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Combination of black cumin seeds with *Curcuma xanthorrhiza* extract as an antioxidant and immune-modulator agent in the COVID-19 pandemic era

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ABSTRACT

The COVID-19 pandemic demands changes in using of medicinal plants. Empirically, the medicinal plants of *Curcuma xanthorrhiza* (CX) and black cumin (BC) have been used massively in the era of the COVID-19 pandemic. *Curcuma xanthorrhiza* and black cumin (CXBC) preparations have been developed with the main ingredients of BC oil and CX extract. The purpose of the study was to determine the antioxidant and immunomodulatory activities of CXBC preparations active substances (polyphenols, flavonoids, thymoquinone). We conducted experimental laboratory research. The immunomodulatory activity test was carried out on human large lung cancer cell line (HTB-183 cells) by observing the expression of tumor necrosis factor alfa (TNF- α) and interleukine 10 (IL-10). The results showed that the CXBC preparation contained 4% thymoquinone, 25.87 mg/ml polyphenols, and 41.86 mg/dl flavonoids. CXBC preparations contain vitamins (A, C, and E) and minerals (potassium, calcium). The antioxidant activity of the CXBC preparation was included in the strong category with IC₅₀=54.87 ppm. CXBC preparations increased TNF- α expression and decreased IL-10 expression in HTB-183 cells. Based on the study results, it can be concluded that the CXBC preparation contains 4% thymoquinone, 25.87 mg/ml polyphenol, 41.86 mg/dl flavonoid, and a high level of vitamin and minerals. CXBC preparations have potent antioxidant activity, increase TNF- α and decrease IL-10 expression.

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1. INTRODUCTION

The COVID-19 pandemic has affected changes in people's medical behavior [1], one of which is the use of medicinal plants [2]. As a country with an area located in the equatorial region, Indonesia has various herbs with medicinal properties [3]. Indonesia has various medicinal plants with potential as immunomodulators and antioxidants [3]. *Curcuma xanthorrhiza* (CX) and black cumin seeds (BC) are medicinal plants that have been empirically used as immunomodulators and antioxidants by the Indonesian people [4], [5]. CX is one of the plants that has been empirically used as an immunity booster, anti-inflammatory, and antioxidant [6].

Traditionally, CX has been used as an appetite enhancer in children who have difficulty eating [7]. Xanthorhizol is one of the main active ingredients of CX, in addition to curcumin and curcuminoids. Xanthorhizol has been shown to have antioxidant activity [4], [6], [8]. Xanthorhizol has been shown to suppress lipid peroxidation in rat brain homogenates, glutamate-induced neurotoxicity, and reactive oxygen superfamily (ROS) production [9]. Curcumin has been shown to have anti-inflammatory effects, is safe to use, and is well-tolerated [10]. Like CX, BC is a medicinal plant that the community has widely used [11]. Besides being used as a spice in the kitchen, BC has also treated various disorders [12]. In the laboratory, black cumin seed extract and oil have been shown to have anti-inflammatory [13], antibacterial [14], antiviral [15], immunomodulatory [16], and antioxidant activities [17]. BC in vivo has been shown to increase the number of T lymphocytes [18], levels of Interferon- γ (IFN- γ) [19], and macrophage phagocytic activity [16]. Thymoquinone, the main ingredient of BC, can inhibit cyclooxygenase and lipoxygenase enzymes in arachidonic metabolism [20]. It is used for analgesic, anti-inflammatory, anticancer, antioxidant, anti-infective, and antihistamine effects [12], [14], [21], [22]. In the era of the COVID-19 pandemic, the use of these two medicinal plants by the Indonesian people experienced a spike [23], [24]. Alveolar epithelial cells are the target cells of the severe acute respiratory syndrome coronavirus 2 (SARS CoV-2 virus) [25]. Damage to alveolar epithelial cells causes an inflammatory reaction followed by an increase in several cytokines, including IL-6, IL-10, IL-15, and Granulocyte colony-stimulating factor (GCSF) [26]. Alveolar tissue damage also causes oxidative stress due to oxidative explosions in phagocytes to destroy germs [27]. The use of antioxidant and immunomodulatory agents is expected to prevent infection and inhibit damage caused by SARS CoV-2 [2], [28].

Empirically, CX and BC are used as immunomodulators and antioxidants both as prevention and complementary therapy for asymptomatic COVID-19 patients [23], [28]. As an immunomodulator, the consumption of medicinal plants is expected to improve the innate immune status to prevent infection from the SARS CoV-2 virus, which is easily transmitted [29]. The cytokine tumor necrosis factor alpha (TNF- α) has been shown to play an essential role in developing rapid antiviral immune responses [30]. TNF- α increases the phagocytic activity of macrophages and neutrophils to eliminate viruses that survive physical traps [31], [32]. Immunomodulators are also expected to prevent the occurrence of cytokine storms in patients with COVID-19 [2], [33]. COVID-19 patients have higher levels of plasma cytokines, namely interleukin family (IL-2, IL-6, IL-7, IL-10), and a high risk for Cytokine Storm Syndrome [34]. Antioxidant agents reduce lung tissue damage due to oxidative stress [35]. Infection with the SARS CoV-2 virus is accompanied by oxidative stress and decreased endogenous antioxidant capacity [36], thus requiring external antioxidant supplementation [37]. So far, research has been conducted to provide a scientific basis for using these medicinal plants by the public in dealing with the COVID-19 storm. When the COVID-19 pandemic storm still threatens and requires joint vigilance, research related to medicinal plants related to efforts to prevent and strengthen therapy in patients with COVID needs to be carried out. Combining two or more medicinal plants empirically has become a tradition in utilizing medicinal plants [37]. Javanese herbal preparations "jamu" use a combination of several medicinal plants as a traditional medicinal preparation for specific indications [3], [38]. The combination preparation of CX and BC extract is thought to contain active polyphenols, flavonoids, and nutrients with potential activities as antioxidants and immunomodulators [39]. CXBC preparations have been developed as immunomodulatory and antioxidant agents. CXBC preparation is thought to contain micro and macronutrients that act as antioxidants and immunomodulators. The purpose of this study was to determine the levels of flavonoids, polyphenols, and nutrients in CXBC preparation and the potential of antioxidant and immunomodulatory activities.

2. RESEARCH METHOD

2.1. Materials and instruments

This research was experimental laboratory research. We used several methods to achieve the research objectives: to determine the levels of flavonoids, polyphenols, and nutrients of CXBC preparation and its potential activity as an antioxidant and immunomodulator. We used CXBC preparation as the primary test material. CXBC preparation has been provided by the licensed traditional medicine industry (obtained permission from the Food and Drug Supervisory Agency of the Republic of Indonesia). To determine flavonoid levels, we used UV-Vis spectrophotometry, pure quercetin, and 10% aluminum chloride reagent. Folin-Ciocalteu reagent, UV-Vis spectrophotometer (Shimadzu 1240), gallic acid, and Na₂CO₃ p.a to determine polyphenol content. Pure thymoquinone and densitometry were used to determine thymoquinone levels. α , α -diphenyl- β -picrylhydrazyl (DPPH) reagent was used to examine the potential for antioxidant activity. TNF- α and IL-10 Fluorescein isothiocyanate (FITC) mAbs for testing potential immunomodulatory activities.

2.2. Research procedure

2.2.1. Examination of total flavonoid, total polyphenol, thymoquinone, and nutrient composition

Quantitative phytochemical evaluations of the CXBC preparation were carried out to determine the amount of total phenolic content (TPC) and total flavonoid content (TFC). We determined TPC by the Folin-Ciocalteu method, and total flavonoids content (TFC), whereas we used the aluminumchloride colorimetric assay, as described in the previous study. The Folin-Ciocalteu method was employed for total phenolic content determination following previously reported paper with slight modifications using gallic acid as standard (0–200 mg/L). Briefly, 0.5 mL of the CXBC preparation in methanol (1,000 ppm) was mixed with 2.5 mL of 10% aqueous Folin-Ciocalteu solution, stirred, and left for 5 min. A 2.0 mL of 5% aqueous Na₂CO₃ solution was then added. The mixture was further incubated at 40 °C for one hour. The absorbance was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer (Genesys UV-Vis Spectrophotometer, Thermo Fisher Scientific, Madison, WI, USA) with methanol as the blanks. The TPC was expressed as milligrams of gallic acid (Sigma- Aldrich Chemie) equivalent per gram of extract [40].

Pure thymoquinone and a densitometer were used to determine the thymoquinone assay. The TFC in the studied samples was estimated by the aluminum chloride colorimetric method described by the previous study, with minor modifications. In brief, 50 L of the preparation (2.5 mg/mL) was mixed with 4 mL of distilled water, 300 L of 5% (w/v) of sodium nitrite (Sigma), and 300 L of 10% (w/v) aluminum trichloride (Sigma). The mixture could stand for 6 min at room temperature; then, 2 mL of sodium hydroxide (1 M) was added to stop the reaction. The final volume of the mixture was adjusted to 10 mL with sterile-distilled water, and the absorbance was measured at 510 nm after 10 min against the reagent blank. The TFC was calculated from a calibration curve using rutin standard solution, and the result was mentioned as ppm of rutin equivalent of CXBC preparation. We determined the levels of thymoquinone by using a densitometer as was done by previous researchers [41], [42]. Meanwhile, the energy, carbohydrates, protein, fat, vitamin A, vitamin C, vitamin E, calcium, and potassium content of CXBC preparation were examined at the Nutrition Laboratory, Faculty of Agricultural Technology, Gadjah Mada University.

2.2.2. Examination of antioxidant and immunomodulatory activity of CXBC preparation

Examination of the potential of preparatic X asan antioxidant was carried out using the 3,6 DPPH (2,2'-diphenyl-1-picrylhydrazyl radical) method. The radical scavenging ability with DPPH radical was determined according to a previously published method. In brief, a volume of 20 µL of each sample at different concentrations (2-fold dilution; 2,500–1.22 g/mL) was mixed with 180 µL of 80 µM DPPH solution in ethanol in a 96-well plate. The plate was shaken and allowed to reach a steady state at room temperature in the dark for 30 min. DPPH bleaching was measured by monitoring the absorbance at 520 nm. The potential immunomodulatory activity was tested on HTB-183 cells (HTB-183 or NCI-H661 is lung carcinoma from humans (*Homo sapiens*)). The cells were cultured in a Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, then cultured in a CO₂ incubator, containing 5% CO₂, at 37 °C. The cell growth status was observed regularly, the inoculum was replaced according to the cell growth condition, and the cell passage was performed when the cell density reached 90%. All experiments were completed independently, and three parallel tests were set up. Testing the potential activity of CXBC preparation as an immunomodulator was carried out by the fluorescence-activated cell sorting (FACS) flow cytometry method by observing the expression of TNF- α and IL-10 in HTB-183 cells [38].

2.3. Data analysis

We performed univariate analysis to present data on total flavonoid content, total polyphenols, and nutrient content of CXBC preparation. We also performed a univariate analysis to present antioxidants of CXBC preparation. We performed the bivariate analysis with one-way ANOVA to determine the difference in the mean expression of TNF- and IL-10 based on the concentration of the CXBC preparation in HTB-183 cells.

3. RESULTS AND DISCUSSION

3.1. Results of examination of total flavonoid levels, total polyphenol levels, thymoquinone levels, and nutrient content of CXBC preparations

The results of the examination of total flavonoid levels, total polyphenols, thymoquinone levels, and nutrient content levels of CXBC preparations are presented in Table 1.

Table 1. Content of micro and macronutrients in CXBC preparations

The parameters	Result	Content of nutritional value per 5 ml	Nutritional adequacy rate (4-6 years)
Protein	0.73(%)	365 mg	25 gr
Carbohydrate	69.68(%)	3.48 gr	220 gr
Calcium	0.36(%)	18 mg	1000 mg
Potassium	0.20(%)	10 mg	2700 mg
Vitamin A	34789.32(mcg/100g)	1.74 mg	450 RE
Vitamin E	14879.551(mcg/100g)	743.95 mcg	7 mcg
Vitamin C	265.851(mcg/100g)	13.29 mcg	45 mcg
Flavonoid	47.86 (mg/mL)	239.3 mg	
Polyphenol	31.87(mg/mL)	159.35 mg	
Thymoquinone	4.00(%)	200 mg	

The CXBC preparation contains the expected micro and macronutrients. This herbal immunomodulator contains high vitamins A, C, and E levels. Besides vitamins, it also contains calcium and potassium, two minerals that are important for the growth and development of children. Phenolics and flavonoids are major antioxidant components available in the CX extracts and BC seed oil [43], [44].

The results of this study follow the latest trends in the use of medicinal plants [2]. Phytotherapy, Jamu or traditional Javanese medicine, traditional Chinese medicine, Ayurveda (Hinduism) are based on a holistic approach, using a mixture of medicinal plants instead of a single herbal component [3], [45], [46]. Several recent studies have supported the tradition of using herbal medicines as mixed preparations [47]. It has been disclosed about the clinical importance of the synergistic effect produced by the application of a multicomponent herbal mixture in patients with chronic diseases (such as diabetes mellitus, cancer, hypertension) and several infectious diseases (SARS COV-2, severe acute respiratory syndrome (SARS), tuberculosis, human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), malaria) [48], [49].

3.2. The results of the examination of the potential activity of CXBC preparations as antioxidants

The results of examining the potential activity as an antioxidant for CXBC preparations by measuring the ability to inhibit DPPH activity are presented in Table 2. Based on Table 2, the higher the concentration of CXBC preparations, the greater the ability to inhibit DPPH activity.

Table 2. Results of examination of the antioxidant activity of the preparation against DPPH

No	Controlled absorbance	Concentration (ppm)	Absorbance	% Inhibition	Regression	R2	IC50
1	0.687	5	0.676	1.601164	Y=4.33x-20.69	0.98	54.78 ppm
2	0.687	6	0.654	4.803493			
3	0.687	7	0.621	9.606987			
4	0.687	8	0.598	12.95488			
5	0.687	9	0.554	19.35953			
6	0.676	5	0.676	1.601164			
7	0.654	6	0.654	4.803493			
8	0.621	7	0.621	9.606987			
9	0.598	8	0.598	12.95488			
10	0.554	9	0.554	19.35953			

Based on the examination results of CXBC preparation's ability to inhibit DPPH as shown in Table 2, a regression formula can be drawn up the relationship between the concentration of CXBC preparation and the percentage of inhibition on DPPH activity ($y=4.33x-20.69$). Based on the regression equation of the relationship between the concentration of CXBC preparation and the ability to inhibit DPPH (% inhibition), we found that the IC50 value of CXBC preparation as an antioxidant was 54.78 ppm. IC50 of CXBC preparation was 54.78 ppm, which means that at a concentration of 54.78 ppm, CXBC preparation could inhibit 50% of DPPH activity in generating free radicals.

DPPH is a compound that contributes to free radicals [50]. In this study, DPPH was mixed with CXBC preparations as an antioxidant agent that can donate hydrogen to quench free radicals from DPPH. Antioxidants are classified to be very strong (IC50<50 ppm), strong (IC50=50 ppm-100 ppm), moderate (IC50=100 ppm-150 ppm), weak (IC50=150 ppm-200 ppm), and very weak (IC50>200 ppm). Based on the research data, it is known that the CXBC preparation has an IC50=54.78 ppm, which means that the

antioxidant activity of the CXBC preparation is included in the strong category. Compared to previous studies' results, CXBC preparations have lower antioxidant activity than vitamin C (IC₅₀=32.65 ppm) [47], [48].

3.3. Potential activity of CXBC preparations as immunomodulators by increasing TNF- α expression and inhibiting IL-10 expression

The potential immunomodulatory activity of CXBC preparation was tested on HTB-183 cells by observing the expression of TNF- α and IL-10 as shown in Figure 1. The effect of CXBC preparations on the expression of TNF- α and IL-10 in HTB-183 cells was observed at three concentration levels, namely according to the IC₅₀ value as an antioxidant (54.78 ppm), IC₅₀ (27.39 ppm and 1/4IC₅₀ (13.52 ppm). TNF- α and IL-10 in HTB-183 cells are presented in Figure 1.

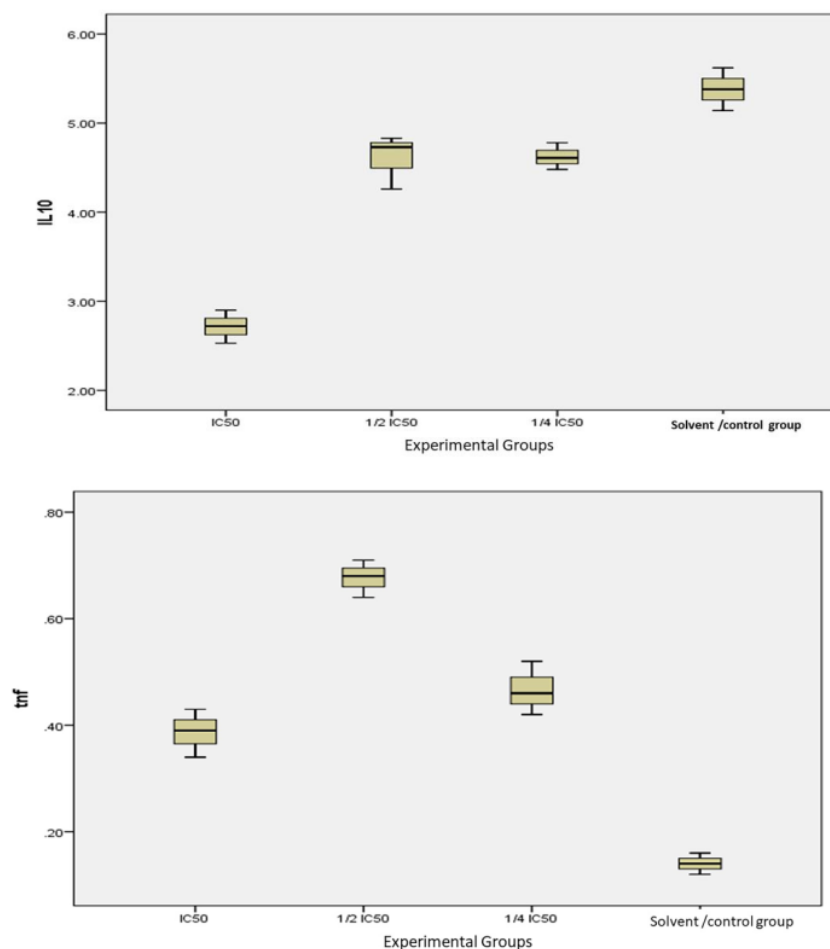


Figure 1. Boxplot image of TNF- α and IL-10 expression examination in HTB-183 cells after induction with CXBC preparations with concentrations according to IC₅₀, IC₅₀, 1/4IC₅₀, and solvent as a control

Based on the data, the results showed that CXBC preparations at concentrations according to IC₅₀ increased TNF- α expression but inhibited IL-10 expression. TNF- α expression in the treatment group was higher than TNF- α expression in the solvent control group. The IC₅₀ concentration group had the highest TNF- α expression. The IL-10 expression in the treatment group was lower than the IL-10 expression in the

solvent control group. The IC50 concentration group had the lowest IL-10 expression. Based on the research data, it is known that the CXBC preparation acts as an immunomodulator, which can increase the expression of TNF- α and decrease the expression of IL-10. TNF- α is one of the pro-inflammatory cytokines, and IL-10 is an anti-inflammatory cytokine so that the preparation can stimulate inflammatory reactions [49]. IL-10 elicits significant suppressive effects on myeloid cells by inhibiting proinflammatory cytokines, antigen-presenting cells (APCs), and other functions [30]. IL-10 also has a direct inhibitory effect on memory Th17 and Th2 cells while promoting the survival and action of Foxp3+ regulatory T cells (Tregs). Signaling the IL-10 pathway is associated with inflammatory diseases such as inflammatory bowel disease (IBD) and is often accompanied by immunopathology during infections [50]. Conversely, high, or dysregulated productions of IL-10 may contribute to chronic infection. In patients with Covid-19, elevated levels of IL-10 increase the risk of cytokine storm syndrome and the need for ICU care. The higher the vascular epithelial cell damage level was associated with an increased IL-10 level [34], [51].

The results showed that the CXBC preparation increased TNF- α expression and inhibited IL-10 expression. The research data showed that the CXBC preparation contained 4% thymoquinone. Thymoquinone, an active compound of BC, has acted as an antioxidant and immunomodulatory [11], [21]. Thymoquinone, via Toll-like receptor-4 (TLR-4), has been shown to increase the phagocytic activity and secretion of TNF-, and IFN- γ by macrophages. Thymoquinone has also been shown to increase the proliferation and differentiation of CD4Th into Th1 and Th2. Decreased expression of IL-10 is associated with an increase in the number of Th1, which produces pro-inflammatory cytokines, thereby inhibiting Th2 activity in producing IL-10 [52], [53]. The antioxidant activity of thymoquinone was demonstrated through activation of the aryl hydrocarbon receptor (AhR), activation of the transcription factor Nrf-2 and increased production of glutathione S transferase [13], [54]. The thymoquinone level in this study was higher than the thymoquinone level in the black cumin seed oil (BCSO) from the previous study (2.7%) [55].

Curcumin is an active compound that belongs to the curcuminoid group. Curcuminoid compounds are polyphenols with a yellow color like turmeric, CX, and other Zingiberaceae [8], [56]. Curcumin is a compound with several biological effects: anti-dyslipidemia, anti-oxidant, anti-inflammatory, anti-viral, and anti-fungal [56]. Curcumin has also been shown to inhibit the formation of atherosclerotic plaques [55], cancer chemoprevention, and hepatoprotective. In contrast to thymoquinone, curcumin is an antagonist of Toll-like receptor 4 (TLR4). TLR-4 is the innate immunity receptor of bacterial endotoxins and plays a pivotal role in inducing inflammatory responses [37], [56]. CX also contains xanthorrhizol (XNT) [8]. Previous studies demonstrated that XNT reduced the serum levels of free fatty acid and triglyceride in high-fat diet-(HFD)- induced obese mice. It has also been shown to promote cardiovascular health through anti-hyperglycemic, vasorelaxation] and low density lipoprotein (LDL) oxidation inhibitory effects [57]. The structural changes of polyphenols cause the loss of antioxidant capacity depending on the free phenols are higher than the glycosides, and iron-phenol chelates and the phenolic acids inter-react with the other molecules in the food matrix [9]. In vitro and in vivo conditions, previous studies indicate that BCSO and C. xanthorrhiza had great potential to reduce oxidative stress and immunomodulator [58].

4. CONCLUSION

CXBC preparation contains 4% thymoquinone, 25.87 mg/ml polyphenol, 41.86 mg/dl flavonoid, and a high level of vitamins and minerals. CXBC preparations have potent antioxidant activity, increase TNF- α and decrease IL-10 expression. Based on the results of this study, it is necessary to conduct research to test the effectiveness and safety of CXBC preparations so that CXBC preparations can be used as antioxidant and immunomodulator in the era of the COVID-19 pandemic.

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