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Optimization of curcumin extraction from *Curcuma domestica* Vahl. rhizome with microwave-assisted extraction technique in inhibiting nitric oxide production and cell viability

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Abstract

Curcumin is a yellow dye derived from the turmeric plant (*Curcuma domestica* Vahl.). In turmeric rhizome, there are active compounds that are often used as traditional medicine in the form of curcuminoids. The microwave-Assisted Extraction (MAE) technique is an extraction technique that utilizes microwave radiation to heat the solvent quickly and efficiently and is also very suitable for extracting compounds that are not resistant to heat. This research was conducted to determine the level of curcumin from *C. domestica* Vahl. Extract with MAE using virgin coconut oil (VCO) as solvent. Extraction of curcumin was carried out using *C. domestica* Vahl powder with various weights (1 g, 1.5 g, 2 g, and 2.5 g) and VCO as solvent (mL), with an extraction time of 10 mins and power of 270 W. Curcumin from the extract was measured using High-Performance Liquid Chromatography (HPLC). The results showed that the method has a limit of detection (LOD), which is 3.57±0.01 µg/mL, a limit of quantification (LOQ) of 11.91±0.04 ppm, and good linearity with a correlation coefficient (r) of 0.9993. The curcumin level was determined with HPLC from each various weight (1.27±0.03 mg/g); 1.5 g (1.20±0.02 mg/g); 2 g (3.45±0.02 mg/g) and 2.5 g (3.44±0.01 mg/g) respectively. The results showed that the optimum weight for curcumin extraction with MAE is 2 g with 20 mL of VCO with 10 mins extraction time and power (270 W). The best results were shown at a 1.56 µg/mL concentration and had the highest % of living cells, cell viability testing showed that curcumin was not toxic to RAW 264.7 cells. The nitric oxide (NO) production results show that cells stimulated with LPS had higher nitrite levels at 31.25 g/mL and lower nitrite levels at 62.5 µg/mL; 125 µg/mL; 250 µg/mL, implying that curcumin MAE extract can inhibit NO production, whereas extract-stimulated cells showed a decrease in nitrite levels.

1. Introduction

The mechanism of the cellular immune response against pathogenic elements is highly dependent on the immune system's ability to recognize foreign molecules (antigens) present on the surface of pathogenic elements and the ability to carry out appropriate reactions to eliminate antigens (Marshall *et al*., 2018). Immunomodulators are substances or drugs that can modulate the function and activity of the immune system. Based on how it works, immunomodulators are divided into two groups, namely immunostimulators and immunosuppressors. In the search for safer immunomodulators, many secondary metabolites such as terpenoids, phenolics, and alkaloids have been investigated for their ability to modulate the immune system. The selection of immunomodulatory agents from natural ingredients that can affect the immune system is a future opportunity (Yuandani and Suwarso, 2017; Auliafendri *et al*., 2019).

Indonesia is an agricultural nation where most of its citizens are engaged in the agricultural sector, with various products. Turmeric, which is one of the agricultural commodities in Indonesia, has a relatively high content of curcumin (Cahyono *et al*., 2011; Hewlings, 2017). Turmeric (*Curcuma domestica*, Vahl.) is a plant widely cultivated in tropical and subtropical countries and regions, especially in China, India,

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Indonesia, and several Latin American countries such as Brazil and Peru (Felipe Osorio-Tobón *et al*., 2013; Ghoreishian *et al*., 2013)

Conventional natural extraction methods generally use organic solvents with negative impacts, such as toxic residues, chemical changes in extract compounds, and waste that is difficult to degrade. Therefore, the need for cleaner extraction methods and the use of safe solvents is increasing. Extraction methods that are more environmentally friendly (green extraction), such as Microwave-Assisted Extraction (MAE), are expected to be a solution (Yuandani *et al*., 2016). This research aimed to determine the Curcumin content of Curcuma domestica Vahl. Rhizome uses a Microwave Assisted Extraction technique and knows Curcumin's ability in nitric oxide production and cell viability.

2. Materials and methods

2.1 Materials

The turmeric rhizome used is a large and old turmeric rhizome that has a rhizome thickness of 4.06 cm. Turmeric (*Curcuma domestica* Vahl.) is cleaned of impurities by washing it thoroughly with running water, then draining and weighing the total weight as wet weight. Dexamethasone (Harsen), Griess reagent and Nitrite Standard Solution (Biotium), RAW 264.7 cells were taken from the Parasitology Laboratory of Gadjah Mada University's Faculty of Medicine. The cells were cultured at 37°C and 5% CO_2 in humidified air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum and 100 units/mL penicillin and streptomycin.

2.2 Preparation of extraction

Turmeric that has been cleaned and then sliced thinly is dried in a drying cabinet and then pureed using a blender, sieved to make sure no lumps of powder are followed. The powder that passes through the sieve is turmeric powder which will be extracted, and the turmeric powder left in the sieve can be ground again. The turmeric extraction was performed by dissolving 1 g, 1.5 g, 2 g, and 2.5 g of turmeric powder in 20 mL of VCO solution and heating for 10 mins in the microwave. Microwave power was used for 270 watts, after which the residue and filtrate were separated. The residue is removed after the filtrate is collected (Marin *et al*., 2021).

2.3 Determination of curcumin content in extracts by HPLC

Determination of the curcumin content of the extract was carried out using HPLC with a Photodiode Array (PDA) detector (Shimadzu) with an ODS column of 150 mm × 4.6 mm. The mobile phase system used was isocratic, acetonitrile and 0.1% acid. Formic acid (50:50 v/v) at a flow rate of 1.5 mL/min. The standard solution was injected at 20 L and then identified at a wavelength of 425 nm. Then the sample was injected, as much as 20 L, and detected at a wavelength of 425 nm. Curcumin was identified by comparing the peaks chromatogram to the standard, obtained at almost the same retention time as the curcumin. The determination of the curcumin content was calculated based on the calculation of the regression equation obtained in the calculation of the standard curcumin content and the value of the peak area of the sample that came out at the time of measuring the sample extract (Inoue *et al*., 2008; Badrunanto *et al*., 2019).

2.4 Cell viability

RAW 264.7 cells were grown in DMEM medium with penicillin, streptomycin and fetal bovine serum (FBS) and incubated for 24 hrs to obtain good growth. After 24 hrs, the medium was replaced with a new one, and then the test solution was added (with a concentration of 12.5, 6.25, 3.125, 1.56 µg/mL and incubated at 37°C in a 5% CO_2 incubator for 24 hrs. At the end of incubation, the media and the test solution were removed, and then the cells were removed. Washed with PBS, 100 µL of culture medium and 10 µL of 5 mg/mL MTT were added to each well. To observe the viability of the cells, the cells were incubated again for 4 -6 hrs in a 5% CO_2 incubator at 37°C. The MTT reaction was stopped with a reagent stopper (SDS 10% in 0.1 N HCl). Live cells reacted with MTT to form a purple colour. The test results were read with a microplate reader at a wavelength of 595 nm (Yuandani *et al.*, 2016).

2.5 Nitric oxide

For 24 hrs, RAW 264.7 cells were plated in 96-well plates at a density of 3×10^3 cells/mL. The cells were subsequently cultured for additional 24 hrs with test samples (12.5 and 25 µg/mL) and dexamethasone (1.25 and 2.5 g/mL), followed by stimulation with LPS (1 µg/mL). After 24 hrs of incubation at 37°C, 5% CO_2, the amount of nitric oxide produced was evaluated by measuring the amount of nitrite in the medium using Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid and 1% sulfanilamide). Approximately, 100 mL of Gries reagent was added to the culture supernatant and incubated in the dark for ten minutes. The absorbance at 595 nm was determined using a microplate reader, and the nitrite concentrations were calculated using a reference solution of sodium nitrite. Nitrite concentrations were evaluated

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in samples using a sodium nitrite standard curve (Auliafendri *et al*., 2019).

3. Results and discussion

3.1 Curcumin levels by HPLC method

The levels of curcumin in the samples varied, as shown in Table 1. This study attempts to dissolve curcumin using oil, specifically VCO, and this clearly distinguishes the levels of curcumin produced due to variations in turmeric weighing. From the data, it can be seen that the levels obtained by extracting turmeric weighing 1 g, 1.5 g, 2 g and 2.5 g with VCO using the MAE method at 270 W microwave power for 10 mins resulted in levels of 1.27±0.03 mg/g; 1.20±0.02 mg/g; 3.45±0.02 mg/g; 3.44±0.01 mg/g respectively. The results of data collection on curcumin levels show that weighing factors have an impact on curcumin levels, with the highest levels being 3.45±0.02 mg at 2 g curcumin. Compared to traditional extraction methods, where the longer the extraction process, the longer the contact between the solvent and the solute, the analyte dissolution process will continue and stop until the solvent is saturated with the analyte. The MAE method is faster and prevents thermolabile compounds from degrading. When using the MAE method, the extraction process takes less time. Microwave heating that occurs in direct contact with the material results in higher levels of extract and more heat energy transfer (Chemat *et al*., 2012).

3.2

Table 1. Results of curcumin levels by HPLC.

No	Sample	Weight (g)	X±SD (mg/g)
1	1 g	0.102	1.27±0.03
2	1.5 g	0.1036	1.20±0.02
3	2 g	0.1042	3.45±0.02
4	2.5 g	0.1023	3.44±0.01

Cell viability

The test results of cell viability values in Table 2 show that the higher the concentration, the lower the viability value or, the higher the extract concentration, the fewer living cells, which means the higher the extract concentration, curcumin's cytotoxic effect on culture cells tested cell viability test results were higher than the cytotoxic effect on the culture cells tested. The best results were shown at a 1.56 µg/mL concentration and had the highest % of living cells. Colourimetry is the measurement of colour intensity that occurs due to living cells converting a substrate into coloured products. In this test, MTT salt was used, which was involved in the dehydrogenase enzyme's work. The succinate tetrazolium reductase system, including mitochondria from living cells, will convert MTT to formazan, MTT will be reduced to formazan by the succinate tetrazolium reductase system, including mitochondria from living cells (Kupcsik and Martin, 2011). Cytotoxic test using the MTT method is a cellular reduction reaction based on the yellow MTT tetrazolium salt to blue-purple crystals. The colour change method is an indicator of cell proliferation. In proliferating cells, mitochondria will absorb MTT, and the cells will turn purple due to the formation of tetrazolium crystals (Formazan). More living cells means more cells that are actively metabolizing. The number of formazan crystals formed is also increasing, which causes the intensity of the purple colour on the plate to increase. MTT salts cannot stain dead cells, so they do not form purple like living cells. As a result, the dead cells do not make a purple format, but the quality remains yellow like the medium (Freshney, 2000). Cell viability testing showed that curcumin was not toxic to RAW 264.7 cells. With these results, the sample concentration that produced the highest % of live cells (>90%) was selected for the next experiment (Joo *et al.*, 2014).

3.3 Nitric oxide

The NO production test results in Table 3 show that cells stimulated with LPS experienced an increase in nitrite levels, whereas cells produced with extracts experienced a decrease in nitrite levels, as seen at a concentration of 31.25 µg/mL greater nitrite levels and a concentration of 62.5 µg/mL; 125 µg/mL; 250 µg/mL decreased, implying that curcumin MAE extract can inhibit NO production. The best concentration is seen at a concentration of 31.25 µg/mL for the extract (Price *et al*., 2011). The NO production test was conducted using the Griess reagent. In an acidic medium, nitrite was combined with a diazotizing reagent such as sulfanilamide to form a transient diazonium salt. This intermediate is then reacted with the coupling reagent N-naphthyl-ethylenediamine (NED) to form a stable azo compound. The purple colour produced enables highly sensitive nitrite analysis (Sun *et al*., 2003). MAE curcumin extract contains flavonoid compounds,

Table 2. Results of cell viability.

Concentration	12.5	6.25	3.125	1.56
Mean±SEM	45.52±0.59	63.42±0.16	99.22±0.22	100.72±0.23

Table 3. Results of nitric oxide production.

Concentration	250	125	62.5	31.25
Mean±SEM (µg/mL)	1.31±0.09	1.89±0.08	2.18±0.11	2.73±0.20

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saponins, tannins, steroids/triterpenoids, and glycosides that can inhibit NO production in RAW 264.7 cells that have been stimulated with LPS. Previous studies have shown that some flavonoids and steroids/triterpenoids can inhibit NO production in response to inflammatory stimulation (Durga *et al*., 2014; Venkatesha *et al*., 2016). Saponins and glycosides can also inhibit NO production (Deng *et al*., 2015; Jang *et al*., 2016; Dewi *et al*., 2017).

4. Conclusion

Curcumin extraction with MAE requires 2 g of curcumin, 20 mL of VCO, and a 10-minute extraction time and power of 270 W. According to cell viability testing, Curcumin MAE did not cause toxicity in RAW 264.7 cells and had a high potential to reduce nitric oxide production.

Conflict of interest

The authors declare no conflict of interest in conducting this study.

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