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In Vitro Immunomodulatory Activity of Calamansi Orange Peel (*Citrus microcarpa* Bunge) Essential Oil on Mice Lymphocyte Cell Proliferation

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Immunomodulators remain the most crucial part of the prevention and even treatment process for various diseases related to the body's immune system. Calamansi orange peel is a waste from the calamansi syrup industry which is widely available in Bengkulu. This can be used to extract the essential oil from the peel of calamansi orange which is known to have high antioxidant activity. The purpose of this study was to determine the potential immunomodulatory activity of calamansi orange peel essential oil on mouse lymphocyte cell proliferation in vitro. The essential oil was obtained by steam-water distillation method. immunomodulator test was carried out by taking essential oils with varying concentrations, namely 6.25- 12.5- 25- 50- and 100 g/mL. Isolated lymphocyte cells were obtained from the spleen of Swiss Webstern strain mice. Lymphocyte cell proliferation test was performed using MTT Assay and absorbance was measured using an ELISA reader at 550 nm. In this study, the essential oil yield was 2.13%. The results of the immunomodulator activity test analysis showed that the essential oil of calamansi orange peel has potential immunomodulatory activity against lymphocyte cell proliferation. The highest immunomodulatory activity was obtained at an essential oil concentration of 50 g/mL with an optical density (OD) value of 0.348.

Keywords: *In vitro*; calamansi orange peel; *Citrus microcarpa* Bunge; lymphocyte cell

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Immunomodulator is the most important part of the treatment process related to the immune system. Immunomodulators help the body to optimize the function of the immune system, which is the main system that plays a role in the body's defense from viruses. These immunomodulatory products can be made from synthetic and natural materials, such as from plants. one of the plants that is empirically used as a source of immune-boosting drinks is calamansi orange (*Citrus microcarpa* Bunge).

Calamansi are classified as citrus fruits, including lemons and limes, have orange flesh, watery, sour, and have a similar taste to lime [1]. Calamansi orange peel is a waste from the calamansi syrup industry which is widely available in Bengkulu. This can be used to extract the essential oil from the peel of calamansi orange which is known to have high content of flavonoids [2], antibacterial activity [3], and contains antioxidant compounds [4].

Therefore, this research needs to be carried out in the hope that it can add scientific evidence that this plant can be used as an immunomodulator for companion therapy in infectious diseases that can reduce immune responses (such as COVID-19). The purpose of this study was to determine the immunomodulatory activity of calamansi orange peel against the proliferation of mouse lymphocyte cells in vitro.

METHODS

This research was carried out in the workshop of the science laboratory study program, the faculty of mathematics and natural sciences and the biomedical and research laboratory of the faculty of medicine and health sciences, Bengkulu University. This research method consists of three stages, namely calamansi orange peel essential oil extraction, calamansi orange peel essential oil characterization test and immunomodulatory activity test.

Essential oil extraction of calamansi orange peel was steam-water distillation method. Calamansi orange peel that has been cleaned cut into small pieces. Furthermore, the calamansi peel pieces are dried in the sun to reduce the water content in the sample.

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Table 1. Yield of calamansi orange peel essential oil.

	Yield (%)
This Research	2,31%
[9]	0.326%
[10]	0.49 %

The sample is then mashed using a blender to reduce the surface area. Then the sample is put into a steam-water distillation apparatus, then heated at a temperature of 100 degrees Celsius for 4 hours until there is a distillate. The distillate that comes out is then separated between oil and water using a separatory funnel, then the oil is purified using anhydrous Na_2SO_4 [5].

Furthermore, the characterization of calamansi orange peel essential oil, including the calculation of yield, refractive index, Density, optical rotation, and solubility in 70% alcohol was carried out. Finally, the immunomodulator test was carried out with the following stages: Preparation of Test Solution, Isolation of Mice Lymphocyte Cells, Cell Proliferation Test Through MTT Assay Using ELISA Reader [6-8].

RESULTS AND DISCUSSION

The Yield Calamansi Orange Peel Essential Oil

In this study, the yield of calamansi orange peel essential oil was 2.13%. The yield produced in this study was higher than the yield obtained by [9-10]. this can happen due to several things such as the distillation method, distillation temperature, distillation time, dosage form, location for taking raw materials, and others.

Calamansi Orange Peel Essential Oil Characterization

The color of the calamansi orange peel essential oil is clear white. And turbidity is transparent (Figure 1a). While for the smell, calamansi orange peel essential oil has a distinctive smell of Calamansi orange (Table 2). Density is an important criterion for the quality and purity of essential oils. In general, the density of essential oils is less than 1.00. From this research, the density of the essential oil from the peel of the calamansi orange is 0.8135. not much different from previous studies (Table 2).

Refractive Index is a physical property that is useful in fluid analysis. Often a liquid can be identified by determining its index of refraction. The refractive index can also measure the level of purity of a liquid being analyzed by comparing the value of the sample's refractive index with pure liquid in the literature. From the table 2, it can be seen that the refractive index of the orange peel essential oil is close to the refractive index in other literatures.

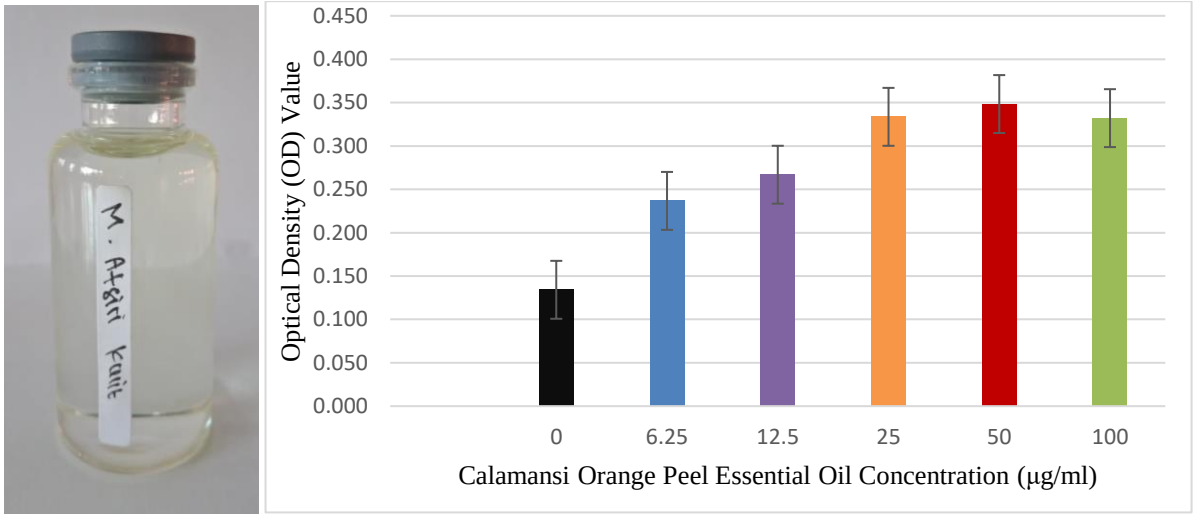
Optical rotation is a physical property of a substance or compound which is a measure of optical activity caused by the interaction of light with the substance or compound being analyzed. Most essential oils when irradiated will rotate the plane of polarization to the right (dextroratory) or to the left (laevorotatory). From this research, it is known that the essential oil of calamansi orange peel undergoes an optical rotation to the right of 88.4 degrees (Table 2).

Most essential oils are slightly soluble in water and soluble in absolute alcohol, so it is possible to determine the volume of dissolved alcohol required to completely dissolve one unit volume of essential oil. Determination of solubility is a fast and good method to evaluate the quality of essential oils. In this study, it was found that the solubility of essential oil in alcohol was 1:2, which indicates that the essential oil of calamansi orange peel is rich in oxygenated compounds.

Table 2. Physicochemical Properties of Calamansi Orange Peel Essential Oil characterization.

Physicochemical Properties	**This Research**	**[9]**	**[11]**
Color	clear white	-	-
Turbidity	transparent	-	-
Smell	Calamansi orange smell	-	-
Density (g/mL)	0,8135	0,8428	0,824
Refractive index	1,4738	1,4653	1,4762
Solubility in 70% alcohol	1:2	1:7	-
Optical rotation	+88,4	-	-

The Test of Immunomodulatory Activity on the Proliferation of Mice Lymphocyte Cells



(a) (b)

Figure 1. The color of the calamansi orange peel essential oil (a) and value of optical density (OD) calamansi orange peel essential oil on mice lymphocyte cell proliferation (b).

The immunomodulatory activity is proportional to the obtained optical density value. The results show that the OD value will increase with increasing essential oil concentration, which indicates that the number of living cells is also increasing. Essential Oil for each concentration (6.25 until 50 µg/ml) increased the OD value compared to the negative control, and decrease on the concentration 100 µg/ml. This proliferation-increasing activity is probably due to the content of flavonoids, limonene from Kalamasi Orange Peel. Flavonoids can increase the proliferation of lymphocyte cells because they can increase the production of IL-2.IL-2 has a significant role in activating T lymphocyte cells to proliferate. The binding of IL-2 regulates Antigen-stimulated T lymphocyte proliferation to its receptor. In addition, IL-2 also stimulates the proliferation and differentiation of B lymphocytes and Natural Killer (NK) cells [6].

Calamansi Orange Peel Essential Oil with 50 µg/mL can produce the highest proliferative activity (optimum concentration), and the average OD value reaches 0.3465 ± 0.003. This result is higher than proliferative activity from VCO by same methode [8] This result is nearly the same as the positive control, Phytohemagglutinin (PHA), which can reach an OD value of 0.408 [6].

CONCLUSION

It can be concluded that the essential oil of calamansi orange peel has potential immunomodulatory activity against lymphocyte cell proliferation. The highest immunomodulatory activity was obtained at an essential oil concentration of 50 g/mL with an optical density (OD) value of 0.348.

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