune system by increasing the production of interferon- $\gamma$  (IFN- $\gamma$ ) in peripheral blood leukocytes and reducing the production of TNF- $\alpha$  and IL-10 in bone marrow cells.

Such fatty acid compounds as undecanoic acids have also been reported as immunomodulators, being able to affect both the innate and adaptive responses. Fatty acids are able to enhance neutrophil aggregation, neutrophil-endothelial cell attachment, and phagocytic and candidacidal capacities (Di Sotto *et al.*, 2020). Other studies also reported that fatty acids are able to boost the production of anti-inflammatory cytokines like IL-4, in spite of a reduction of proinflammatory TNF- $\alpha$  (Kumar *et al.*, 2019).

*Hibiscus sabdariffa* L. fractions contain many flavonoids such as 5,7,2',5'-tetrahydroxy-flavone and kaempferol derivatives. Flavonoids have been widely known as immunomodulators. They

have the potential of working against the lymphokines produced by T cells, thereby stimulating phagocytes to exhibit phagocytosis responses (Hosseinzade *et al.*, 2019). Flavonoids have proven to be able to boost IL-2 and lymphocyte proliferation. Lymphocyte proliferation will influence CD4<sup>+</sup> cells, which in turn will cause Th1 cells to be activated. Activated Th1 cells will influence specific macrophage-activating factors (SMAFs). SMAFs *per se* are multiple molecules, one of which is IFN- $\gamma$ . IFN- $\gamma$  will activate macrophages, leading them to undergo an increase in phagocytosis. This will result in the macrophages being capable of killing bacteria faster. Flavonoids also have a working mechanism by activating NK cells to stimulate IFN- $\gamma$  production. IFN- $\gamma$  is a main macrophage-activating cytokine, which will activate macrophages and trigger the elevation of phagocytosis activity. Activated macrophages and neutrophils will produce some proteolytic enzymes in phagolysosomes such as elastase and cathepsin G that function in destroying bacteria (Mendes *et al.*, 2019; Sulistiani *et al.*, 2015).

Steroid compounds have also been reported to act as immunomodulators. Heroor *et al.* (2020) reported that steroid compounds had higher activity in stimulating phagocytes when compared to alkaloids, flavonoids, and tannins. Steroid compounds are also known to induce a Th1 response in T-helper cells (Brüll *et al.*, 2009).

The immunomodulatory activity of Fractions A, B, C, D, F, and G *H. sabdariffa* L. flower contains compounds that act as immunomodulators. Fraction A as the most active fraction contains 3-tert-butyl-4-methoxyphenol (phenol) and stigmastan-3,6-dione (steroid) compounds. The activity of Fraction A may be caused by the synergistic effect of the two compounds, especially steroid compounds which have high immunomodulatory activity.

*Hibiscus sabdariffa* L. flower fractions exhibit many compounds that have potential as immunomodulators. Immunomodulatory activity can result from the synergetic effect of the compounds. For further research, it is suggested to carry out active compounds isolation to determine the active compounds.

## CONCLUSION

Fractions A, B, C, D, F, and G of the *H. sabdariffa* L. flower showed immunomodulatory activity. Fraction A displayed the best activity among all the fractions. The results of the identification of the chemical compounds in the *H. sabdariffa* L. flower fractions showed that some of the compounds contained have potential as immunomodulators.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## CONFLICT OF INTEREST

The authors confirm that this article's content has no conflicts of interest.

## ETHICAL APPROVALS

Animal testing was carried out with the approval of the Animal Ethics Committee with Letter No. 177a/UN29.20.1.2/PG/2021.

## LIST OF ABBREVIATIONS

TLC	Thin-layer chromatography
VLC	Vacuum liquid chromatography
Na-CMC	Sodium carboxymethyl cellulose
LC-MS	Liquid chromatography-mass spectroscopy
IL	Interleukin
LPS	Lipopolysaccharide
IFN	Interferon

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