HPTLC ANALYSIS OF *Curcuma mangga* Val. EXTRACTS AND THEIR IMMUNOMODULATORY EFFECTS ON DELAYED-TYPE HYPERSENSITIVITY RESPONSE

Yuandani^{1,✉}, Sony Eka Nugraha², Lia Laila³, Denny Satria² and Rony Abdi Syahputra¹

¹Department of Pharmacology, Faculty of Pharmacy/Universitas Sumatera Utara, Medan-20155, Indonesia

²Department of Pharmaceutical Biology, Faculty of Pharmacy/Universitas Sumatera Utara, Medan-20155, Indonesia

³Department of Pharmaceutical Technology, Faculty of Pharmacy/ Universitas Sumatera Utara, Medan-20155, Indonesia

✉Corresponding Author: yuandani@usu.ac.id

ABSTRACT

The purpose of the study was to analyze several extracts of *Curcuma mangga* rhizomes by HPTLC and determine their immunomodulatory effects on the Delayed-Type Hypersensitivity (DTH) response in Wistar rats. The *C. mangga* rhizomes were macerated with 73; 83 and 99% ethanol separately. HPTLC analysis was performed to evaluate the content of curcumin in various extracts of *C. mangga*. The immunomodulatory potential on DTH response was investigated by measuring the paw volume. All extracts were tested at the dose of 400 mg/kg bw. Quantitative analysis using HPTLC revealed that among the samples tested, 99% ethanol extract of *C. mangga* contained the highest amount of curcumin (9.94 µg/mL). All the samples tested stimulated DTH response. Amongst the extracts, 99% ethanol extract of *C. mangga* rhizomes demonstrated the highest stimulation on DTH response but it was not different significantly ($P>0.05$). However, the stimulatory activity of 99% ethanol extract of *C. mangga* was higher than levamisole as a positive control ($P<0.05$). The results indicate the presence of curcumin in *C. mangga* extract may contribute to its immunostimulatory activity.

Keywords: *Curcuma mangga*, Immunostimulatory, Delayed-Type Hypersensitivity (DTH) response, Ethanol Extract.
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INTRODUCTION

The immune response system should be modulated to manage and treat a variety of disorders, including inflammation, immunodeficiency, and infectious infections.¹ Substances that are used to increase or decrease the immune system are called Immunomodulators.² There are a variety of chemical immunomodulators on the market. Unfortunately, some of these over-the-counter medications have negative side effects.³ Natural products remain one of the valuable sources of innovative and safe immunomodulatory compounds.⁴ Ethnopharmacological data can be used to give preliminary data in the search for novel pharmaceuticals to discover safer drugs.⁵ The active compounds such as terpenoids, phenolics, and alkaloids are present in medicinal plants.⁶ Some medicinal plants have been proposed to have effects on immune response.⁷ Medicinal plants have been the subject of several ethnobotanical and phytochemical studies. Previous studies have indicated that plants in Genus *Curcuma* such as *Curcuma mangga*, *Curcuma longa*, *Curcuma aeruginosa* and *Curcuma xanthorrhiza* are able to modulate certain lineages of the immune response, thus they can be used to treat various diseases related to immune response.⁸⁻¹¹

Curcumin is one major component present in *Curcuma species*. Our previous study reported that *C. mangga* rhizome contains curcuminoid (curcumin, desmethoxycurcumin and bidesmethoxycurcumin).¹² Curcumin has a diverse set of biological properties, including anti-inflammatory and antibacterial properties.¹³ However, the investigation on curcumin content in *C. mangga* after extraction using various grades of ethanol as well as curcumin concentration and immunomodulatory action has rarely been linked in previous

studies. The current study was conducted to perform High-Performance Thin Layer Chromatography (HPTLC) analysis to evaluate the amount of curcumin in *C. mangga* extracts obtained by maceration using different grades of ethanol as a solvent. Furthermore, the stimulatory effects on delayed-type hypersensitivity response of those extracts were investigated to correlate the activity with curcumin content.

EXPERIMENTAL

Materials and Instruments

The reagents used in this current study were natrium carboxymethylcellulose (Na CMC) (Sigma, USA), ethanol (SmartLab, Indonesia). Mobile phase consists of benzene: chloroform ethanol absolute (Merck, Germany) was also used in this study. A rotary evaporator (Heidolph, Germany), Linomat 5 TLC spotter (Camag, Muttenez, Switzerland), glass chamber 20x20 cm, TLC scanner 3 (Camag, Muttenez, Switzerland), TLC documentation system (Camag, Muttenez, Switzerland) were also used in this study.

Extraction Procedure

The rhizomes were macerated using various grades of ethanol (73%, 83% and 99% ethanol). The dried material (1500 g of *C. mangga* rhizomes) was separated into three parts and steeped in 73 percent ethanol for the first part, the second part was macerated with 83% ethanol, meanwhile, the last part was soaked with 99% ethanol. The extraction using each solvent was repeated two times. A rotary evaporator was used to remove the solvent, yielding different extracts of *C. mangga* rhizomes.

Quantitative Analysis of *Curcuma mangga* extracts using High-Performance Thin Layer Chromatography (HPTLC)

Curcumin was determined in three separate extracts (73 percent, 83 percent, and 99 percent ethanol extracts) using the High-Performance Thin Layer Chromatography technique.¹⁴ Briefly, 10 mg curcumin (PT. FitoLab 99%) was dissolved in a standard solution in 10 mL of methanol. Sample solutions were prepared by dissolving 100 mg *C. mangga* extract (73%, 83% and 99%) in 10 mL of ethanol absolute. Then, the samples were spotted (10 μ L) on a precoated silica gel glass plate 60F245 (20 cm x 20 cm) (E. Merck, Darmstad, Germany) with a micro liter syringe, the space between two spot was 10 mm. The mobile phase was made up of benzene: chloroform : ethanol absolute (45:45:10). The length of neach run was 17 cm and performed in appropriate conditions with a temperature was $25 \pm 2^{\circ}\text{C}$ and % Relative Humidity (%RH) was 60 ± 55 . After that, the plates were dried in the open air. A TLC scanner was used to do densitometric analysis at 425 nm.

Calibration Curve of Curcumin

A standard compound stock solution (1000 $\mu\text{g/mL}$) was dissolved in methanol and spotted on TLC plate 10; 8; 6; 4; 2 μL , then a linear regression equation was used to analyze the data of peak area versus curcumin concentration. The levels of curcumin were determined by the substitution peak area of samples in the regression equation of standard (Curcumin).

Antigen Preparation

The antigen used was activated bacteria. *Staphylococcus aureus* was cultivated in nutrient broth agar (NBA) and incubated for 24 h. Then, the cell concentration was adjusted to 1×10^8 cells/mL using spectrophotometry. OD was measured in 580 nm with 20%T. Thereafter, it was centrifuged (10.000 rpm; 10 minutes; 25°C). The supernatant was discarded. Finally, the cells were resuspended with 1 mL of PBS.

Delayed-Type Hypersensitivity (DTH) Response

The response of DTH was evaluated by measuring paw volume according to a modified previous method.¹⁵ Animals were divided into 5 (five) groups for each condition, including:

1. Na CMC 0.5% suspension
2. 73% ethanol extract of *C. mangga* 400 mg/kg BW
3. 83% ethanol extract of *C. mangga* 400 mg/kg BW
4. 99% ethanol extract of *C. mangga* 400 mg/kg BW
5. Levamisole 25 mg/kg BW

Animals were given a suspension of *C. mangga* extract in Na CMC at a concentration of 0.5%, 72 hours prior sensitization with *S. aureus* (1×10^8 cells/mL) by intraperitoneal injection and continued until 14 days. Meanwhile, the group of negative control received vehicle only (Na CMC 0.5%). As a positive control, levamisole (25 mg/kg bw) was employed. On day 14, the paw volume was measured, then, all the rats were challenged with *S. aureus* (1×10^8 cells/mL) in the hind footpad. Then, after 24 hours the paw volume was measured again and the DTH response was determined. The procedure has been approved with approval number 0260/KEPH-FMIPA/2019.

Statistical Analysis

The data were reported as the mean standard error of the mean (SEM). To determine the normality of the data, the Kolmogorov Smirnov and Shapiro Wilk tests were used. Then, Kruskal-Wallis and Mann-Whitney tests were performed to identify the difference.

RESULTS AND DISCUSSION

Quantitative analysis of *Curcuma mangga* Extracts using HPTLC

Our previous study demonstrated that *C. mangga* rhizomes contain curcuminoids.¹² The purpose of this study was to determine the curcumin content in several extracts produced with various ethanol/water solvents. Curcumin was eluted with an R_f value of 0.69, whereas 0.68, 0.71, and 0.68 were found in 73, 83 and 99% ethanol extracts, respectively (Fig.-1). Calibration curves plotted were linear with a correlation coefficient (r^2) of 0.9964 (Fig.- 2), indicating the linearity of the method used in this study. Table-1 shows that amongst the samples, 99.99% ethanol extract of *C. mangga* rhizomes was found to have the highest amount of curcumin (9.94 $\mu\text{g/mL}$). However, the 73% ethanol extract contained a higher amount of curcumin as compared to 83% ethanol extract of *C. mangga* rhizomes.

Fig.-1: Representative HPTLC Chromatograms of (a) *C. mangga* 99% Ethanol Extract (b) *C. mangga* 83% Ethanol Extract (c) *C. mangga* 73% Ethanol Extract

Table-1: Curcumin Content in *C. mangga* Extracts obtained from HPTLC Measurement

Samples	Rf	Area	Concentration ($\mu\text{g/mL}$)
<i>C. mangga</i> 99% ethanol extract	0.68	30296.2	9.94
<i>C. mangga</i> 83% ethanol extract	0.71	851.7	1.78
<i>C. mangga</i> 73% ethanol extract	0.68	15296.8	5.78

Fig.-2: Calibration Curve of Curcumin at Rf 0.69, detected at wavelength 425 nm

Delayed-Type Hypersensitivity (DTH) Response

Various extracts of *C. mangga* were tested for their effects on delayed-type hypersensitivity response using the paw edema method as described previously¹⁵. All the samples were able to stimulate the cellular immune response as shown in Table-2. Amongst the sample extracts, *C. mangga* 99% ethanol extract demonstrated the highest stimulation in DTH response (Table-2). However, the difference of ethanol/water solvent did not affect the DTH response significantly ($P > 0.05$). In addition, the immunostimulatory activity of *C. mangga* 99% ethanol extract was higher than those of levamisole, as a positive control. DTH response is facilitated by the interaction of T lymphocytes-antigen that causes activation and cytokines release. This type of immune response requires sensitized T cells that respond 24–48 h after exposure to antigens. Thus, in this study, the paw volume was measured 24 h after *S. aureus* injection. T cell activation is followed by proliferation and differentiation. The Th-1, Th-2, Th-17, and CD4 T regulatory (Treg) cells are four subpopulations of CD4 T cells that perform different tasks.¹⁶ Th-1 induces inflammation by stimulating macrophages by producing IFN.¹⁷ The result was in accordance with a prior study that found *C. mangga* ethanol extract to be effective in boosting phagocytosis activity.⁸ The highest amount of curcumin was found in the 99% ethanol extract of *C. mangga*, according to HPTLC analysis. The results indicate that curcumin appears to play a significant role in the immunostimulatory activity of *C. mangga* rhizomes. Curcumin's immunostimulatory effect on dendritic cells (DSc) was also discovered in a prior study.¹⁸⁻¹⁹

Table-2: The Effect of *C. mangga* Extract on delayed-type hypersensitivity (DTH) Response (mean $\pm$ SD)

Samples	Paw Volume (ΔV)
CMC Na 0.5%	0.41 $\pm$ 0.13
Levamisole 25 mg/ kg bw	1.63 $\pm$ 0.09*
<i>C. mangga</i> extract 99% 400 mg/kg bw	2.18 $\pm$ 0.20*
<i>C. mangga</i> extract 83% 400 mg/kg bw	1.61 $\pm$ 0.27*
<i>C. mangga</i> extract 73% 400 mg/kg bw	1.71 $\pm$ 0.43*

* $P < 0.05$ significant to respective control

CONCLUSION

The highest amount of curcumin was found in the 99% ethanol extract of *C. mangga* rhizomes according to HPTLC quantitative analysis. Furthermore, the *C. mangga* 99% ethanol extract demonstrated the highest

stimulation on DTH response, although it was not different significantly as compared to other extracts. 99% ethanol extract of *C. mangga* revealed higher stimulation on cellular immunity as compared to positive control. Thus, further studies are required to elaborate on the mechanism of components in *C. mangga* to modulate the immune response.

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