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Research Article
Phytochemical Analysis and Immunomodulatory Potential on Diabetic-Infected Tuberculosis by Fruit *Etlingera rubroloba* A.D. Poulsen

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Abstract

Background and Objective: *Etlingera rubroloba* (*E. rubroloba*) A.D. Poulsen is an endemic plant in South-East Sulawesi and is a newly discovered species. This plant is expected to have the potential as an immunomodulator in patients with diabetes mellitus (DM), which can prevent tuberculosis infection by increasing the phagocytic function of macrophage cells and interleukin-12 (IL-12) levels. **Materials and Methods:** Phytochemical analysis of the ethanolic extract of the fruit of *E. rubroloba* A.D. Poulsen using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) was carried out. The immunomodulatory potential *in vivo* on BALB/c mice model DM was carried out by oral induction of TB antigen with extract dose, control positive, negative and normal groups. Furthermore, the phagocytic activity of macrophage cells can be seen with a microscope and the levels of IL-12 with the Elisa kit. **Results:** The results showed the ethanol extract of the fruit of *E. rubroloba* contained eight chemical compounds and had potential as immunomodulators in BALB/c DM mice induced by TB antigen by increasing the phagocytic activity of macrophage cells and levels of IL-12, which were significantly different from the negative control ($p<0.05$). **Conclusion:** The chemical composition of the ethanol extract of the fruit of *E. rubroloba* has the potential as an immunomodulator in TB antigen-induced DM *in vivo*.

Key words: *Etlingera rubroloba*, phagocytosis, interleukin-12 (IL-12), Diabetes Mellitus-Tuberculosis (DM-TB), Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The prevalence of diabetes mellitus (DM) globally is still increasing and the reported prevalence of type 2 DM with complications of Tuberculosis (TB) is around 1.9-45%[1-3]. The presence of a decreased immune response in DM sufferers results in easy conflict with infectious disease, TB, caused by the bacterium *Mycobacterium tuberculosis* (Mtb)[3,4].

Preventing DM patients from becoming infected with TB can be done by an optimal innate immune system mechanism from the host through an effective phagocytosis process from phagocytic cells such as macrophages in the early invasion of microorganisms against Mtb bacteria[5,6] Macrophages are professional phagocytes responsible for destroying cells infected with intracellular pathogens and targets for Mtb bacteria[4,5,7]. Macrophages produce proteolytic enzymes and cytokines, which in turn kill bacteria, such as secreting Interleukin-12 (IL-12). IL-12 has a role in inducing T helper-1 lymphocyte and IFN-g secretion from T helper-1 and natural killer cells[4,8] Improving the function of macrophage phagocytic activity with immunomodulatory substances is one of the efforts to prevent DM patients from being easily infected with TB[9].

Giving natural immunomodulatory agents in DM and TB should be considered as supportive therapy for the main drug in these diseases so that the healing period is shorter due to the optimal role of the immune system and the side effects of using immunosuppressant drugs[10,11].

Natural immunomodulators are sourced from natural ingredients, one of which is the genus *Etlingera*. Plants of the *Etlingera* genus have been shown to have immunomodulatory potential, such as *E. elatior* (Jack) R.M. Smith[12]. Another species of *Etlingera* is *Etlingera rubroloba* (*E. rubroloba*) A.D. Poulsen, which grows endemic to South-East Sulawesi and has a taxonomic affinity with *E. elatior* (Jack) R.M. Smith and its pharmacological activity or chemical content of this plant which is thought to have an immunomodulatory effect[13]. The pharmacological activity of *E. rubroloba*, fruit ethanol extract, has an immunomodulatory effect by increasing the activity of T helper lymphocyte (CD4) cells[14] and isolating a compound from methanol stem extract of *E. rubroloba* has pharmacological activity as an antioxidant and inhibits xanthine oxidase[15,16], In addition, fraction from the ethanol extract of the fruit of *E. rubroloba* which has an immunomodulatory effect on mice infected with Mtb antigen by increasing the phagocytic activity of macrophage cells and level of Interleukin-12 (IL-12)[8].

Seeing the potential and the lack of scientific reports on this plant, especially the immunomodulatory effect on diabetes mellitus, is not yet known. Hence, researchers are interested in testing the immunomodulatory potency of the ethanolic extract of *E. rubroloba* fruit on macrophage cell phagocytosis and IL-12 levels in male BALB/c mice modelling diabetes mellitus, which is induced by the Mtb antigen. The results of this study can be further developed as a natural immunomodulator in DM patients so that they are not easily conflicted with infectious diseases such as TB.

MATERIALS AND METHODS

Study area: This study was conducted Pharmacy Laboratory, Faculty of Pharmacy and Medicine Laboratory, Faculty of Medicine, Universitas Halu Oleo Kendari, Southeast Sulawesi, Indonesia from August, 2021 to January, 2022.

Materials: The material used in this study was the fruit of *E. rubroloba*, BALB/c strain mice, BCG vaccine (Bio Farma®), 96% ethanol (Mercks®), 70% alcohol (Mercks®), barbiturate anaesthetic (Thiopental Sodium KF®), streptozotocin (Sigma®), 5% dextrose (Otsu-NS®), Na.CMC 0.5% (Food Grade®), Elisa rat IL-12 kit (BT LAB®), 1 cc syringe (OneMed®), glucose strip (Easy touch®), EDTA Vacutainer tube (BD Vacutainer®), phosphate-buffered saline/PBS (Sigma®), Na buffer. Citrate pH 6.0 (Sigma®), Giemsa (Merck®), oil immersion (Merck®) and commercial meniran extract (Dexa Medica®).

Methods

Sample preparation and extraction: The sample used was the fruit of *E. rubroloba* A.D. Poulsen is from Punggaluku Village, Punggaluku District, South Konawe Regency, Southeast Sulawesi. A wet sample of 17.5 kg was made of 3.3 kg of dry simplicia and then extracted by the maceration method using 96% ethanol in a ratio of 1:2 (3×24 hrs). The obtained filtrate was concentrated with a rotary vacuum evaporator at 50°C to get a thick extract[8,14]. The extract was then weighed to determine its weight.

Phytochemical analysis: The content of secondary metabolites of the ethanolic extract of the fruit of *E. rubroloba* was determined using Liquid Chromatography-Mass Spectrometry (LC/MS-MS) analysis Xevo G2-XS QTOF (Milford, USA). The LC-MS/MS analysis used a mobile phase of a mixture of acetonitrile in 0.1% formic acid and 0.1% formic acid in water. The sample is introduced into the mobile phase stream by injecting 1 µL of the sample. The compounds in the sample will be separated based on their polarity and different retention times, which will then be observed in a spectrum

with separate peaks in the form of a chromatogram. The molecular weight, structure, identity and amount of components in the sample were known from the LC/MS-MS data[17-19].

Modeling of diabetes mellitus BALB/c mice: The health research ethics committee has approved this research, Institute for Research and Community Service, Universitas Halu Oleo number: 706/UN29.20/PPM/2020. Thirty male BALB/c strain mice were modelled for DM by inducing diabetogenic Streptozotocin (Stz) at a dose of 150 mg kg^{-1} body weight intraperitoneally dissolved in Na. buffer citrate pH 6.0 in a dark glass container[20-22]. The overall DM condition of mice was obtained two days after Stz induction with an average fasting glucose level of >200 mg dL^{-1} [23,24].

Animals treatment: Thirty-five healthy DM BALB/c male mice were divided into six groups randomly. The treatment of the test animals was carried out once a day for 7 days orally according to the volume of administration[8], Then on the eighth day, 0.1 mL of BCG antigen was induced by IP in mice in the extract group, with positive and negative control, then waited for 3 hours and then surgery was performed to collect peritoneal fluid and blood[25].

The distribution of treatment groups is as follows:

P_1 : Stz (150 mg kg^{-1} b.wt.) + ethanol extract dose of 100 mg kg^{-1} b.wt. + BCG antigen

P_2 : Stz (150 mg kg^{-1} b.wt.) + ethanol extract dose 200 mg kg^{-1} b.wt. + BCG antigen

P_3 : Stz (150 mg kg^{-1} b.wt.) + ethanol extract dose 300 mg kg^{-1} b.wt. + BCG antigen

K^+ : Stz (150 mg kg^{-1} b.wt.) + Phylantii extract 0.067 mg kg^{-1} b.wt. + BCG antigen

K^- : Stz (150 mg kg^{-1} b.wt.) + Na. CMC 0.5% + BCG antigen

N : Group without treatment

Phagocytosis assay: Mice were anaesthetized with thiopental sodium and then dissected. Peritoneal fluid was taken, then stained on an object glass and fixed with methanol for 5 min, then 10% Giemsa staining was performed for 20 min, rinsed with running water and dried. Phagocytic activity was observed under a microscope with magnification (100-1000x)[8,9]. The phagocytic activity of macrophage cells was determined by calculating the value of phagocytic activity (SPA), the percentage of macrophage cells that actively perform phagocytosis among 100 macrophage cells[12]:

$$\text{Phagocytic activity} = \frac{\text{Amount of active macrophage cells}}{\text{Total macrophage cell amount}} \times 100\%$$

IL-12 level assay: Mice that had been euthanized were taken from the heart, collected in a vacutainer tube containing 0.1% EDTA anticoagulant and centrifuged at 3000 rpm at 25°C for 20 min. The plasma obtained was put into an Eppendorf tube and then the levels of IL-12 were checked using the Elisa mouse IL-12 kit (BT LAB®) following the protocol in the kit. Absorbance measurement on Elisa's plate reader at a wavelength of 450 nm[8,26].

Statistical analysis: Immunomodulator test results data are interpreted in the form of average percentages of phagocytic and Levels of IL-12. A One-way Analysis of Variance (ANOVA) followed by a *post hoc* LSD test showed significant differences between groups. Statistical analysis with a $p<0.05$ is considered significant[8].

RESULTS

Fresh fruit of *E. rubroloba* A.D. Poulsen obtained 17.5 kg from Punggaluku Village, Laeya District, South Konawe Regency, with a dry weight of 3.3 kg. It then carried out the extraction process, obtaining 424.6 grams of thick extract with a yield of 12.86%.

The Phytochemical analysis test of *E. rubroloba* ethanol extract used the LC-MS/MS method to determine the molecular weight, molecular structure and types of compounds in the extract, as shown in Table 1.

There are eight types of compounds, five compounds whose names and chemical structures have been detected and three compounds in the form of candidate mass in compounds (Fig. 1a-e). Those may be new compounds from this plant, so further investigation is needed to prove the possibility that is a new compound.

The results of determining the phagocytic activity of macrophage cells in Fig. 2 showed the highest average % of macrophage cell phagocytic activity in the dose group of 300 mg kg^{-1} b.wt. (68.00±2.94%) and 200 mg kg^{-1} b.wt. (59.33±2.89) compared to Phylantii extract commercial (positive control) 0.067 mg kg^{-1} b.wt. (55.00±2.65).

Statistical data analysis in Fig. 2 using ANOVA on the phagocytic activity of macrophages obtained a significant (*$p<0.05$), indicating a significant difference in the increase in the phagocytic activity of macrophages. Furthermore, do the test post hoc LSD so that the results are obtained, namely, the dose group of 300 and 200 (mg kg^{-1} b.wt.) significantly different from the positive control (*$p<0.05$), which means that the extract dose has different immunomodulatory potential with positive control in increasing phagocytic

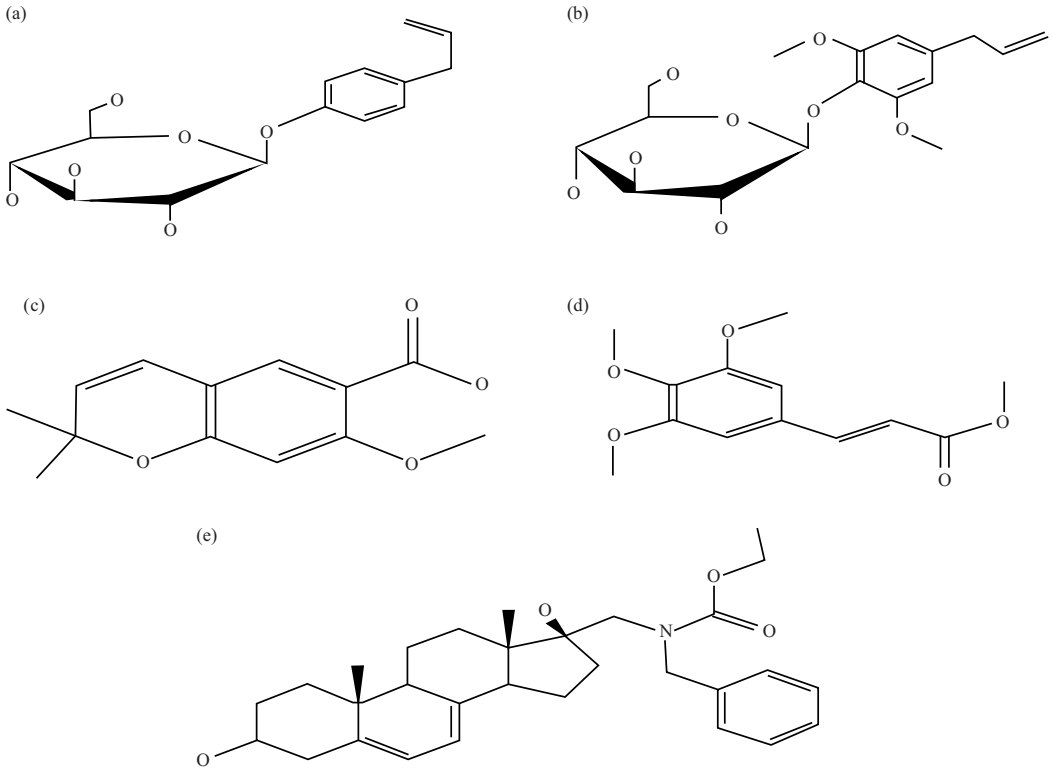

Fig. 1(a-e): Molecular structure of *E. rubroloba* fruits ethanol extract using LC-MS/MS method, (a) Chavicol-β-D-glucoside, (b) Erigeside II, (c) 2-Methoxyanofinic acid, (d) Methyl 3,4,5-trimethoxycinnamate and (e) Ethyl benzyl [((3beta,17beta)-3.17-dihydroxyandrosta-5,7-dien.1,7-yl) methyl] carbamate

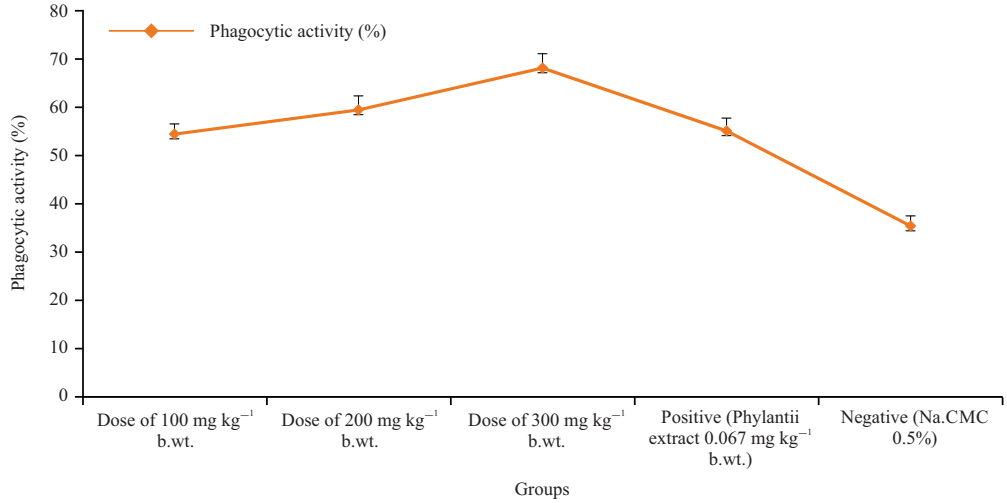

Fig. 2: Phagocytic activity of macrophage

activity in macrophage cells. In contrast, the 100 mg kg^{-1} b.wt., dose group had no significant difference from the positive control (*p>0.05).

Assay for IL-12 levels using the Elisa sandwich method, the FITC anti-IL-12 anti-rat kit (BT LAB®). The measurement of the average level of IL-12 in each treatment in Table 2 showed the highest average level of IL-12 was in the dose group of 300 mg kg^{-1} b. wt., followed by the dose group of 200 mg kg^{-1} b.wt., compared to the positive dose group as a positive control. The results of the measurement of IL-12 levels can be seen in Table 2.

Table 1: Compounds identified in the ethanol extract of the fruit of *E. rubroloba* by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method

Compounds	Observed m/z (Mr)	Chemical formula	Observed RT (minutes)	Compound group
2-Methoxyanofinic acid	235	$C_{13}H_{14}O_4$	9.09	Terpenoids
Chavicol-β-D-glucoside	319	$C_{15}H_{20}O_6$	9.19	Phenylpropanoid-glycoside
Erigeside II	379	$C_{17}H_{24}O_8$	6.26	Phenylpropanoid-glycoside
Methyl 3,4,5-trimethoxycinnamate	253	$C_{13}H_{16}O_5$	7.12	Phenylpropanoid
Candidate mass. C9H12O8	249	$C_9H_{12}O_8$	1.57	Terpenoids
Candidate mass. C3H8O7	157	$C_3H_8O_7$	0.87	Terpenoids
Candidate mass. C35H46N2O5	597	$C_{35}H_{46}N_2O_5$	5.39	Alkaloids
Ethyl benzyl[((3beta,17beta)-3,17-dihydroxyandrosta-5,7-diem-17-y) methyl] carbamate	480	$C_{30}H_{41}NO_4$	9.31	Alkaloids

Table 2 : Average levels of IL-12

Groups	Average levels of IL-12 (ng mL^{-1})
Normal	8.09±0.36*
Negative control (Na.CMC 0.5%)	10.03±0.31
Positive control (Phylantii extract 0,067 mg kg^{-1} b.wt.)	12.22±0.23*
Dose of 100 mg kg^{-1} b.wt.	11.32±0.45*
Dose of 200 mg kg^{-1} b.wt.	17.2±0.19**
Dose of 300 mg kg^{-1} b.wt.	19.41±0.53**

Results are shown as Mean±SEM (n = 5), *p<0.05 and **p<0.01 significantly different from negative control group

The results of the one-way ANOVA test for IL-12 levels showed that all treatment groups gave significant differences in IL-12 levels (p<0.05) and the *post hoc* LSD test showed that all groups of extract doses were significantly different from the negative control group (*p<0.05) and in the dose groups 200 and 300 (mg kg^{-1} b.wt.) were very significantly from the negative control group (**p<0.01). It showed that the ethanolic extract of the fruit of *E. rubroloba* has very potential as an immunomodulator based on IL-12 levels.

DISCUSSION

The researchers test the immunomodulatory potential of the ethanolic extract of *E. rubroloba* fruit *in vivo* by measuring the parameters of macrophage cell phagocytic activity and IL-12 levels in the BCG antigen-induced DM model mice[8]. Induction of BCG antigen to stimulate innate immune cells, especially macrophage cells as the first line of the innate immune system and at the same time as a target for Mtb bacteria, so that macrophages can respond about 0-12 hrs after Mtb antigen infection by phagocytosis[27].

Phylantii extract commercial was used as a positive control because it was proven to have immunomodulatory activity by optimizing the function of the immune system[28]. In addition, is a phytopharmaceutical product that has gone through preclinical and clinical tests and is often used as a comparison material in research on immunomodulatory tests of natural ingredients[10,29].

The phagocytic activity of macrophages and levels of IL-12 in DM mice induced by BCG antigen in Fig. 2 and Table 2 showed that the ethanolic extract of *E. rubroloba* fruit had immunomodulatory potential by increasing macrophage phagocytic activity and IL-12 levels. This increase was thought to be due to the role of chemical compounds in the ethanolic extract of *E. rubroloba* fruit by improving the immune system work. It was supported by the phytochemical analysis in Table 1, which showed that the fruit extract of *E. rubroloba* contains the chemical compound 2-Methoxyanofinic acid and Chavicol-β-D-glucoside as antioxidants by increasing the proliferation of lymphocyte cells[11,18,30]. Besides contains a class of compounds flavonoids, alkaloids and triterpenoids that have been shown to have potential as immunomodulators[9,14].

Alkaloids, triterpenoids and flavonoids have immunostimulating effects because they are mitogens by increasing the proliferation of T and B lymphocytes, which are thought to be through the production of IL-12 cytokines, where IL-12 will trigger the activation of macrophage cells to carry out phagocytosis of pathogens[26,31]. The proliferation of T lymphocyte cells will further enhance the function of phagocytic cells such as macrophages in phagocytosing Mtb antigens[8,31]. In addition, it increases the activation of T helper lymphocytes, which differentiate into T helper-1 lymphocytes. Then, it produces IFN-γ and TNF-α cytokines and stimulates natural killer cells, activating macrophages to produce IL-12 and nitric oxide compounds beneficial for phagocytosing antigens[32,33].

The current study found an immunomodulatory effect of *E. rubroloba* fruit extract by increasing the phagocytic activity of macrophage cells and IL-12, thereby potentially improving immune system function in DM patients and preventing TB infection. Previous studies supported the findings of this study by Ilyas *et al.*[8,14] that *E. rubroloba* fruit increased the activity of T helper lymphocytes (CD4), IL-12 and cytotoxic T lymphocytes (CD8) cells.

The immunomodulatory activity of the ethanolic extract of *E. rubroloba* has implications for the discovery of new

immunomodulatory agents for DM so that they are not easily infected with TB disease. However, further studies are needed to provide broader pharmacological data. Phytochemical analysis data in the extract contained several compounds that have the potential as immunomodulators to provide immunomodulatory activity and to find out the compounds that provide immunomodulatory activity, it is necessary to isolate the compounds contained in the extract.

CONCLUSION

Ethanol extract of the fruit of *E. rubroloba* A.D. Poulsen has immunomodulatory potential in BCG antigen-induced DM *in vivo* by increasing the phagocytic activity of macrophage cells and IL-12 levels. The identification results by LC-MS/MS contained eight chemical compounds in the fruit extract of *E. rubroloba* A.D. Poulsen. They showed that some of the compounds contained had potential as immunomodulators. The results of this study can be used as scientific information for the development of *E. rubroloba* A.D. Poulsen as an immunomodulatory agent to prevent TB infection in DM patients.

SIGNIFICANCE STATEMENT

This study found the immunomodulatory potential of *E. rubroloba* A.D. Poulsen, which can be helpful in DM patients, increases the function of the immune system to prevent infection with TB disease. This study will be the basis for further development in finding new candidate immunomodulatory agents. Thus, possible drug candidates from these extracts may be discovered.

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