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Metabolite profile of false elder leaves (*Peronema canescens* Jack.) based on development levels

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ABSTRACT

At each stage of leaf development, the formation of compounds in the leaves will occur, thus allowing the chemical components in it to also experience changes, both the properties possessed by a plant and the quantity of metabolites it has. This study aims to determine the differences in metabolites and the abundance of metabolites of Sungkai or False Elder leaves (*Peronema canescens* Jack.) based on the level of leaf development with a non-target metabolomics analysis approach using GC-MS. Obtained metabolite compounds were acquired using a quantitative descriptive method. Obtained differences and abundance of detected metabolites. Sungkai young, mature, and old leaves show 17 types of metabolites. The abundance of each type of compound from each leaf development was different, and the total abundance of metabolite compounds was found in mature Sungkai leaves at 75.64%, followed by young Sungkai leaves at 71.55% and old Sungkai leaves at 51.27%. The results show that there are unique compounds such as phenol, 2,6-dimethoxy-4-(2-propenyl)- and ursodeoxycholic acid which are detected which are known to have pharmacological effects as natural immunomodulators for the body

Keywords: Metabolite profile, Leaf development, *Peronema canescens* Jack. (Sungkai, False Elder), GC-MS

INTRODUCTION

The Corona Virus Disease 2019 (COVID-19) outbreak is a new type of disease that can be transmitted from human to human. According to the World Health Organization (WHO) in 2020 in Indonesia, the spike in cases reached 8.9%, the highest mortality in Southeast Asia. There is no known cure for this type of disease, so steps that can be taken are to prevent transmission by maintaining the body's immune system (Antari *et al*., 2021).

The body's immune system can be strengthened by consuming healthy and balanced nutrition, regular exercise and supplements. However, some supplements with active ingredients in their use that are not on target will pose a dangerous risk to health (Wati *et al*., 2014). Indonesia is a country with great potential in the supply of traditional medicines. One of the plants that has the potential as herbal medicine is sungkai leaf (*Peronema canescens* Jack.) from the Verbenaceae family which is known to have metabolites in the form of alkaloids, phenols, and other metabolite compounds both primary and secondary (Ibrahim and Kuncoro, 2012).

The chemical content in the metabolism of leaf development is related to the process of photosynthesis. This is because chlorophyll levels will increase with age until the leaves are fully developed and then chlorophyll levels decrease as the leaves get older. When the leaves are old it is indicated that there are other compounds that act as the main barrier for oxidation reactions (Tinungki *et al.*, 2018). Therefore the order of the leaves on the stalk can determine the young or mature leaves, this affects the quantity of the metabolite compounds contained therein (Ningtyas *et al.*, 2012). Leaves store many secondary metabolite products which function to carry out the synthesis of organic compounds as a source of energy needed in the process of photosynthesis (Putri *et al.*, 2018). The need for sunlight, water and nutrients as nutrients is needed for growth and development in every plant organ as well as the chemical components in it (Sumenda, 2011).

Several studies have shown that the methanol extract of Sungkai leaves contains alkaloids, phenols, tannins, flavonoids, saponins, steroids, and terpenoids (Ibrahim and Kuncoro, 2012). Then the administration of infusion from the extraction of Sungkai leaf aquadest contains secondary metabolites that have the potential to have an immunomodulatory effect that can work to increase the number of leukocytes in male mice (Rahman *et al.*, 2021).

This study aims to determine the differences in metabolites and the abundance of sungkai leaf metabolites based on the level of leaf development with a non-target metabolomics analysis approach using GC-MS which is expected to provide initial information regarding the metabolite profile and the abundance of sungkai leaf metabolites obtained based on the level of leaf development and as material for consideration in efforts to standardize traditional medicinal ingredients, so that these herbs can be used as natural immunomodulators for the body.

MATERIAL AND METHOD

Material

The sungkai leaf samples used have been observed, with the following criteria: young sungkai leaves are at number 1 – 3 from the shoot, growing at the ends of branches that face each other including leaves that are still in the bud phase, red-purple to greenish in color (light green). For the mature leaf category, on one main petiole (petiolus comunis) the color of all the leaves is light green, usually in the order number 4 – 6 from the shoot. As for the old leaves, the criteria are dark green, the surface of the leaves is rougher and stiffer, they are located at the bottom or near the base of the branch or are in the order number 7 – 8 if counted from the shoots. In one branch of the sungkai plant there are usually 7- 8 pairs of main petiole that cross each other (folia opposita or folia decussata) on one branch of the branch.

Method

The stages of the research were carried out through the following stages:

1. Extraction

Extraction of simplicia powder from young, mature and old Sungkai leaves was carried out using the maceration method (Ahmad and Ibrahim, 2015). Extraction using pro-analyst methanol solvent with a ratio of 1:5 (w/v) for 3×24 hours in a dark room at room temperature then filtered with filter paper and remaceration 2 times with the aim of optimizing extraction. Then the filtrate obtained was concentrated using a vacuum rotary evaporator at 50 C until a thick extract was obtained.

2. Metabolite profiling using GC-MS

Sample preparation was carried out using methanol solvent. The methanol extract that had been added with 10 mL methanol was injected as much as 1 μL into the GC-MS according to the method instrument based on the working protocol of the GC-MS TraceTM1310 ISQ.

3. Data Analysis

The results of the identification of metabolites were analyzed by descriptive quantitative analysis which was expressed in the form of peaks on the chromatogram based on the level of leaf development. Furthermore, metabolite compounds were determined based on their area percentage and compatibility with plants, and biosynthetic pathways were traced using the CheBI, KEGG, PlantCyc, PubChem, Spectrabase, and Greenmolbd websites.

RESULT AND DISCUSSION

Analysis and characterization of the metabolic compounds obtained by the results of GC-MS obtained information that the components of chemical compounds in the methanol extract of Sungkai leaves (*P.canescens*) contained as many as 17 compounds at each stage of development of young, mature and old Sungkai leaves as seen by peak -the peak that appears on the chromatogram image. Characterization by GC-MS aims to determine the molecular weight and components of chemical compounds in the methanol extract of Sungkai leaves from the GCMS analysis presented in (Figure 1) and (Table 1).

Based on the data obtained, it is known that the GC-MS results of the methanol extract of Sungkai leaves, it is known that it predominantly contains a class of fatty acid compounds consisting of methyl tetradecanoate; hexadecanoic acid, methyl ester; hexadecanoic acid, 14-methyl-, methyl ester; 6-octadecenoic acid, methyl ester, (z)-; eicosanoic acid, methyl ester; butyl 4,7,10,13,16,19docosahexaenoate; methyl stearate; 9,12,15-octadecatrienoic acid, 2,3-bis [(trimethylsilyl) oxy]propyl ester, (z,z,z). The steroid compound consists of ethyl iso-allocholate; pregnan-20-one, 3-(acetyloxy)-5,6:16,17-diepoxy-. While the carbohydrate compounds consist of resibufogenin and 2-deoxy-d-galactose which are seen based on the total percent area content of the compounds detected in each stage of development of Sungkai leaves.

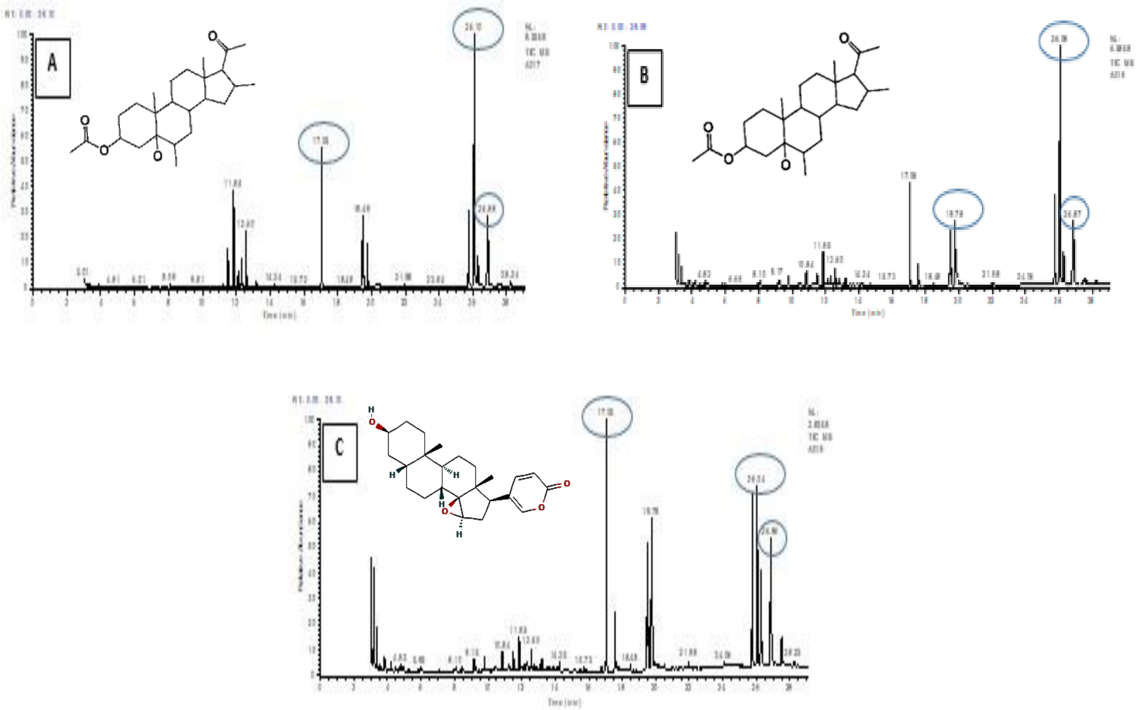

Figure 1. Chromatogram of Methanol Extract of Sungkai (*Peronema canescens* Jack.) Leaves: (A) Young Sungkai Leaves, (B) Adult Sungkai Leaves, (C) Old Sungkai Leaves based on GC-MS

Based on these data, it is known that there are differences in metabolite profiles at the developmental level of Sungkai (*P.canescens*) leaves which are characterized by differences in the types of compounds contained at each stage of leaf development and the total abundance produced. Adult sungkai leaves had the largest total area of abundance, namely 75.64%, followed by young sungkai leaves 71.55% while old sungkai leaves 51.27%. This is presumably because it is influenced by the development of leaf age where as the age of the leaf increases, the amount of metabolite compounds produced will increase and decrease when the leaves are old.

Sungkai Leaf Metabolite Compounds, Based on the results of the interpretation of the data that has been obtained, it can be seen that there are several major or dominant compounds, namely compounds that have a higher percentage area compared to other compounds. The dominant compounds in sungkai are hexadecanoic acid, pregnan-20-one, 3-(acetyloxy)-5,6;16,17-diepoxy -, butyl 4,7,10,13,16,19 docosahexaenoate, resibufogenin and methyl stearate which can seen in (Table 1).

Table 1. Metabolism profile of young, mature and old Sungkai (*Peronema canescens* Jack.) leaves based on GC-MS

Young Sungkai leaves					
Peak	**Molecular formula**	**Metabolite compound**	**Class compound**	**RT (min)**	**Area (%)**
1	C_5H_6O	*2-Cyclopenten-1-one*	Ketones	3.85	0,28
2	$C_4H_6O_2$	*Butyrolactone*	Cyclic Esters (Lactones)	4.81	0,27
3	$C_6H_{15}NO_3$	*Trolamine*	Cyclic Esters (Lactones)	6.31	0,18
4	$C_{10}H_{18}O$	*1,6-Octadien-3-ol, 3,7-dimethyl-*	Terpenes (Monoterpene)	8.09	0,38
5	C_8H_8O	*Coumaran*	Coumarins (oilvolatile)	9.81	0,43
6	$C_{15}H_{24}$	*Caryophyllene*	Terpenes(Sesquiterpene)	11.85	4,13
7	$C_{15}H_{24}$	*Naphthalene, 1,2,3,5,6,8a- hexahydro-4,7-dimethyl-1 -(1-methylethyl)-, (1S-cis)-*	Terpenoids (Sesquiterpenoids)	2.60	2,67
8	$C_{15}H_{30}O_2$	*Methyl tetradecanoate*	Fatty acid	14.24	0,26
9	$C_{22}H_{42}O_2$	*Phytol, acetate*	Terpenoids (Diterpenoids)	15.72	0,17
10	$C_{17}H_{34}O_2$	*Hexadecanoic acid, methyl Ester*	Fatty acid (Methyl Esther)	17.05	10,95
11	$C_{18}H_{36}O_2$	*Hexadecanoic acid, 14-methyl-, methyl ester*	Fatty acid	18.48	0,14
12	$C_{19}H_{36}O_3$	*6-Octadecenoic acid, methyl ester, (Z)-*	Fatty acid (Methyl Esther)	19.49	7,68
13	$C_{21}H_{42}O_2$	*Eicosanoic acid, methyl ester*	Fatty acids (Acid arachidonic)	21.98	0,29
14	$C_{26}H_{44}O_5$	*Ethyl Iso-allocholate*	Steroids	23.84	0,14
15	$C_{23}H_{34}O_4$	*Pregnan-20-one, 3- (acetyloxy)-5,6:16,17-diepoxy-, (3á,5à,6à,16à)-*	Steroids (phytosterols)	26.10	33,85
16	$C_{26}H_{40}O_2$	*Butyl 4,7,10,13,16,19-Docosahexaenoate*	Fatty acid	26.88	9,16
17	$C_{30}H_{48}O_2$	*3,12-Oleandione*	Terpenoids (Triterpenoids)	28.24	0,57
	Total Abundance of Compounds (%)				**71,55**

Mature Sungkai leaves					
Peak	**Molecular formula**	**Metabolite compound**	**Class compound**	**RT (min)**	**Area (%)**
1	$C_4H_6O_2$	*Butyrolactone*	Cyclic Esters (Lactones)	4.82	0,46
2	$C_7H_8O_2$	*Phenol, 2-methoxy--4-vinylphenol*	Phenol (Phenolics)	6.68	0,31
3	$C_{10}H_{18}O$	*1,6-Octadien-3-ol, 3,7-dimethyl-*	Terpenes (Monoterpenes)	8.10	0,63
4	$C_8H_{16}O_2$	*Octanoic acid*	Fatty acid	9.17	0,53
5	C_8H_8O	*Coumaran*	Coumarins (oil volatile)	9.78	0,90
6	$C_9H_{10}O_2$	*2-Methoxy-4-vinylphenol*	Phenol (Phenolics)	10.84	0,86
7	$C_{15}H_{24}$	*Caryophyllene*	Terpenes (Sesquiterpenes)	11.85	1,60

8	$C_{15}H_{24}$	*Naphthalene, 1,2,3,5,6,8a- hexahydro-4,7-dimethyl-1 - (1-methylethyl)-, (1S-cis)-*	Terpenoids (Sesquiterpenoids)	12.60	1,10
9	$C_{15}H_{30}O_2$	*Methyl tetradecanoate*	Fatty acid	14.24	0,29
10	$C_{27}H_{44}O_3$	*24,25- Dihydroxycholecalciferol*	Vitamins (Cholecalciferol)	15.73	0,25
11	$C_{17}H_{34}O_2$	*Hexadecanoic acid, methyl Ester*	Fatty acid (Methyl Esther)	17.05	8,46
12	$C_{16}H_{32}O_2$	*l-(+)-Ascorbic acid 2,6-dihexadecanoate*	Vitamins (Ascorbic acid)	18.48	2,48
13	$C_{19}H_{38}O_2$	*Methyl stearate*	Fatty acid	19.78	9,60
14	$C_{26}H_{44}O_5$	*Ethyl iso-allocholate*	Steroids	21.98	0,30
15	$C_{15}H_{24}O_2$	*Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)-*	Sesquiterpenoids	24.06	8,59
16	$C_{23}H_{34}O_4$	*Pregnan-20-one, 3- (acetyloxy)-5,6:16,17-diepoxy-, (3á,5à,6à,16à)-*	Steroids (Phytostrerols)	26.08	29,64
17	$C_{24}H_{32}O_4$	*Resibufogenin*	Carbohydrate (Cardiac glycosides)	26.87	9,64
	Total Abundance of Compounds (%)				**75,64**

Old Sungkai leaves					
Peak	**Molecular formula**	**Metabolite compound**	**Class compound**	**RT (min)**	**Area (%)**
1	$C_4H_6O_2$	*Butyrolactone*	Cyclic Esters (Lactones)	4.83	0,39
2	$C_6H_{12}O_5$	*2-Deoxy-D-galactose*	Carbohydrates (Monosaccharides)	5.95	0,52
3	$C_{10}H_{18}O$	*1,6-Octadien-3-ol, 3,7-dimethyl-*	Terpenes (monoterpene)	8.10	0,49
4	$C_8H_{16}O_2$	*Octanoic acid*	Fatty acid	9.15	0,64
5	C_8H_8O	*Coumaran*	Coumarins (Oil volatile)	9.78	0,80
6	$C_9H_{10}O_2$	*2-Methoxy-4-vinylphenol*	Phenol (Phenolics)	10.84	0,72
7	$C_{15}H_{24}$	*Caryophyllene*	Terpenes (Sesquiterpene)	11.85	0,98
8	$C_{15}H_{24}$	*Naphthalene, 1,2,3,5,6,8a- hexahydro-4,7-dimethyl-1 -(1-methylethyl)-, (1S-cis)-*	Terpenoids (Sesquiterpenoids)	12.60	0,91
9	$C_{15}H_{30}O_2$	*Methyl tetradecanoate*	Fatty acid	14.25	0,38
10	$C_{20}H_{40}O_2$	*Ethanol, 2-(9- octadecenyloxy)-, (Z)-*	Alcohol (Ether)	15.73	0,33
11	$C_{17}H_{34}O_2$	*Hexadecanoic acid, methyl Ester*	Fatty acid (Methyl Esther)	17.05	11,82
12	$C_{19}H_{38}O_2$	*Methyl stearate*	Fatty acid	19.78	8,6
13	$C_{18}H_{36}O_2$	*Hexadecanoic acid, 14-methyl-, methyl ester*	Fatty acid	21.98	0,54
14	$C_{27}H_{52}O_4Si_2$	*9,12,15-Octadecatrienoic acid,2,3-bis[(trimetylsyl)oxy]propy ester, (Z,Z,Z)*	Fatty acid (Linolenic acid)	24.06	0,73
15	$C_{23}H_{34}O_4$	*Pregnan-20-one, 3- (acetyloxy)-5,6:16,17-diepoxy-, (3á,5à,6à,16à)-*	Steroids (Phytosterols)	26.04	10,8
16	$C_{24}H_{32}O_4$	*Resibufogenin*	Carbohydrate (cardiac glycosides)	26.86	12,11
17	$C_{30}H_{48}O_2$	*1-Monolinoleoylglycerol trimethylsilyl ether*	Fatty acid (Linolenic acid)	28.23	0,51
	Total Abundance of Compounds (%)				**51,27**

The compound hexadecanoic acid (palmitic acid) is one of the most common saturated fatty acids found in animals and plants. It is found in the form of esters (glycerides) in vegetable or animal oils and fats. According to Karunia *et al.* (2017) hexadecanoic acid, methyl ester (palmitic acid) including fatty acids which have antibacterial properties by damaging the structure of cell walls and membranes with a synergistic mechanism with various active compounds so as to increase the effect of antibacterial activity.

The compound methyl stearate from linoleic acid which is classified as a free fatty acid is known to have functions as an antimicrobial, anti-inflammatory and antioxidant. According to Hamzah *et al.* (2021) stated that several types of free fatty acids such as linoleic and linolenic have strong antibacterial activity against certain gram-positive and gram-negative bacteria.

Other compound components such as pregnan-20-one, 3-(acetyloxy)-5,6:16,17-diepoxy- which have the highest area percent content in young and mature Sungkai leaves belong to the steroid group. Compounds of the steroid class are cyclic or acyclic and often have alcohol, aldehyde or carboxylic acid groups. According to Novitasari *et al.* (2016) steroids have important bioactivity, for example in forming membrane structures, forming hormones and vitamin D, as repellents and attracting insects and as antimicrobials.

Phenolic group compounds such as phenol, 2-methoxy-4-vinylphenol, caryophyllene, phytol (Table 1) is known to have antioxidant, antimicrobial and anti-inflammatory bioactivity. These compounds are categorized as phenols and have strong antioxidant activity. One of the antioxidant compounds can donate electrons or hydrogen to prevent the formation of free radicals. According to Baky *et al.* (2021) phytol compounds are flavorful essential oils that have various biological activities such as cytotoxins, antioxidants, inducing apoptosis, antinociception, anti-inflammation, antimicrobials and immune modulation. Young, mature and old Sungkai leaves are differentiated based on the level of leaf development, each of which has unique metabolite compounds which are included in organic compounds. Unique metabolites are metabolites that are only detected in certain types of Sungkai leaf samples that only have one level of leaf development. Young sungkai leaf samples showed the presence of unique metabolites such as eicosanoic acid; octadecane, 3-ethyl-5-(2-ethylbutyl)-; heptadecanoic acid; butyl 4,7,10,13,16,19docosahexaenoate (belongs to the class of fatty acids); nonanal (aldehyde); trolamine (alcohol); fenretinide; à-cubebene; alloaromadenrene; à-acorenol (terpenes); ylangene; guaia1(10),11-diene; cubedol; ç-muurolene; 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-, z,z,z-; and 3,12-oleandione (a terpenoid). Compounds that have known pharmacological roles are eicosanoic acid; butyl 4,7,10,13,16,19-docosahexaenoate; nonanal; and 3,12-oleandione. According to Beschi *et al.* (2021) eicosanoic acid or also known as ARA functions to activate syntaxin-3 (STX-3), a protein involved in the growth and repair of neurons. Nonanal compounds according to Ramya *et al.* (2015) confirmed the presence of constituents known to exhibit medicinal value as well as pharmacological activities that act as antioxidants, free radical scavengers, anti-inflammatory, antiviral (HIV), and antitoxin.

Unique metabolite compounds in mature sungkai leaf samples, namely pentadecanoic acid (included in the fatty acid class); cholestan-3-ol,2-methylene-,(3á,5à)-(steroids); propanoic acid, 2oxo- (ketone); 1,6-cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethy l); patchoulene; naphthalene, 1,2,3,5,6,7, 8,8a-octahydro-1, (terpenes); carbolic acid (phenol); 1,6,10dodecatrien-3-ol, 3,7,11-trimethyl-; nerolidyl acetate; 1,6,10-dodecatrien-3-ol,3,7,11trimethyl-,(e)-; and 1,6-octadien-3-ol,3,7-dimethyl- (terpenoid). pentadecanoic acid and nerolidyl acetate are compounds with known pharmacological roles. According to Beschi *et al.* (2021) pentadecanoic acid has a function as a pharmacological agent that can act as an antimicrobial and antifungal. Patil and Jadhav (2014) nerolidyl acetate has antibacterial activity.

Old Sungkai leaves have unique metabolites, namely 9-hexadecenoic acid, methyl ester,(z)-; methyl hexadecanoate; heptadecanoic acid, 10-methyl; 7,10-octadecadienoic acid, methyl ester; 9,12,15-octadecatrienoic acid,2,3-bis [(trimethylsilyl) oxy] propyl ester; 1monolinoleoylglycerol trimethylsilyl ether; nonanoic acid; oxiraneundecanoic acid, 3-pentyl- (belongs to the class of fatty acids); glycine, n-[(3à,5á)-24-oxo-3-[(trimethylsilyl)oxy]cholan24-yl]- (amino acid); ethanol,2-(9-octadecenyloxy)-,(z)- (alcohol); phytol; 2(3h)-furanone,3butyldihydro- (terpenes); ursodeoxycholic acid (steroids); 2-deoxy-d-galactose (carbohydrate).

Ursodeoxycholic acid is a compound from the steroid class which is only found in old Sungkai leaf samples. According to Cha (2017) Ursodeoxycholic acid (UDCA), a compound that is used as a traditional medicine for the treatment of liver disease and has been shown to provide anti-inflammatory and protective effects on cells which are associated with regulation of immunoregulatory responses with regulation of cytokines. Hidayah and Kawuryan (2018) stated that UDCA was able to prevent hepatocyte apoptosis, change bile acid components, and have an immunomodulatory effect.

Tannin compounds found in plants are classified as polyphenol compounds with astringent properties which cause a dry and chelating taste in the mouth which react with and agglomerate proteins or various other organic compounds including amino acids and alkaloids. Phenol, 2,6-dimethoxy-4-(2-propenyl)- is one of the compounds detected in sungkai leaves from the Caffeic acid group or caffeic acid belonging to the polyphenol (pseudotannin) compound group which is one of the strong antioxidant bioactive components derived from plants -Lower molecular weight plants with a simpler structure compared to actual tannins (hydrolyzed tannins) which have effects as anticancerogenic, anti-inflammatory, antioxidant and immunomodulatory in a broad spectrum. According to Hanifah and Kiptiyah (2020) the compound groups of polysaccharides, terpenoids, alkaloids, flavonoids, polyphenols vitamins C and E, limonoids, curcumin and catechins are compounds that have bioactivity as immunostimulating agents. Immunostimulant agents or immunomodulators are substances capable of inducing, strengthening and inhibiting any component or phase of the immune system. Immunomodulators are especially needed for conditions where the body's immune system status begins to decline which is usually caused by many factors.

CONCLUSION

From the research that has been done, it can be concluded that the abundance of sungkai leaf metabolites is influenced by the level of leaf development, where young, mature and old sungkai leaves have a total abundance of compound types of 71.55%, 75.64% and 51, respectively. 27%. It was found that the dominant metabolites of other compounds were detected in young, mature and old Sungkai leaf samples belonging to the class of fatty acids, steroids and carbohydrates such as hexadecanoic acid, pregnan-20-one, 3-(acetyloxy)-5,6:16, 17-diepoxy-, butyl 4,7,10,13,16,19-docosahexaenoate, resibufogenin, and methyl stearate. As well as phenol compounds, 2,6-dimethoxy-4-(2-propenyl)- and ursodeoxycholic acid, which are compounds that have potential as immunomodulators for the body.

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