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Synergistic anti-inflammatory effects of Curcuma xanthorrhiza rhizomes and Physalis angulata herb extract on lipopolysaccharide-stimulated RAW 264.7 cells

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ABSTRACT

Activation of macrophages stimulates the release of various inflammatory mediators which become targets for the development of anti-inflammatory drugs. The combination of *Curcuma xanthorrhiza* and *Physalis angulata* extracts was investigated for its anti-inflammatory properties and synergistic effects. Separate extraction with ethanol was applied for both materials, followed by the determination of the characteristics of each extract. To assess the production of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6), each extract and its combination was tested on lipopolysaccharide- (LPS)-induced RAW 264.7 cells using the enzyme-linked immunosorbent assay method. Nitric oxide production was determined by measuring the nitrite content using the Griess method. The cell viability was determined using the MTT method. Both extracts were also able to inhibit inflammatory mediators such as TNF-α, IL-6, and NO. The combination of the two extracts was dominated by a synergistic effect ($C < 1$) in all concentration ratios, but increasing the concentration ratio gave a cytotoxic effect. It can be concluded that both extracts possessed an anti-inflammatory activity on the LPS-induced RAW 264.7 cells model, and the combination of these extracts displayed synergistic anti-inflammatory activity.

INTRODUCTION

Inflammation is a cellular event in response to infection and tissue damage. The response of inflammation in increasing vascular permeability and migration of immune cells at the damaged site is also characterized by the production of nitric oxide (NO) and cytokines, such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) (Kim *et al*., 2015). Production of more proinflammatory molecules and nitric oxide (NO) may damage the structure, function, and integrity of lipids, proteins, and nucleic acids, leading to various chronic diseases (Ben-Baruch, 2006). These inflammatory mediators are widely involved in the pathogenesis of human diseases (Chen *et al*., 2018). Traditional medicine with a holistic concept that has been tested in clinical practice has a definite therapeutic effect. Compared to Western medicine, it is less toxic in treating various diseases, especially complex chronic diseases (Yuan *et al*., 2017). Ethnomedicine often uses blended ingredients of several plant extracts to exploit the biological activity of different phytochemical assemblages through synergistic interactions (Komape *et al*., 2017; Liu *et al*., 2016; Williamson, 2001).

Research on the interactions between synergistic phytomedicines plays a very important role because it explains the efficacy of low doses of active constituents in herbal products. The concept of synergistic interaction suggests that plant

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extracts offer more advantages than isolated single ingredients (Williamson, 2001). There is evidence supporting synergies in phytomedicines; for example, the use of a combination of extracts made of *Strophanthus hispidus* (root) and *Aframomum melegueta* (seeds) significantly provides a synergistic anti-inflammatory effect (John Kenneth *et al*., 2019). The combination of *Ocimum bacilicum* L. leaf extract and *Syzygium polyanthum* is more effective as an anti-inflammatory agent than each used separately (Sukmawati *et al*., 2018). There was also a similar report regarding the combination of *Euphorbia hirta* L. (herb) and *Carica papaya* L. (leaf) extracts (Dermiati *et al*., 2018).

Curcuma xanthorrhiza has long been used as a traditional medicine and has been confirmed to have pharmacological properties such as anti-inflammatory effects (Rahmat *et al*., 2021). Although *C. xanthorrhiza* has demonstrated anti-inflammatory properties, clinical trials have shown that curcumin exhibits poor bioavailability. An approach to solving this problem is to combine two or more phytochemicals to obtain a synergistic effect. Therefore, there is no need to increase the dose (Anand *et al*., 2007; Zhang *et al*., 2019). One way to increase its anti-inflammatory activity is to combine it with other plants such as *Physalis angulata.* This plant is easy to grow in the tropics and has been used in different countries in the treatment of various diseases such as inflammatory diseases, including dermatitis and arthritis, pain, malaria, leishmania, asthma, tuberculosis, fluid retention, and cancer (Saldago *et al*., 2012). The selection of the two plants was also based on taxonomic family differences. This will provide a diversity of phytoconstituents, and the interactions between phytochemicals will cause a synergistic effect.

This study aimed to investigate the combination of the two plant extracts in providing anti-inflammatory effects. The results of this study expectedly could provide information on strategies for treating inflammation. In this study, the anti-inflammatory activity of the extracts made of *C. xanthorrhiza* and *P. angulata* was studied using lipopolysaccharide- (LPS-) stimulated RAW 264.7 macrophages. LPS-stimulated macrophages have been widely used to study anti-inflammatory compounds *in vitro* (Dong *et al*., 2017). The synergistic effect of the combination of these extracts was calculated using the combination index (CI) equation (Chou, 2010). This study provides scientific evidence regarding the use of a combination of herbal medicines to enhance anti-inflammatory effects.

MATERIALS AND METHODS

Materials

Curcuma xanthorrhiza rhizomes were collected in March 2020 in Singaraja, while *P. angulata* herbs were collected in April in the same year in Gianyar. Both regencies are in Bali, Indonesia. The two plants were identified at the Bali Botanical Gardens, Indonesian Institute of Sciences, with plant identification numbers of B-198/IPH.7/AP/VII/2020 for *C. xanthorrhiza* and B-199/IPH.7/AP/VII/2020 for *P. angulata*. Curcumin standard was obtained from the isolation carried out by Prof. apt. Suwijiyo Pramono., DEA, with 88.65% purity. Quercetin (Sigma), Folin–Ciocalteu reagent, aluminum chloride, vanillin sulfuric acid reagent, potassium hydroxide, Liebermann–Burchard reagent, ferric chloride reagent, Dragendorff reagent, gallic acid, hydrochloric acid, Thin Layer Chromatography (TLC) plate of silica gel 60 F254, toluene, ethanol, methanol, chloroform, acetone, acetic acid, diethyl ether, n-hexane, and ethyl acetate were purchased from Merck.

The RAW 264.7 murine macrophages were obtained from the Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. Other materials used were Dulbecco's modified Eagle's media (DMEM) (Gibco), fetal bovine serum (FBS) (Sigma), phosphate-buffered saline (PBS) (Sigma), penicillin-streptomycin (Gibco), Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco), [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Bio Basic), LPS from *Escherichia coli* (Sigma), sodium dodecyl sulfate (SDS) (Merck), TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Sigma), and dexamethasone (Kimia Farma).

Equipment

The equipment used consisted of TLC-Densitometry CAMAG TLC Scanner 3, CAMAG Linomat 5 Sample Applicator, Spectrophotometry Hitachi UH5300, Inverted Microscope Leica/DM IL LED, CO_2 Incubator Thermo Science-341/8000DH, Cytotoxic Safety Cabinet LABCONCO Purifier Class II, Microplate Reader TECAN SPARK 20M, and ELISA Reader Bio-Rad 680XR, Centrifuge Mini Refrigerated Eppendorf 5424R.

Extraction

The *C. xanthorrhiza* rhizomes and *P. angulata* herbs were cleaned under running water and drained at room temperature separately. After the *C. xanthorrhiza* rhizomes were sliced thinly and *P. angulata* was cut, they were then oven-dried at 50°C for 2 days and ground to a fine powder. The dry powder of both plant materials was extracted using the maceration method. One kilogram of dry plant powder was macerated with 10 l of ethanol (70%) for 24 hours, and the mixture was stirred occasionally. This process was repeated three times. The collected macerate was then concentrated using a rotary evaporator and put into a desiccator (Kemenkes Republik Indonesia, 2017).

Characterization of the extracts

The characteristics of the ethanolic extract of *C. xanthorrhiza* (EECX), including the contents of curcumin and essential oil, were determined. The total phenolic and flavonoid contents of the ethanolic extract of *P. angulata* (EEPA) were determined. The extraction yield and moisture content of both extracts were also determined. All the extract characteristic parameters were assessed in accordance with the Indonesian Herbal Pharmacopoeia (Kemenkes Republik Indonesia, 2017). Preliminary screening for secondary metabolites such as steroids, phenols, alkaloids, anthraquinones, saponins, flavonoids, and monoterpenes was carried out according to Indonesian Herbal Pharmacopoeia and Harborne (1998). A test solution was prepared by dissolving 10 mg of the extract with 10 ml of chloroform–ethanol (1:1) v/v. The eluted TLC plate was then tested with various spray reagents. The color of the spots was observed under ultraviolet (UV 254 nm and UV 365 nm) and visible light (Wagner and Bladt, 2001).

Cell culture

RAW 264.7 cells were grown in DMEM with 10% FBS supplementation and a 2% penicillin-streptomycin solution. The

cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 until the cells were confluent.

Anti-inflammatory activity on LPS-activated cells

For TNF-α and IL-6 determinations, RAW 264.7 cells were seeded in a 24-well plate with a cell density of 1.5×10^5 cells per well. The cell line was incubated in a 37°C and 5% CO_2 incubator. After 24 hours of incubation, the supernatant was discarded and the adherent cells were washed with PBS. RAW 264.7 cells were incubated with LPS from *E. coli* at 1 µg/ml. In addition, nontoxic concentrations of EECX and EEPA and controls (0.5% Dimethyl sulfoxide (DMSO) and 5 µg/ml dexamethasone) were added to the wells. After 24 hours, the cells or their supernatants were collected and analyzed.

Measurement of nitric oxide (NO) concentration

The cell supernatant was mixed with an equal volume of Griess reagent, 1% sulfanilamide (Sigma), and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid (Merck) and incubated at room temperature for 15 minutes. Sodium nitrite (Sigma) was used as the reference standard. The nitrite concentration was then determined at a wavelength of 540 nm (Kim *et al*., 2015).

Measurement of proinflammatory cytokines TNF-α and IL-6

The TNF-α and IL-6 levels in the cell supernatants were quantified using sandwich-enzyme immunoassays, in accordance with the manufacturer's protocol (Sigma) with catalog Nos. RAB0477-IKT for TNF-α and RAB308-IKT for IL-6.

Evaluation of cell viability

The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The supernatant was taken for analysis, and then each well was washed using 500 µl PBS/well. After this, a 500 µl MTT solution was added to each well, which was then incubated for 4 hours in a 37°C CO_2 incubator. Formazan crystal formation was observed under an inverted microscope. After formazan crystals were formed, a 500 µl/well stopper reagent was added (10% SDS in 0.1 N HCl) and incubated at room temperature in the dark overnight. Cell density was determined by reading the absorbance at a wavelength of 550 nm (Braga *et al*., 2019; Kim *et al*., 2015). The percentage of viable cells was calculated using the following formula:

$$\text{Viability cell}(\%) = \frac{(\text{Abs sample} - \text{Abs media})}{(\text{Abs control} - \text{Abs media})} \times 100\%.$$

Inhibitory activity of inflammatory mediators

The inhibitory concentration (IC_{50}) value of all samples was calculated based on the linear regression equation $y = bx + a$, which was generated from the plot of sample concentration versus %inhibition. The percentage of inhibition of the formation of NO, TNF-α, and IL-6 in each sample was obtained using the following formula:

$$\text{Percentage inhibition}(\%) = \frac{(An - As)}{(An)} \times 100\%.$$

The value of A_n was obtained from the absorbance of the negative control (0.5% DMSO with 1 µg/ml LPS) minus the absorbance of the control (0.5% DMSO without LPS), while the value of A_s was obtained from the absorbance of the sample minus the absorbance of the control (0.5% DMSO without LPS).

Determination of anti-inflammatory synergism

The combined effect was determined based on the combination of the IC_{50} of every single extract. For this purpose, a series of solution concentrations was made, resulting in inhibition percentages of 12.5%, 25%, 37.5%, and 50%. The four concentrations were combined based on the checkerboard method to produce a total of 16 combinations. The combinations were then tested to obtain the percentage of the inhibition of each proinflammatory mediator parameter. The CI was used to determine whether a combination provided a synergistic effect. The CI was calculated using the following formula:

$$\text{CI} = \frac{(D)1}{(Dm)1} + \frac{(D)2}{(D\text{m})2}.$$

CI is the combination index, where CI = 1 indicates additive effect, CI < 1 synergism, and $C > 1$ antagonism. $(D)_1$ and $(D)_2$ are the concentration of each combined extract that produces the x effect, while $(D_m)_1$ and $(D_m)_2$ are the concentration of every single extract that produces the x effect.

RESULTS AND DISCUSSION

The chemical compositions of the extracts made of these two species varied in both quality and quantity due to environmental factors that affect plant metabolism (Rahmat *et al*., 2021; Sandeep *et al*., 2015; Zhang *et al*., 2016). The presence of secondary metabolites in both plants was determined, and the results are summarized in Table 1. Several secondary metabolites have been identified in the genus *Physalis*, such as alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, physalins, and withanolides (Ferreira *et al*., 2019). Ethanol organic extracts have shown the presence of more groups of compounds than aqueous extracts. The EECX mainly consisted of terpenoids, phenols, flavonoids, saponins, cardiac glycosides, alkaloids, and coumarins (Halim *et al*., 2012). The characteristics of the EECX and EEPA can be seen in Table 2. Based on the spectrophotometric analysis, the total phenol and flavonoid contents on EEPA were 8.172% ± 0.340% Gallic acid equivalents (GAE) and 0.513% ± 0.014% Quercetin equivalents (QE), respectively. Various biological activities attributed to *Physalis* are due to the different metabolic and structural compounds in this plant (Bhat *et al*., 2008). The levels of total phenolics, flavonoids, and the concentration of some of the individual flavonols were significantly correlated to antioxidant properties (Cobaleda-Velasco *et al*., 2017). The curcumin content in EECX was analyzed with thin-layer chromatography with curcumin as a standard; the curcumin content in the extract was measured with the winCATS software version 1.4.3.6336. The analyte concentration was determined from the intensity of the reflected light, which was indicated as the peak area. These results were then correlated with the analyte concentrations using the standard curcumin. The mobile phase used n-hexane: ethyl acetate (1:1 v/v). The Retardation factor (Rf) value of the curcumin compound was reported as 0.46 (Fig. 1). The

Table 1. Preliminary screening of secondary metabolites from EECX and EEPA.

Test	EECX	EEPA
Steroids	+	+++
Phenols	+++	++
Alkaloids	+	−
Anthraquinones	−	−
Saponins	+	+
Flavonoids	−	+
Terpenoids	+++	++
Tannins	+	++

+++ = highly positive; ++ = positive; + = mildly positive; − = negative.

Table 2. The characteristics of the EECX and EEPA.

Parameters	EECX	EEPA
Yield (%)	19.70%	26.55%
Water content (% ml/g)	9.113% ± 1.071%	8.269% ± 1.084%
Total phenolic content (% GAE)	Nd	8.172% ± 0.340%
Total flavonoid content (% QE)	Nd	0.513% ± 0.014%
Curcumin (%)	5.991% ± 0.090%	Nd
Volatile oil (% ml/g)	6.429% ± 0.119%	Nd

Nd: not determined; the data represent the mean ± SD of triplicate experiments.

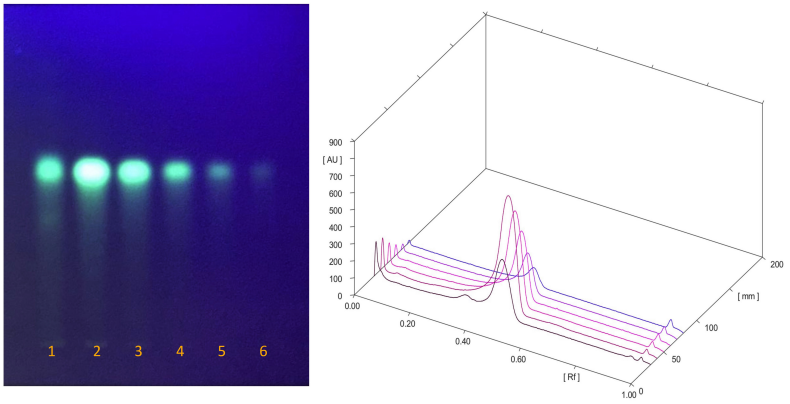

Figure 1. TLC profile of EECX (Track 1) curcumin standard at different concentrations: 188, 94, 47, 23.5, and 11.75 µg/ml (Tracks 2–6) 3D view of densitogram at 425 nm.

curcumin content in EECX was 5.991% ± 0.0903%. The essential oil content was obtained by a distillation method, with the result of 6.429% ± 0.119% ml/g. Curcumin yellow pigment is a phenolic compound and the main phytochemical constituent of the *Curcuma* species (Pulido-Moran *et al*., 2016). The essential oil extracted from the *C. xanthorrhiza* rhizomes showed that xanthorrhizol was the main compound, followed by camphene pinene, thujene, and myrcene (Mary *et al*., 2012). Curcumin and xanthorrhizol potent natural bioactive compounds have protective health effects mainly through anti-inflammatory and antioxidant mechanisms (Oon *et al*., 2015; Pulido-Moran *et al*., 2016).

In previous research, both plants were indicated to have a cytotoxic activity. The phytochemical content of *P. angulata* has cytotoxic activity in A549 (human non-small cell lung cancer cell lines), Hela (human cervical cancer cell lines), and p388 (human leukemia cell lines) (Meng *et al*., 2019). Preclinical studies have shown that curcumin has a cytotoxic activity against various types of cancer cells by modulating multiple molecular targets (Bimonte *et al*., 2016). Before testing the activity of EECX and EEPA *in vitro* cell assays, a cytotoxic analysis of both extracts was performed. As shown in Figure 2, the cell viability decreased as the concentration of the extract increased. The results showed that the concentration required for RAW 264.7 cells to have 80% viability for each of the EECX extracts was 24.411 ± 2.214 µg/ml and that of EEPA was 26.062 ± 2.062 µg/ml. For this reason, a concentration of 20 µg/ml was used to evaluate the anti-inflammatory activity of each extract. To evaluate the anti-inflammatory activity, the ability to inhibit the production of NO, TNF-α, and IL-6 using LPS-stimulated RAW

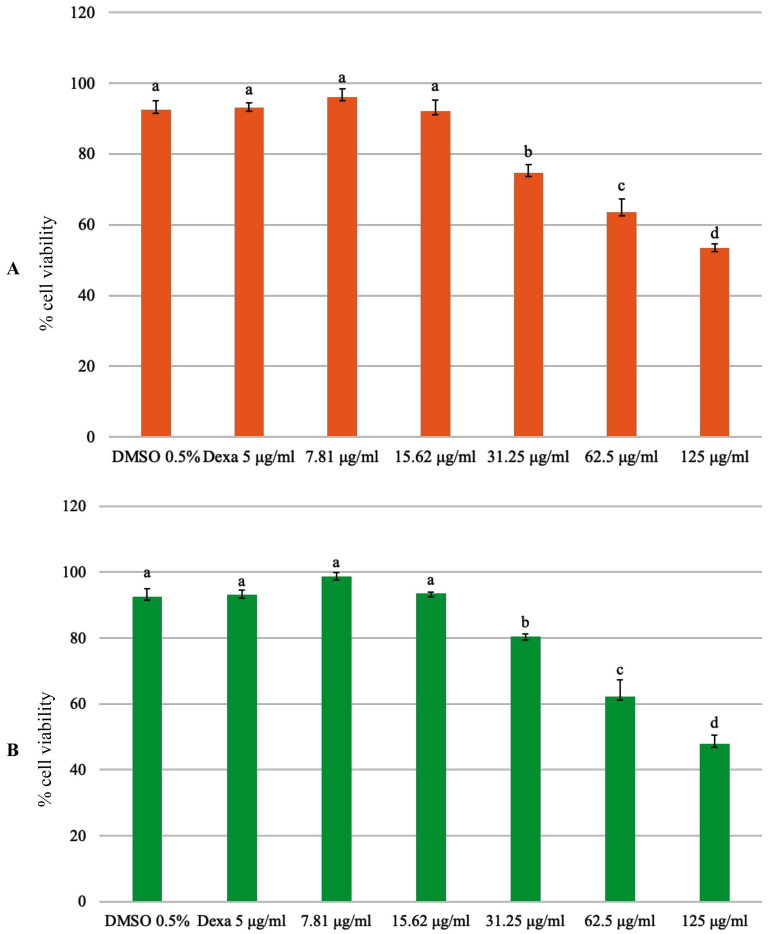

Figure 2. Cell viability assay using the MTT assay; RAW 264.7 cells were treated with (A) EECX and (B) EEPA at different concentrations (7.81, 15.62, 31.25, 62.5, and 125 µg/ml). The control group was cultured in the presence of 0.5% of DMSO and dexamethasone 5 µg/ml (Dexa). Error bars represent the standard deviation (n = 3). Different letters (a–d) indicate significant differences in analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$).

264.7 cells of both extracts was tested. LPS is a component of the outer cell membrane of Gram-negative bacteria that induce macrophages to produce proinflammatory mediators (Lee *et al*., 2017; Park *et al*., 2014).

After being activated by endotoxins or cytokines, macrophages produce NO, which can help kill and inhibit the growth of microorganisms. However, the presence of excess NO may cause the development of cancer (McAdam *et al*., 2012). TNF-α is a cytokine mainly produced by monocytes, macrophages, and T cells and is triggered by endotoxins from other pathogens or substances. TNF-α is a strong inducer for IL-1, IL-2, and IL-6 (Kitaura *et al*., 2013). In addition, TNF-α is involved in various inflammatory and infectious diseases. At the cellular level, TNF-α strengthens body responses such as inflammation, immunoregulation, proliferation, apoptosis, and antiviral activity (Lam *et al*., 2000). When infection or tissue injury occurs, IL-6 is immediately produced by monocytes and macrophages. IL-6 contributes to the elimination of infectious agents and the restoration of damaged tissues through the activation of immune responses, but excessive and uncontrolled IL-6 production plays a pathological role in the development of various inflammatory diseases (Tanaka *et al*., 2016). The release of proinflammatory mediators is like a double-edged sword for the host. If not controlled, this may lead to the development of various chronic diseases.

Curcuma xanthorrhiza rhizomes have long been used in traditional medicine and to treat diseases. In this study, EECX can inhibit the production of proinflammatory mediators (Fig. 3). Research using extracts of *C. xanthorrhiza* and xanthorrhizol was carried out to prevent the recruitment of immune cells to adipose tissue through the downregulation of inflammatory cytokine genes (Kim *et al*., 2014). In another study, the methanol extract made of *C. xanthorrhiza* rhizomes has the activity of inhibiting the production of nitric oxide (NO) in LPS-induced RAW 264.7 cells where the active compounds are curcuminoids and xanthorrhizol (Park *et al*., 2014). Curcuminoids vary in their ability to suppress Nuclear Factor Kappa Beta (NF-κβ) activation. Curcumin has the highest activity of inhibiting TNF-α compared to demethoxycurcumin and bisdemethoxycurcumin (Sandur *et al*., 2007). Xanthorrhizol can reduce the production of TNF-α, IL-6, and IL-1β by turning off the activation of NF-κβ, thus providing an anti-inflammatory effect (Oon *et al*., 2015). The mechanism of curcumin in lowering the proinflammatory cytokine levels is by inhibiting NF-κβ activation while increasing anti-inflammatory cytokines. Curcumin drives antioxidant defense mechanisms by increasing the transcription and expression levels of antioxidant enzymes and by enhancing mitochondrial function (Gounden *et al*., 2017; Trujillo *et al*., 2014).

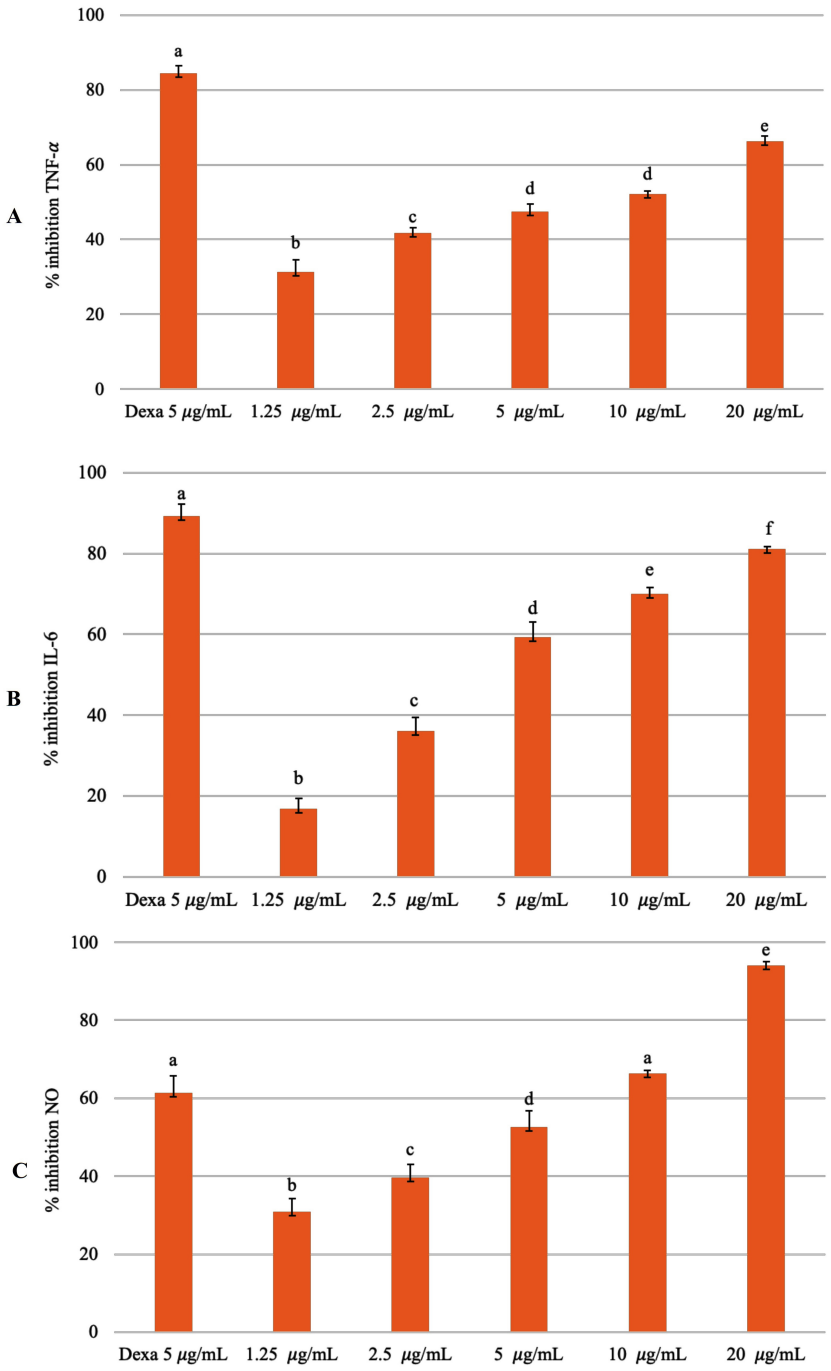

Figure 3. Inhibitory percentage against (A) TNF-α, (B) IL-6, and (C) NO from LPS-stimulated RAW 264.7 cells treated with different concentrations of EECX (1.25, 2.5, 5, 10, and 20 µg/ml). The positive control group: dexamethasone 5 µg/ml (Dexa). Error bars represent the standard deviation ($n = 3$). Different letters (a–f) indicate significant differences in ANOVA, followed by Tukey's test ($p < 0.05$).

The plant *P. angulata* produces a triterpenoid withanolide, which contains a steroid lactone with 28 carbon atoms (Huang *et al*., 2020; Tuan Anh *et al*., 2021). These compounds have antimicrobial, anticancer, anti-inflammatory, hepatoprotective, and immunomodulatory properties (Saldago *et al*., 2012; Sun *et al*., 2017b). The pharmacological activity of EEPA can reduce NO production because the aqueous extract of *P. angulata* is associated with inhibition of nitric oxide synthase (Bastos *et al*., 2008). The physalin E component of *P. angulata* can suppress TNF-α and IL-6 cytokines by inhibiting NF-κβ activation (Yang *et al*., 2017). Physalin B, F, G compounds contained in *P. angulata* may inhibit the production of TNF-α *in vitro* and *in vivo*. The protective effect of physalin not only inhibits the action of TNF-α but also suppresses other cytokines such as IL-6, IL-12, and NO (Soares *et al*., 2003). This is in accordance with previous studies, in which the EEPA can suppress the production

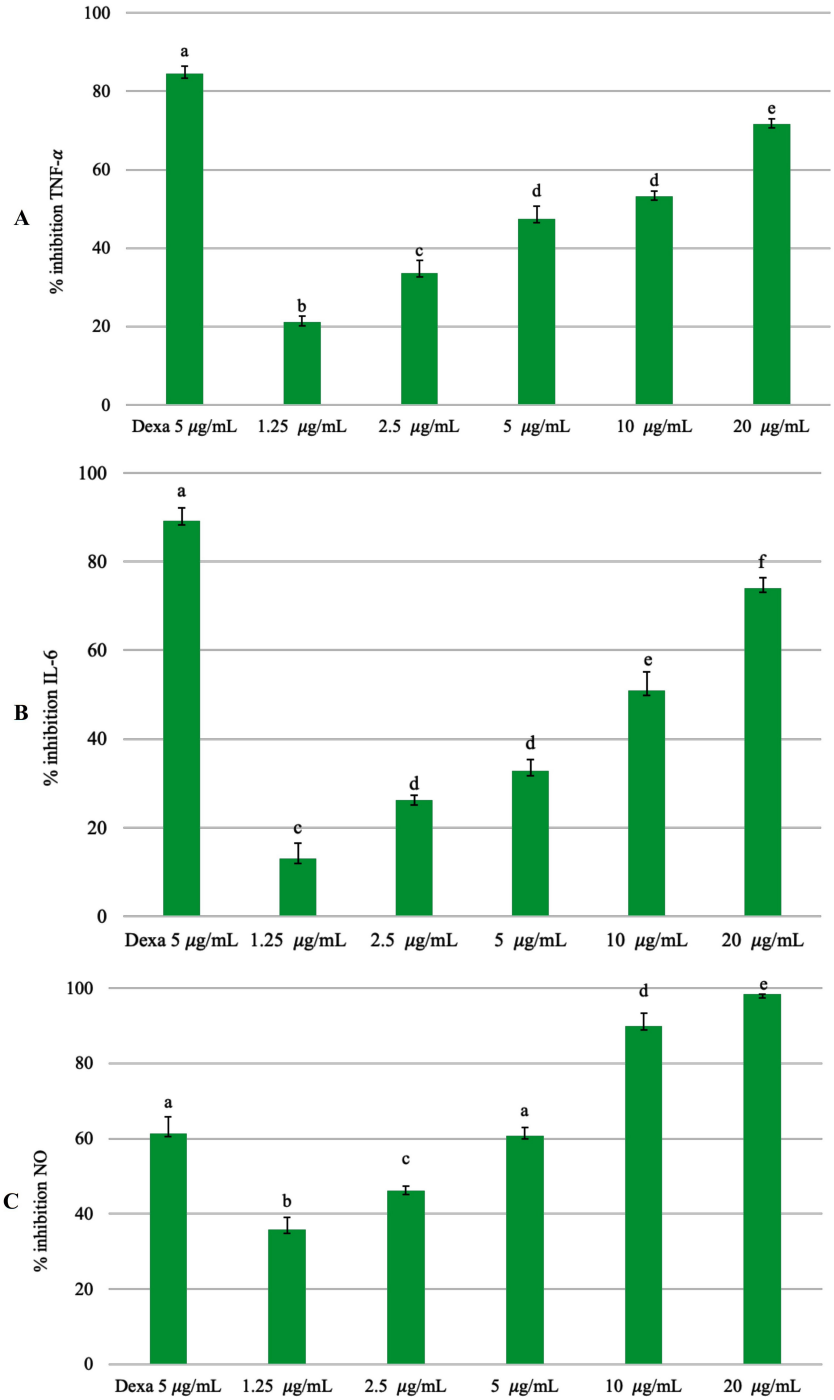

Figure 4. Inhibitory percentage against (A) TNF-α, (B) IL-6, and (C) NO from LPS-stimulated RAW 264.7 cells treated with different concentrations of EEPA (1.25, 2.5, 5, 10, and 20 µg/ml). The positive control group: dexamethasone 5 µg/ml (Dexa). Error bars represent the standard deviation ($n = 3$). Different letters (a–f) indicate significant differences in ANOVA, followed by Tukey's test ($p < 0.05$).

of inflammatory mediators (Fig. 4). The ethanol extract of *P. angulata* can also inhibit COX-2 activity in MCF-7 cells (Sutrisna *et al*., 2012).

Both extracts were able to reduce the production of inflammatory mediators. The quantification of inflammatory mediators such as the levels of NO, IL-6, and TNF-α in the supernatant from LPS-stimulated RAW 264.7 cells can be seen in Table 3. The quantification of the inflammatory mediator levels for the positive control (dexamethasone 5 µg/ml with LPS 1 µg/ml) was as follows: TNF-α = 823.60 ± 244.221 pg/ml; IL-6 = 107.706 ± 5.463 ng/ml; and NO = 3.049 ± 0.181 µM. The quantification of the inflammatory mediator levels for the negative control (DMSO 0.5% with LPS 1 µg/ml) was as follows: TNF-α = 4,713.600 ± 424.276 pg/ml; IL-6 = 341.706 ± 50.634 ng/ml; and NO = 6.325 ± 0.133 µM. The quantification of the inflammatory mediator levels for the control (DMSO 0.5% without LPS 1 µg/ml) was as

Table 3. The viability and quantification of inflammatory mediators in supernatants from LPS-stimulated RAW 264.7 cells.

Concentration (µg/ml)	EECX				EEPA			
	TNF-α concentration (pg/ml)	IL-6 concentration (ng/ml)	NO concentration (µM/ml)	Cell viability (%)	TNF-α concentration (pg/ml)	IL-6 concentration (ng/ml)	NO concentration (µM)	Cell viability (%)
1.25	3,276.267 ± 291.221	297.440 ± 37.849	4.672 ± 0.329	114.593 ± 7.830	3,738.600 ± 340.137	307.773 ± 42.795	4.412 ± 0.305	110.474 ± 6.099
2.5	2,788.933 ± 227.062	247.573 ± 27.392	4.209 ± 0.151	104.770 ± 6.072	3,155.600 ± 130.399	274.106 ± 42.700	3.861 ± 0.230	107.548 ± 5.398
5	2,537.600 ± 301.045	187.573 ± 21.934	3.513 ± 0.314	98.650 ± 6.473	2,528.267 ± 244.347	256.573 ± 36.120	3.078 ± 0.261	102.669 ± 9.123
10	2,314.933 ± 168.075	160.373 ± 23.408	2.788 ± 0.280	98.879 ± 2.493	2,260.933 ± 195.115	210.573 ± 40.737	1.542 ± 0.100	94.884 ± 5.613
20	1,664.933 ± 75.481	131.240 ± 18.686	1.310 ± 0.305	95.647 ± 6.819	1,415.600 ± 151.248	150.306 ± 27.323	1.078 ± 0.314	94.768 ± 1.945

The data represent the mean ± SD of triplicate experiments.

Table 4. The IC_{50} values of inflammatory mediator inhibition.

Sample	TNF-α inhibitory activity, IC_{50} (µg/ml)	IL-6 inhibitory activity, IC_{50} (µg/ml)	NO inhibitory activity, IC_{50} (µg/ml)
C. xanthorrhiza	6.238 ± 0.163	4.466 ± 0.388	3.697 ± 0.367
P. angulata	6.492 ± 0.185	8.297 ± 0.508	2.567 ± 0.070

The data represent the mean ± SD of triplicate experiments.

follows: TNF-α = 110.933 ± 59.003 pg/ml; IL-6 = 82.640 ± 12.931 ng/ml; and NO = 0.991 ± 0.314 µM.

The inhibition activities against TNF-α, IL-6, and NO in EECX and EEPA were lower than that of the positive control (dexamethasone 5 µg/ml), and interestingly the inhibition against NO production in the extract (doses 10 µg/ml and 20 µg/ml) was greater than that of the positive control. This was possibly due to the presence of a group of phenolic compounds and flavonoids in the extract, which have a strong antioxidant activity (Fakhrudin *et al*., 2016). The presence of reactive oxygen species (ROS) has a fatal effect on oxygen toxicity and cellular dysfunction that exacerbate inflammation. Therefore, antioxidant activity is required to remove ROS (Lee *et al*., 2015). *Curcuma xanthorrhiza* rhizomes, containing curcumin and xanthorrhizol, and *P. angulata,* containing phenol glycoside, are potential for anti-inflammatory treatment because they can inhibit NO production (Park *et al*., 2014; Sun *et al*., 2017a). The results of the anti-inflammatory activity test of each extract determined the IC_{50} value (Table 4) for each inhibition against NO, TNF-α, and IL-6 production.

The combination of natural compounds present in EECX and EEPA was shown to increase the effectiveness of the anti-inflammatory effect due to the synergistic effect (CI < 1). The percentage of inhibition of inflammatory mediators also increased with increasing concentrations, but unfortunately, there was a decrease in cell viability, especially in the TNF-α and IL-6 assays (Table 5). The use of the combination of these two extracts at high doses can cause toxic effects to RAW 264.7 cells. The NO test showed cell viability above 80% because the dose used was relatively lower than the dose in the TNF-α and IL-6 tests. Interestingly, the combination of EECX and EEPA with cell viability above 80% had more synergistic than antagonistic effects.

The combination test of EECX and EEPA extracts showed a synergistic effect, along with an increase in the concentration ratio. The mechanism of why synergism occurs is probably due to antioxidant regeneration. The combination of curcumin and quercetin provides anti-inflammatory and antioxidant effects that also fight oxidative stress (Abdel-Diam *et al*., 2019). The role of oxidative stress is very important because it is responsible for chronic development and chronic disease (Miller *et al*., 2018). The synergistic effect may result from the increase in antioxidant capacity by protecting each other from oxidative agents and differences in the solubility of the phytochemical constituents (Becker *et al*., 2007).

Chemical compounds have very specific interactions with inflammatory markers and signaling pathways. The use of the combination reached the threshold level of signaling pathway activation, which cannot be reached by single components (Zhang *et al*., 2019). This combination also had an effect in regulating NF-κβ; high NF-κβ will affect the expression of proinflammatory cytokines (Güran *et al*., 2019). The NF-κβ pathway is responsible for the development of chronic inflammation as activated NF-κβ increases the production of cytokines, chemokines, and adhesion molecules as well as leukocyte recruitment. Therefore, weakening the NF-κβ pathway can be a treatment target (Lawrence, 2009).

A combination with a low concentration ratio did not show a synergistic effect. However, at a high concentration ratio, the NO inhibition test caused an antagonistic effect (CI > 1). Antagonistic effects were also found in this combination. This may be because of the presence of phenolic and flavonoid compounds in the combined extract, causing the antioxidant regeneration effect to decrease and causing the two to compete. One of the appropriate reasons is the interference with each other that may cause a reduction in individual activity. Complex stoichiometric

Table 5. The inhibitory activity of inflammatory mediators and cell viability in the combination of EECX and EEPA.

Ratio		Sample code	TNF-α				IL-6				NO			
EECX	EEPA		Inhibition (%)	Cell viability (%)	CI	Remark	Inhibition (%)	Cell viability (%)	CI	Remark	Inhibition (%)	Cell viability (%)	CI	Remark
12.5%	12.5%	Comb. 1	19.201 ± 1.988^{a}	100.774 ± 4.207^{e}	5.146	Antagonism	42.457 ± 1.602^{a}	98.157 ± 2.702^{j}	0.703	Synergism	8.337 ± 1.422^{a}	95.626 ± 2.256^{c}	3.041	Antagonism
25%	12.5%	Comb. 2	28.115 ± 1.590^{b}	95.683 ± 3.360^{de}	4.281	Antagonism	54.334 ± 0.960^{b}	97.200 ± 1.793^{ij}	0.620	Synergism	20.353 ± 1.786^{b}	94.832 ± 2.023^{bc}	2.765	Antagonism
37.5%	12.5%	Comb. 3	52.560 ± 2.676^{d}	99.540 ± 5.652^{de}	0.831	Synergism	64.682 ± 3.082^{c}	88.204 ± 1.611^{f}	0.527	Synergism	35.148 ± 2.722^{cd}	93.693 ± 1.352^{abc}	1.932	Antagonism
50%	12.5%	Comb. 4	60.113 ± 1.925^{e}	97.180 ± 2.851^{de}	0.567	Synergism	76.140 ± 2.165^{e}	77.546 ± 1.143^{de}	0.401	Synergism	41.644 ± 1.975^{e}	93.398 ± 1.742^{abc}	1.814	Antagonism
12.5%	25%	Comb. 5	33.835 ± 0.635^{c}	102.582 ± 1.792^{e}	2.303	Antagonism	57.714 ± 1.834^{b}	94.217 ± 0.922^{ghi}	0.529	Synergism	34.735 ± 0.842^{c}	93.114 ± 3.013^{abc}	1.437	Antagonism
25%	25%	Comb. 6	36.773 ± 0.677^{c}	99.407 ± 5.924^{de}	2.678	Antagonism	66.721 ± 1.494^{cdg}	91.846 ± 3.249^{fgh}	0.473	Synergism	60.665 ± 1.259^{g}	90.801 ± 1.932^{abc}	0.631	Synergism
37.5%	25%	Comb. 7	59.672 ± 0.975^{e}	92.899 ± 0.949^{de}	0.620	Synergism	67.921 ± 1.854^{cdg}	94.126 ± 0.934^{ghi}	0.565	Synergism	67.593 ± 1.409^{h}	91.685 ± 3.512^{abc}	0.581	Synergism
50%	25%	Comb. 8	70.743 ± 1.193^{f}	76.883 ± 3.945^{c}	0.322	Synergism	76.693 ± 1.214^{e}	76.201 ± 1.746^{cde}	0.463	Synergism	51.389 ± 0.705^{f}	90.534 ± 1.427^{abc}	1.412	Antagonism
12.5%	37.5%	Comb. 9	53.455 ± 1.552^{d}	93.030 ± 2.826^{de}	0.805	Synergism	58.010 ± 1.452^{b}	94.645 ± 2.193^{ghi}	0.694	Synergism	53.267 ± 1.803^{f}	95.465 ± 1.748^{c}	0.872	Synergism
25%	37.5%	Comb. 10	59.101 ± 1.170^{e}	90.130 ± 1.780^{d}	0.678	Synergism	68.955 ± 1.540^{cd}	91.352 ± 1.409^{fg}	0.531	Synergism	63.444 ± 2.386^{gh}	90.918 ± 1.533^{abc}	0.704	Synergism
37.5%	37.5%	Comb. 11	77.490 ± 1.648^{g}	68.936 ± 3.022^{bc}	0.226	Synergism	77.986 ± 1.321^{ef}	73.005 ± 2.212^{bcd}	0.429	Synergism	67.104 ± 1.571^{h}	93.361 ± 1.071^{abc}	0.716	Synergism
50%	37.5%	Comb. 12	81.151 ± 1.632^{gh}	64.854 ± 1.134^{ab}	0.194	Synergism	84.959 ± 1.201^{gh}	70.281 ± 1.695^{ab}	0.370	Synergism	62.974 ± 1.574^{gh}	92.109 ± 1.201^{abc}	0.996	Synergism
12.5%	50%	Comb. 13	62.554 ± 1.826^{e}	96.693 ± 3.925^{de}	0.572	Synergism	69.594 ± 1.509^{c}	95.015 ± 1.827^{ghi}	0.507	Synergism	61.585 ± 0.670^{g}	93.565 ± 1.436^{abc}	0.769	Synergism
25%	50%	Comb. 14	76.742 ± 2.809^{g}	68.100 ± 2.629^{bc}	0.266	Synergism	70.180 ± 1.441^{d}	79.636 ± 0.872^{e}	0.599	Synergism	64.345 ± 2.160^{gh}	91.844 ± 3.391^{abc}	0.815	Synergism
37.5%	50%	Comb. 15	82.803 ± 1.249^{h}	63.478 ± 1.021^{ab}	0.197	Synergism	82.473 ± 1.977^{fg}	71.293 ± 1.553^{abc}	0.405	Synergism	40.255 ± 1.998^{de}	88.585 ± 1.800^{a}	2.659	Antagonism
50%	50%	Comb. 16	84.370 ± 0.813^{h}	57.072 ± 2.835^{a}	0.191	Synergism	89.656 ± 1.898^{h}	66.766 ± 0.509^{a}	0.339	Synergism	52.309 ± 1.449^{f}	89.195 ± 1.014^{ab}	1.811	Antagonism

The data represent the mean ± SD of triplicate experiments.
Different letters (a–i): differences in ANOVA, followed by Tukey's test ($p < 0.05$).

factors should be investigated further because they can affect antioxidant activity (Peyrat-Maillard *et al*., 2003). Hydroxy groups are protected from steric effects that, in combination with curcumin, impair removal capacity and reduce activity (Naksuriya and Okonogi, 2015).

CONCLUSION

Both extracts showed inhibitory effects against proinflammatory mediators such as TNF-α, IL-6, and NO *in vitro* tests. The combination of these extracts produced a synergistic effect in inhibiting inflammatory mediators. Further tests need to be conducted to see the effectiveness and toxic effects, so it can be used as an alternative treatment for chronic inflammatory diseases.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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