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Immunostimulant Activity of Moringa Leaves (Moringa Oleifera Lam.) Combined with Lime Peel (Citrus Aurantifolia) in Vitro

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ABSTRACT

Moringa leaves (Moringa Oleifera Lam.) are known to have many chemical contents such as vitamin A, vitamin C, flavonoids, phenolic compounds, carotenoids, tannins and triterpenoids with high uses, one of which is as an immunostimulant. Previous researchers reported that moringa leaves have been developed in various preparations but have not been able to cover the problem of the smell and taste of moringa leaves that are less accepted by consumers. In this study, moringa leaves were combined with lime peel ginger (Citus aurantifolia) to cover the taste and smell of moringa leaves. This study aims to determine the immunostimulant activity of moringa and lime peel in three formulas. Formula F1= moringa:lime peel (1:1); F2= moringa:lime peel (2:1) F3= moringa:lime peel (3:1); F4= moringa extract. The research conducted was experimental research with the research design used was a completely randomised design and three different treatments with three repetitions. Each treatment was repeated 3 times so that 12 experimental units were obtained. Statistical analysis using the probit test. The results of plant identification showed true moringa leaves and lime peel with chemical content of flavonoids, tannins, terpenoids, alkaloids, saponins based on the results of colour recreation. The IC50 value of immunostimulant activity test of the best formula of tea as immunostimulant is F4, F3, F2 and F1 respectively: 701.83 ± 113.36; 701.83 ± 113.36; 488.08 ± 139.60; 488.08 ± 139.60 µg/mL. Overall, the formulas have the potential to be developed as immunostimulants.

Keywords: *Immunostimulant, Lime Peel, Moringa*

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INTRODUCTION

Immunostimulants are a form of immunomodulators that function to boost the immune system against viral infections, which have proven to be an alternative therapy in handling covid-19 and are very much explored lately while still implementing health protocols. Various drugs can be used to modulate the immune system, but the high price of synthetic drugs makes medicinal plants an affordable source as an immunostimulant development material. Indonesia has high biodiversity, one-third of the 6000 species used in traditional medicine have been equipped with scientific data. This encourages most Indonesians to start using medicinal plants to maintain health and treat diseases.

One of the plants used for treatment is the Moringa plant. This plant is very easy to grow in East Nusa Tenggara and especially on the mainland of Timor island which is recorded to have the second best quality after Spain. Moringa leaves contain many active compounds such as vitamin A, vitamin C, flavonoids, phenolic compounds, carotenoids, and triterpenoids. Research using moringa leaf extract showed an increase in macrophage activity in its role as an immunostimulant. In another study, 4 new compounds were found in the methanol extract of moringa leaves, namely: 9,12,15-octadecatrienoic acid ethyl, 6-octadecenoic acid, cis-vaccenic acid and 2-octyl-cyclopropaneoctanal which have potential as immunomodulators.

People usually use moringa leaves by boiling them to make vegetables. Modifications of moringa leaf preparations have been made, among others, in the form of dry powder as additional nutrients in children's food and additional formulations into processed food products to increase their nutritional value such as soy meatball, moringa candy, and served in the form of tea. Moringa leaf tea made by steam blanched method at 650C for 4 hours has an aroma, taste that is preferred by all panelists.

The widespread use of moringa in the community but there is no scientific data on the immunostimulant activity of moringa tea, so it is important to assess the immunostimulant effect of the preparation. Moreover, the availability of moringa, which is easily available in the Kupang-NTT area and the technology used is easy to apply, also supports this research. Moringa leaf tea will be made as a product packaged in bags made of filter paper as teabags. Moringa leaf tea is formulated by adding ginger and orange peel to increase immunostimulant potential and give a distinctive flavour. Testing of its immunostimulant activity was conducted in vitro.

RESEARCH METHODOLOGY

The research method used is experimental research method. The research design used was a Randomised Group Design (RAK) consisting of 4 different treatments including: F1= moringa:orange peel (1:1); F2= moringa:orange peel (2:1) F3= moringa:orange peel (3:1); F4= moringa extract. Each treatment was repeated 3 times to obtain 12 experimental units. Statistical analysis used probit test. This study aims to assess moringa leaf herbal teabags in terms of their activity as immunostimulants. Several stages were carried out to achieve these objectives, among others: determination of moringa and lime plants, preparation of simplisia, making powder of moringa leaves and lime peel, phytochemical screening, making herbal teabags with a combination of

moringa leaves and lime peel, making extracts and testing immunostimulant activity as a quantitative test parameter.

Plant determination and preparation of moringa and lime leaf powder

Moringa and lime leaves used came from the Penfui area, Kupang Regency, East Nusa Tenggara in May 2022. Moringa leaves collected were green, not too old or young, fresh and intact. Lime rhizomes were taken fresh. Plant identification certification was obtained from Jatinangor Herbarium, Plant Taxonomy Laboratory, Department of Biology FMIPA UNPAD based on determination letter No.47/HB/02/2022.

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Identification of chemical compounds

The material used for chemical content analysis was steeped teabags. The teabags of each formula were dipped up and down with 200 mL of warm water at 40oC for 5 minutes, then the bags were removed from the solution and cooled to room temperature and analysed for chemical content.

a. Flavonoids

   The flavonoid test was carried out using the Wilstater test, namely 2 grams of sample weighed and put into a test tube. The sample was dissolved in 2 mL of warm water and then added a spatula of Mg powder and four drops of HCl2%. If there is a colour change to dark red, it is positive for flavonoids.

b. Identification of triterpenoids

   A sample of 1 mL was added to 3 mL of 70% ethanol and 2 mL of concentrated H2SO4 and 2 mL of anhydrous acetic acid (Liebermann-Burchard reagent). The colour change from purple to blue or green indicates the presence of steroids, while the formation of brownish red colour on the inter-surface indicates the presence of triterpenoids.

c. Identification of tannins

   Samples taken 2mL put into a test tube and added FeCl3 1% as much as 2-3 drops. The formation of a blackish green or dark blue solution indicates the sample contains tannin.

d. Alkaloids

   Alkaloid identification is done by reacting the sample in chloroform and ammonia each 1 mL. The result of the recreation is then heated on a bunsen flame, then shaken and filtered. The filtrate was divided into three equal parts. In each part, 3 drops of H2SO4 2N were added, shaken and allowed to separate. The supernatant was taken to be reacted with several reagents and observed the colour of the

precipitate. The sample was positive for alkaloids if the Meyer reagent identified an orange precipitate, a brown precipitate on the Wagner reagent and on the Dragendorf reagent formed a white precipitate.

e. Saponins
The sample is heated to boiling using 5 mL of distilled water and filtered. The filtering results were shaken and then allowed to stand for 15 minutes. Positive samples of saponins are indicated by the formation of foam.

Immunostimulant activity test

a. RAW 264.7 Cell Subculture
Subculture of RAW 264.7 cells begins with thawing the cells taken from the nitrogen tank at room temperature. DMEM complete medium (MK-DMEM) was prepared consisting of sterile DMEM medium, 10% FBS and 1% penicillin-streptomycin.
A total of 3 mL of MK-DMEM was put into a conical tube and 1000 µL of liquid cell suspension was added. The mixture was resuspended first, then MK-DMEM was added up to 14 mL and centrifuged for 10 minutes at 9000 rpm. The supernatant was discarded and the pellet obtained was added 4 mL MKDMEM and resuspended until homogeneous. RAW 264.7 cells were then grown in 8 mL MK-DMEM in a culture flask and observed for cell condition, then stored in a 5% CO2 incubator at 37℃. After the media became yellow, the culture media was replaced and the cells were grown until confluent until the number was sufficient for the next treatment.

b. Harvesting of RAW 264.7 Cells
Cells were observed under an inverted microscope, if the number was 80% confluent the cell media was removed and the confluent cells were washed using PBS solution.
Cells are washed with 1 mL PBS (for flasks that are rather turbid, washing is done twice) then the solution is discarded. The function of PBS solution is as a cell washer to remove serum and remove cells that have died. Then a cell scrapper was used to release cells from the matrix. MK-DMEM media was added 2-5 mL and resuspended. Cells were transferred to a conical tube and centrifuged at 1200 rcf for 5 min. The supernatant was discarded and the pellet containing the cells was added 1 mL of media and resuspended again, then cell counting was done.

c. RAW 264.7 Cell Viability Test
RAW 264.7 cells were grown in MK-DMEM. RAW 264.7 cells (1 x 104 cells/well) were grown in 96-well plates and incubated for 24 hours in a 5% CO2 incubator. After 24 hours, cells were ready for treatment when they were 80% confluent. Remove the cell media (turn the microplate 180°) on a paper towel gently pressed the microplate and added 100 µl of PBS into all wells filled with cells, then remove the PBS by turning the microplate over the paper towel.

A total of 100 µL of test solution with a concentration of 0.781-200 µg/mL was added to the microplate wells except for the media control and cell control wells. In the media control, DMSO control and cell control wells, 100 µL of MK-DMEM was added, then the microplate was incubated for 24 hours in a 5% CO2 incubator at 37°C. After 24 hours at the end of incubation, the media and test solution were removed and the cells were washed with PBS. In each well, 100 µL of culture medium and 10 µL of 5 mg/mL MTT were added. To observe viability, the cells were incubated again for 4-6 hours in a 5% CO2 incubator at 37°C. The MTT reaction was stopped with stopper reagent (10% SDS in 0.1 N HCl), then the microplate was wrapped with aluminum foil to make it opaque at room temperature and left overnight. Living cells react with MTT to form a purple color. The test results were read with a microlempenge reader at a wavelength of 595 nm.

Cell viability was calculated by the formula:
% Live Cells = ((Sample Absorbance-Media Control Absorbance))/ ((Cell Control Absorbance-Media Control Absorbance)) x 100.

RESULT AND DISCUSSION

Determination of moringa and lime plants, making powder of moringa and lime leaves, making herbal tea bags combined with moringa and lime leaves, quality testing of tea bags: phytochemical screening, water content, ash content, shelf life, hedonic and antioxidant activity as quantitative test parameters.

Determining the characteristics of ingredients is important to assess the level of quality of ingredients according to standards based on two parameters, namely specific and non-specific26. Assessment for specific parameters including identity, organoleptic and chemical content of simplisia as in table 1.

Table 1. Specific parameters of moringa and lime simplisia powder

No	No. Testing parameters	Results
1	Plant identity	1. Sinonim : Moringa zeylanica Burmann Local Name: Kelor Family : Moringaceae 2. Nama ilmiah: *Citrus aurantiifolia* Sinonim : *Zingiber officinale* var. Rubrum Theilade Local Name: Jeruk nipis Family: Rutaceae
2	Organoleptic	Moringa leaf simplisia powder: green color, characteristic odor, tasteless.
3	Chemical content of simplisia powder	Lime peel simplisia powder: brownish green in color, characteristic lime odor, citrus taste.

Plant determination is the first step taken in this study with the aim of determining the correctness of plant identity. Determination of moringa and lime plants was carried out at Herbarium Jatinangor, Plant Taxonomy Laboratory, Department of

Biology FMIPA UNPAD. The determination results showed that the plants used were true moringa plants (Moringa oleifera Lam.) with the Moringaceae family and lime (Citrus aurantiifolia) from the Rutaceae family.

The plant parts used as samples are moringa leaves and lime peel. Each simplisia powder is carried out phytochemical screening with a color reaction to determine the content of active compounds. Screening results showed that both symposia positively contained flavonoids, tannins, terpenoids, alkaloids and saponins.

Immunostimulant test data collection techniques were carried out through laboratory tests. The testing stages include: RAW 264.7 cell sub-culture, RAW 264.7 cell harvesting and RAW 264.7 cell viability test. The test results illustrate the viability of RAW 264.7 cells against the administration of the test formula using a concentration variation of 62.5-1000 µg/mL with the following results:

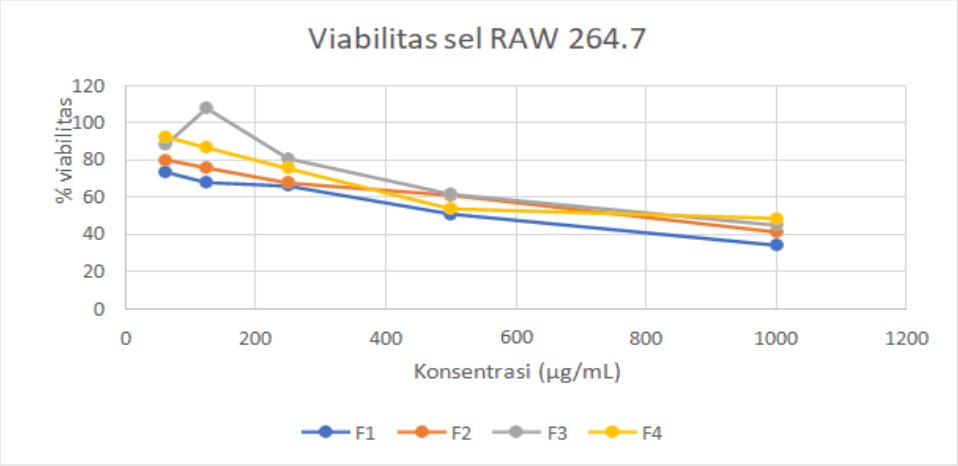

Figure 1. Viability of RAW 264.7 cells against the administration of the test formula

*Data berasal dari 3x pengujian independent F1= kelor:kulit jeruk (1:1)
F2= kelor:kulit jeruk (2:1)
F3= kelor:kulit jeruk (3:1)
F4= ekstrak kelor

Immunostimulant activity is expressed by IC50 which states 50 percent of moringa tea samples inhibit the growth of magrophages with IC50 values of each formula as in the following table:

Formula	IC_{50} (rata-rata ± SE; µg/mL
F1	429,76 ± 41,25
F2	488,08 ± 139,60
F3	663,99 ± 178,39
F4	701,83 ± 113,36

*Data berasal dari 3x pengujian independent
F1= kelor:kulit jeruk (1:1)
F2= kelor:kulit jeruk (2:1)
F3= kelor:kulit jeruk (3:1)
F4= ekstrak kelor

Tea products for each formula have immunostimulant activity with the highest activity in F4 (Moringa extract). In conclusion, the best formulas of tea as immunostimulants are F4, F3, F2 and F1, respectively.

CONCLUSION

Moringa in single form or in combination with lime peel, has the potential to be developed as an immunostimulant with in vivo evidence.

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