Trop J Nat Prod Res, January 2024; 8(1):5794-5800 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Chemical Profiling, Antifungal and Anti-inflammatory Evaluations of Ethanol Extract of *Zingiber officinale* var. *rubrum* and *Curcuma caesia* Mixture from Riau, Sumatera Island, Indonesia

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ARTICLE INFO

Article history:
Received 21 September 2023
Revised 13 January 2024
Accepted 18 January 2024
Published online 01 February 2024

ABSTRACT

Invasive fungal infections can cause inflammation and stimulate immune response. Antifungals like plants possessing inherent anti-inflammatory activity, which can lead to rapid symptomatic relief while providing a mycologic cure is imperative. Plants with evidence of antifungal and anti-inflammatory activities are red ginger (*Zingiber officinale* var. *rubrum*) and black turmeric (*Curcuma caesia*). The study aimed to investigate the chemical profile, the antifungal and anti-inflammatory activities of the ethanol extract of red ginger and black turmeric mixture in *vitro*. Chemical profiling was done using Liquid Chromatography-Mass Spectrophotometry (LC-MS). Antifungal activity was tested using the Kirby Bauer disc diffusion method by measuring the diameter of the inhibition zone. Anti-inflammatory activity was tested by measuring the toxicity and inhibition of nitric oxide (NO) production in macrophage-like RAW 264.7 cells. From the results of the LC-MS analysis, 22 compounds were identified from the ethanol extract of red ginger and black turmeric mixture. The mixture extract exhibited potent antifungal activity; with the 80% concentration being the most effective with an inhibition zone diameter of 11.6 mm. The extract exhibited a dose-dependent anti-inflammatory activity. The 7.5 µg/mL and 15 µg/mL of the mixture extract significantly reduced nitric oxide production in RAW 264.7 cells by 0.62 and 19.854%, respectively. The mixture extract at 1000 µg/mL gave the highest proliferation inhibition of RAW 264.7 cells. On the basis of the results of the study, the extract of red ginger and black turmeric mixture has the potential to be used as an alternative antifungal and anti-inflammatory agent.

***Keywords*:** Antifungal, Anti-inflammatory, *Zingiber officinale* var. *rubrum*, *Curcuma caesia*, Phytotherapy, Nitric oxide

Introduction

Fungi are the leading cause of infectious disease in the tropical nations. Fungal infections in human constitute a serious problem, especially in developing countries including Indonesia.[1] Fungal infections can result in inflammation, and each fungal species has a different response mechanism to evade the host immune system.[2,3] Inflammatory diseases are major health problem worldwide and can increase morbidity and mortality rates each year.[4]

Inflammation is a local protective response resulting from tissue damage caused by physical trauma, damaging chemicals, or microbiological substances. Inflammation is also triggered by harmful foreign stimuli, infected and injured host tissues.[5] Foreign stimuli include pathogenic microbes, toxic chemicals, allergens, mechanical and thermal factors, and others.[6-8]

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Citation: Juariah S, Juliantari E, Dika Hanggara D, Surya A, Endrini S. Chemical Profiling, Antifungal and Anti-inflammatory Evaluations of Ethanol Extract of *Zingiber officinale* var. *rubrum* and *Curcuma caesia* Mixture from Riau, Sumatera Island, Indonesia. Trop J Nat Prod Res. 2024; 8(1):5794-5800. http://www.doi.org/10.26538/tjnpr/v8i1.12

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

If not properly controlled, this physiological response can lead to pathological consequences, which may lead to the development and emergence of various diseases in humans, including asthma, rheumatoid arthritis, inflammatory bowel disease, cancer, atherosclerosis, type 2 diabetes, obesity, and neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, and multiple sclerosis.[9-11] Inflammation destroys, reduces, or localizes (sequesters) both damaging agents and damaged tissues. Signs of inflammation are swelling/edema, redness, heat, pain, and changes in function.[12]

Numerous efficacious agents are available for the topical treatment of superficial fungal infections, like combination steroid/antifungal medication. Although, the judicious use of such products is often quite successful, the practice is questionable on both pragmatic and theoretical grounds. Steroid/antifungal combination medications are used extensively but are associated with potential disadvantages. Steroidal anti-inflammatories can cause peptic ulcers, decreased immunity to infections, osteoporosis, muscle and fat tissue atrophy, increased intra-ocular pressure, and diabetic, while non-steroidal anti-inflammatories can cause peptic ulcers, gastrointestinal bleeding, kidney disorders, and anemia.[13]

Antifungals like plants possessing inherent anti-inflammatory activity that will lead to rapid symptomatic relief while providing a mycologic cure would be very useful. Plants with scientific evidence of antifungal and anti-inflammatory activities are red ginger (*Zingiber officinale* var. *rubrum*) and black turmeric (*Curcuma caesia*). Biological activities of red ginger and black turmeric, such as antioxidant, antimicrobial, and anti-inflammatory properties are due to the presence of various bioactive components. Red ginger and black turmeric are a group of ginger (*Zingiberaceae*) with bioactive compounds such as flavonoids, phenols, terpenoids, and essential oils.[14,15]

The part of red ginger usually used as medicine is the rhizome. Red ginger can cure several diseases, such as sore throat, dry cough, colds, hives, vomiting, and diarrhea. In addition to curing diseases, this plant is also useful as anti-aging and anticancer, relieves for menstrual pain, strengthens the immune system, wards off bacterial and viral infections, and can also overcome digestive problems.[17] Black turmeric is useful as smooth muscle relaxants, and to treat leprosy, diarrhea, wounds, asthma, hemorrhoids, cancer, epilepsy, fever, vomiting, menstrual disorders, helminthes infestation, inflammation, and vaginal discharge. Red ginger has antibacterial activity against pathogenic bacteria.[17] Black turmeric has the potential to be used as antibacterial for nosocomial infections,[18] and as antioxidant.[19] The anti-inflammatory activity of this plant has not been explored, and the antifungal effect of the compounds contained in the plant needs to be evaluated. This study therefore aimed to investigate the chemical profile, the antifungal and anti-inflammatory activities of the ethanol extract of *Zingiber officinale* var. *rubrum* and *Curcuma caesia* mixture.

Materials and Methods

Extraction of plant samples

The plant samples were extracted following the procedure previously reported by Yadufashije *et al*. (2020)[20] and Bhunu *et al*. (2023)[21] with modifications. Fresh samples of red ginger and black turmeric (mixed) were cleaned, peeled, cut into slices, dried at 40°C, and then grind into powder form. The powdered sample (90 g) was extracted by maceration in ethanol (450 mL) (Merck) at room temperature for 72 h. the extract was filtered using Whatman filter paper no. 1, and then concentrated in a rotary evaporator at 78°C. The extract was kept in a sterile container at 4°C until used for the experiments.

Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS for the identification of the secondary metabolite in the extract was performed following a previously reported procedure with modifications.[22] Ultra-High Performance Liquid Chromatography-Mass Spectrophotometry (UHPLC-MS) was performed on the ACQUITY UPLC I-Class system from Waters. The sample extract was injected as much as 1 µL per injection. The analysis stage begins by filtering the sample extract using a 0.2 µm syringe filter and then inserting it into a vial. The sample was then injected into the LC-MS system in the positive ionization mode. Data were generated as chromatograms (LC) and spectra (MS). Each peak in the chromatogram was analyzed one by one to obtain the MS spectra which was matched with the spectra of known compounds in the Chemspider database.

Evaluation of antifungal activity

Candida albicans culture was inoculated and suspended into a test tube containing 5 mL of 0.9% NaCl solution using a syringe and then homogenized until turbidity results obtained was equivalent to 0.5 Mc Farland standard. Thereafter, *C. albicans* strains were cultured on Potato Dextrose Agar (PDA). The extract of red ginger and black turmeric mixture at each concentration was placed on the surface of PDA media smeared with *C. albicans* suspension and incubated for 7 days at 37ºC. The determination was done in triplicates. Then, the inhibition zone diameter was measured.[23]

Evaluation of anti-inflammatory activity

Cells and sample preparation

Cells at 80% confluence were used for the assay. The cells were washed with 1X PBS, after which 1X EDTA-trypsin was added, and then incubated in a 5% CO_2 incubator at 37ºC for 3 min. Then 5 mL of media was added for trypsin inactivation. The cells suspension was transferred into a new sterile conical tube, and centrifuged at 1200 rpm for 5 min, the supernatant was discarded, and 1 mL of culture medium was added to the remaining pellet. Cells were counted using a haemocytometer and trypan blue to determine the number of cells available in 1 mL of media. Cells were diluted with culture medium, transferred to 96-well plates at a volume of 100 µL per well, and incubated overnight. The test sample (10 mg extract) was dissolved in DMSO and diluted to a concentration of 50,000 ppm. The sample was further diluted to the concentrations used for the assay.[24]

Cytotoxicity test on RAW 264.7 cells

The diluted sample (100 µL) was added to the culture medium and incubated at 37ºC for 24 h. After incubation, the cells were washed with PBS (1X6), and 100 µL of MTT reagent was added and incubated for 4 h. Thereafter, 100 µL of 10% SDS was added and incubated overnight. The absorbance was read using an ELISA Reader at a wavelength of 570 nm.[24]

Nitric Oxide (NO) inhibition assay on RAW 264.7 cells

Nitric oxide inhibition assay was performed by using commercial Griess reagent (Sigma, Unites States) for the determination of total nitrate and nitrite concentration as an index of nitric oxide production.[24] Samples of each concentration of the extract were placed into wells and incubated for 2 hours. The lipopolysaccharide (LPS)-inducing agent was added to each well, including the positive control, while medium only was added to the negative control. The supernatant (100 µL) from each well was put into a 96-well plate. Sodium nitrite (100 uL) and 100 µL of Griess reagent were added to the 96-well plates. The absorbance was read using an ELISA Reader at a wavelength of 540 nm. A standard linear regression equation and correlation coefficient curve were made from the absorbance readings obtained. From the linear regression equation, the concentration of NO produced by sample-treated RAW 264.7 cells was calculated with the average absorbance as the y-axis variable and the concentration of NO as the x-axis variable.

Statistical analysis

The determination was done in triplicate and the data analysed by one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS version 16.0, IBM Corp., Armonk, NY, USA).

Results and Discussion

Compounds identified by Liquid chromatography-mass spectrometry (LCMS)

Plants contain a variety of chemical compounds that are known to be physiologically active and responsible for a variety of pharmacological effects. Many of the secondary metabolites found in plants are natural antioxidant sources.[25] LC-MS analysis was conducted for the ethanol extract of *Zingiber officinale* var. *rubrum* and *Curcuma caesia* mixture on the basis of polarity of the solvent used. The chromatogram from the LC-MS analysis is presented in Figure 1, while Table 1 shows the compounds identified in the ethanol extract of red ginger and black turmeric mixture.

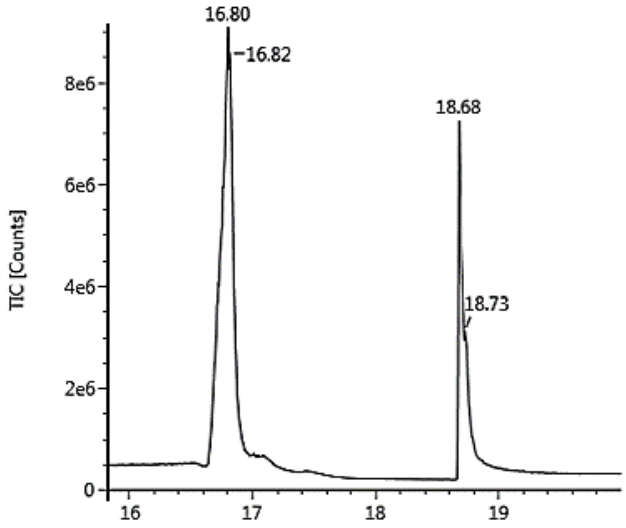

Figure 1: Chromatogram of ethanol extract of red ginger and black turmeric mixture

Table 1: Compounds identified in the ethanol extract of red ginger and black turmeric mixture

Retention time (min)	Compound
18.68	5-(3,4-Dihydroxybut-1-ynyl)-2,2′-bithiophene, Methyl lucidenate Q, Daturametelin J, n-Docosanol, Sarcostin
18.73	Inosine_1
16.80	Potassium quisqualate, α-Terthienyl methanol, 5-Formylxanthotoxol, Digallic acid, 14-Deoxy-11-hydro-xyandrographolide, Mongolicumin A, Arctinone-B, 3,4-Di-O-galloylquinic acid, Isorhamnetin-3-gentiobioside-7-glucoside, Eckol
16.82	3-O-[β-D-Glucopyra-nosyl-(1→2)]-β-D-glucopyranosyl-7-O-β-D-glucopyranosylkaempferol, Glucoraphenin, Picrasidine K, Pedunculagin_1, Salviolone

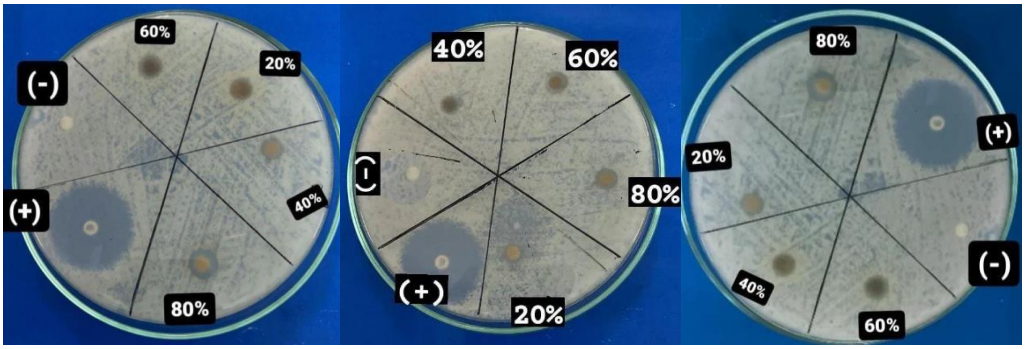

Figure 2: Diameter of inhibition zone of extract of ginger and black turmeric mixture (A) replicate 1), (B) replicate 2, and (C) replicate 3.

The essential oil from the rhizomes of red ginger have been shown to contain high percentages of monoterpenoids (60.6%) consisting mainly 1,8-cineole, geranial, neral, and borneol, and a lower percentage of sesquiterpenes constituents dominated by ar-curcumene, *β*-sesquiphellandrene, and *β*-bisabolene.[25] The essential oil of black turmeric rhizomes consist mainly of Androsta-1,4-dien-3-one, 17-(acetyloxy)-, (17*β*)-Santanol acetate, Eucalyptol, Cycloprop[e]indene1a,2(1*H*)-dicarboxaldehyde,3a,4,5,6,6a,6b-hexahydro-5,5,6btrimethyl,(1a. α.,3a. *β*.,6a. *β*.,6b. *α*), Methyl 7,12-octadecadienoate, (+)-2-Bornanone, L-Bornyl Acetate, Isogermafurene, Isoborneol, Acetic acid, and methyl ester.[26] Differences in chemical profiles in plants of the same species is dependent on the geographical distribution.[27] The essential oil composition of turmeric (*C. longa*) as reported by different researchers has been found to be significantly different, and this has been attributed to the disparity in maturity of the rhizomes as well as the geographical locations.[28]

Antifungal activity against Candida albicans

Candida albicans is a pathogen in the mouth that causes mild opportunistic infections. Several medications, notably anticancer and anti-infective compounds, have been developed using natural ingredients.[29] Ethanol extract of red ginger and black turmeric mixture was evaluated for its antifungal activity, and the results were calculated based on the zones of inhibition using the diffusion method. The extract showed antifungal activity against *C. albicans* in the agar well diffusion assay (Figure 2).

The diameter of the clear zone formed varied according to the concentration used in the treatment. The higher the concentration, the greater the diameter of the inhibition zone formed. The presence of inhibition zones against the growth of *Candida albicans* is attributed to the content of secondary metabolites such as flavonoids, alkaloids, tannins, saponins, terpenoids, steroids, curcumin and essential oils contained in the extract of red ginger and black turmeric mixture. The antifungal activity of flavonoid compounds has been reported to be due to damage to the fungal cell membrane which then lead to increased cell permeability and leakage of cellular components.[30]

The compound α-Terthienyl methanol and 14-Deoxy-11-hydro-xyandrographolide identified in the extract of red ginger and black turmeric mixture have been found to possess antifungal activity. For example, the compound *α*-Terthienyl methanol isolated from *Eclipta prostrata* has been proven to have antifungal activity against *C. albicans*,[31] while the compound 14-Deoxy-11-hydro-xyandrographolide isolated from *Andrographis paniculata* has been shown to be effective in the treatment of fungal skin infections.[32]

Terpenoids and steroids are bioactive compounds that have antifungal properties.[33] These compounds inhibit fungal growth through the cytoplasmic membrane and interfere with the growth and development of fungal spores.[34,35] Alkaloids are primary compounds containing one or more nitrogen atoms, and have alkaline properties. This alkaline nature is likely to suppress the growth of *C. albicans* because the fungus grows in acidic medium (pH 4.5-6.5).[36]

Saponins disrupt the stability of fungal cell membranes, which results in cell membrane damage and cause the release of various important components namely; proteins, nucleic acids, and nucleotides from inside the fungal cell.[36-38]

Tannins are lipophilic compounds that are easily bound to cell walls and cause damage to fungal cell walls. The antifungal mechanism of tannins is attributed to their ability to inhibit the synthesis of chitin used for cell wall formation in fungi, and this leads to damage cell membranes and inhibition of fungal growth.[39-41] Curcumin and essential oils are polyphenol group of compounds that have antifungal activity, they inhibit the growth of *C. albicans* by inhibiting protein synthesis, resulting in inhibition of microbial growth.[42-44]

In this study, nystatin was used as the positive control because nystatin is the main class of drugs against *Candida* sp.[45] Nystatin exhibit antifungal activity by binding sterols (especially ergosterol) in the cell membrane, the cell membrane can no longer function as a selective barrier, and thus potassium and other cell components are lost.[46] The negative control (96% ethanol) did not show any inhibition zone, this of course is due to the absence of secondary metabolites. The antifungal activity exhibited by the extract was concentration-dependent. The diameter of the inhibition zone formed increased with the increase in the extract concentration.

Anti-inflammatory activity

Cytotoxicity of extract against macrophage-like RAW 264.7 cell line

The effect of extract treatment on the cell proliferation of RAW 264.7 cells is presented in Table 2. The highest percentage inhibition of proliferation was shown at 1000 µg/mL of the extract. At high concentrations, the extract of red ginger and black turmeric mixture showed strong anti-inflammatory activity. The resulting anti-inflammatory activity of the extract is attributed to the presence of active metabolites such as flavonoids in the extract. Flavonoids a group of secondary metabolites called polyphenols exhibit anti-inflammatory action by inhibiting the activity of COX enzymes and lipooxygenase, reducing the number of leukocytes and reducing complement activation, inhibiting the release of arachidonic acid and secretion of lysosomal enzymes from neutrophil cells and endothelial cells, and inhibiting the exudation phase and proliferation phase of the inflammatory process.[47,48]

Some flavonoid compounds such as 5-(3,4-Dihydroxybut-1-ynyl)-2,2′-bithiophene and 3-*O*-[*β*-D-Glucopyra-nosyl-(1→2)]-β-D-glucopyranosyl-7-O-β-D-glucopyranosylkaempferol which were identified in the extract of the mixture of red ginger and black turmeric have been found to have anti-inflammatory activity. The compound 5-(3,4-Dihydroxybut-1-ynyl)-2,2′-bithiophene which was isolated from *Echinpos grijsii* has been proven to have inflammatory activity against LPS-stimulated nitric oxide production in RAW 264.7 cell line with IC_{50} value of 20.0 µg/mL.[49] On the other hand, the compound 3-*O*-[*β*-D-Glucopyra-nosyl-(1→2)]-*β*-D-glucopyranosyl-7-*O*-*β*-D-glucopyranosylkaempferol exhibited potent anti-inflammatory activity, with IC_{50} values ranging from 0.46 to 0.79 mg/mL.[50]

The mechanism of anti-inflammatory action of flavonoids occurs via two pathways: (i) by inhibiting capillary permeability and inhibiting arachidonic acid metabolism and secretion of lysosomal enzymes from neutrophils and endothelial cells.[51-53] (ii) by acting on the microvascular endothelium to reduce the occurrence of hyperpermeability and inflammation.[54] Some flavonoid compounds can inhibit the release of arachidonic acid and the secretion of lysosomal enzymes from the membrane by blocking the cyclooxygenase pathway.[55] Inhibition of the cyclooxygenase pathway have a broader effect because the cyclooxygenase reaction is the first step in the pathway leading to the biosynthesis of eicosanoid hormones such as prostaglandins and thromboxanes.[56,57]

Saponins are classified based on the aglycone structure into terpenoidal and steroidal saponins. Both group of compounds have been reported to posses anti-inflammatory activity.[58] The anti-inflammatory mechanism of saponins is by inhibiting the formation of exudates and inhibiting the increase in vascular permeability.[59,60] The terpenoidal saponin such as oleanolic acid exhibit anti-inflammatory activity through antioxidant mechanism, whereas the terpenoidal saponins function as inhibitors of enzyme activity by inhibiting the convertion of arachidonic acid to prostaglandins which are potent inflammatory mediators.[61-63]

Inhibition of Nitric Oxide (NO) production in RAW 264.7 macrophage cells

The anti-inflammatory activity of the extract of red ginger and black tumeric mixture demonstrated by the inhibition of nitric oxide production in RAW 264.7 macrophage cells is presented in Table 3. The anti-inflammatory activity is expressed in log concentration and interpreted as IC_{50} value. The cytotoxicity test of the samples against RAW 264.7 cells (macrophage-like cell line) was conducted in triplicate with six concentration ranges. The percentage of proliferation inhibition is the percentage of cell proliferation after treatment with the extract compared to the cell proliferation in the control. The LPS-stimulated cells treated with the extract exhibited a dramatic decrease in nitric oxide production in a concentration-dependent manner. Specifically, the extract of red ginger and black tumeric mixture at 7.5 and 15 µg/mL significantly reduced the nitric oxide level by approximately 0.62 and 19.854%, respectively.

The results showed that the extract of red ginger and black turmeric mixture inhibited NO production in a concentration-dependent manner. The higher the concentration of the extract used, the greater the ability to suppress the activity of macrophages to produce NO an inflammatory mediator. NO can be produced by macrophages in both physiological and pathophysiological states. Under physiological conditions, NO is produced as a messenger for intercellular communication,[64] while in pathophysiological conditions, it is part of the non-specific immune system to destroy pathogenic cells or infected cells.[65]

During bacterial infection, LPS will be released from the bacterial cell wall to stimulate the production of an enzyme called nitrite oxide synthase (iNOS), which is an enzyme in mammalian cells (macrophages, hepatocytes, and endothelial cells) that converts L-arginine into citrulline and NO.[66,67] Increased production of iNOS in macrophages will lead to increased levels of NO.[68,69] In contrast, inhibition of cell stimulation by LPS will be followed by inhibition of iNOS production and subsequently decreased NO production. The presence of numerous bioactive compounds in red ginger and black turmeric with little or no cytotoxic effect encourages further studies on the potential pharmacological applications of these species as alternative anti-inflammatory agents with antifungal activity.

Table 2: Inhibition of RAW 264.7 cell proliferation by ethanol extract of red ginger and black turmeric mixture

Extract Concentration (µg/mL)	Proliferation Inhibition (%)
15.625	7.228 ± 1.417
31.250	20.215 ± 1.936
62.050	30.025 ± 1.916
125	69.509 ± 2.066
250	99.464 ± 0.818
1000	100.706 ± 0.200

Proliferation Inhibition: Percentage of Cell Proliferation after Sample Addition Compared to Control Cells

Table 3: Inhibition of Nitric Oxide (NO) production in RAW 264.7 macrophage cells by ethanol extract of red ginger and black turmeric mixture

Sample	Concentration (µg/mL)	NO Concentration (µg/mL)	Inhibition of NO/K. LPS (%)	IC_{50} (µg/mL)
Control-	0.062			
Control + (LPS)	1	1.422		
Extract	0.75	1.509	-6.127	47.333
	1.5625	1.433	-0.776	
	3.75	1.517	-6.67	
	7.5	1.413	0.62	
	15	1.14	19.854	
	30	1.045	26.524	

NO Inhibition: Percentage of NO concentration of treatment groups compared awith Control (+) or LPS Control

Conclusion

On the basis of the results obtained from this study, it could be stated that the extract of the mixture of red ginger and black turmeric can be used as alternative to regular antifungal and anti-inflammatory drugs. However, further preclinical and clinical studies are required to substantiate the use of the extract of the mixture of these two plants as topical antifungal agent for the treatment of candidiasis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We would like to thank the Universitas Abdurrab that supported this study financially by an Internal Grant scheme PDUPT_K.

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