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Antifungal Effect Of *Curcuma zedoaria* Ethanol Extract and Fractions Against *Aspergillus niger*

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Abstract : The aim of this study is to investigate the active fraction of *Curcuma zedoaria* ethanol extracts against *Aspergillus niger*. The simplisia of *C. zedoaria* rhizome was extracted with ethanol using a maceration method. The phytochemical screening of ethanol extract was performed to detect antifungal secondary metabolites. All of the antifungal activity test for extracts and fractions were conducted using the agar diffusion method. The thick extracts were then fractionated by liquid-liquid extraction method with a funnel using several solvents such as: n- hexane, ethyl acetate, and water. The minimum fungal concentration (MFC) was determined by a serial macrodilution method, followed by subculturing the overnight minimum inhibitory concentration test result. The antifungal active fraction, then compared with nistatin using the agar diffusion method. The results showed that ethanol extract and n-hexane fraction have antifungal activity against *A. niger*. The MFC value of n-hexane fraction was ranged at 0.25-0.50% w/v. While the antifungal comparative value of n-hexane fraction ad nystatin against *A. niger* was 1:7.22 x 10^{-7}. It can be concluded that the ethanol extract and n-hexane fraction of *C. zedoaria* rhizomes prospects as an antifungal against *A. niger.*
Keywords : *Curcuma zedoaria*, antifungal, *Aspergillus niger*, n-hexane.

Introduction

Aspergillus niger is a successful cosmopolitan saprophyte of common occurrence, and perhaps the most abundant species of *Aspergillus.* It is isolated from house dust, soil, plant litter, dried fruits, nuts and seeds, and untreated textiles such as jute, hemp and cotton bracts[1]. It has been isolated from the thorns of rose bushes[2]. In another study reported that the airborne *A. niger* allergens were found in the highest concentrations (ng/m^3) of four species of *Aspergillus*[3]. It can damage food stuffs such as stored fruits and vegetables, nuts and corn, oil seeds, grains, and dairy products[3]. Diseases caused by *Aspergillus* spp. include clinical allergies (allergic bronchopulmonary aspergillosis, rhinitis, Farmers's lung), superficial and local infections (cutaneous infections, otomycosis, tracheobronchitis), infections associated with damaged tissue (aspergilloma, osteomyelitis), and invasive pulmonary and extrapulmonary infections. Aspergillosis is a common term used to describe infections caused by different species of *Aspergillus*[4]. *Aspergillus niger* was found also as the etiological agent of patient's cavitary lung lesions[5]. *Aspergillus niger* is a mould that is rarely reported as a cause of pneumonia. But a study reported that a case of necrotizing *A. niger* fungal pneumonia that did not respond to voriconazole in a patient on long-term steroid treatment[5]. *Aspergillus* spp. was also reported resistant to itraconazole[6].

The discovery of new antifungal against *A. niger* can be used as a new breakthrough in order to search an anti-aspergillosis, particularly those caused by *A.niger*. Empirically, *C. zedoaria* is a drug based ingredient natural materials that have long been used to treat some infections. *C. zedoaria* rhizome have many chemical

constituents, namely curcuminoid (diarilheptanoid), essential oils, polysaccharides and other groups. The known curkuminoid are as follow: curcumin, curcumin dimethoxy, bisdimetoksikurkumin and 1,7-bis (4-hydroxyphenyl) -1,4,6-heptatrien-3-on, while the main content of essential oil are sesquiterpenes, which contain more than twenty components between other curzerenone (zedoarin) which is the largest component, curzerene, pyrocurcuzerenone, curcumin, curcumemone, epicurcumeno, dehydrocurdione, furadienone, isofuranodienone, furanidiene, zederone, and curdione[7]. The ethyl p-methoxycinnamate was reported as one of *C. zedoaria* component that produced antifungal activity[8]. Based on its antifungal constituents, *C. zedoaria* was proposed to be an antifungal agent for aspergillosis infection caused by *A. niger* and is considered as alternatives to conventional antimicrobial agents especially in this era of antimicrobial drug resistance.

Experimental

Plant Materials

The plant material of the rhizomes of *C. zedoaria* were obtained from the Experimental Field Manaco Balitro, Lembang district, West Java, Indonesia. The determination was done in Determination Institution of Biology Department, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jatinangor, West Java, Indonesia.

Preparation of rhizomes extracts

The process of extraction was using maceration method. The simplicia of *C. zedoaria* rhizomes were sliced and ground to a powder, then put into the maserator chamber and soaked in 70% ethanol for 72 hours. The extracts were evaporated using a rotary evaporator at 50 ^{0}C, then continued to evaporate on a water bath until dried extract with constant weight was obtained. The extract was stored in a refrigerator at 4 ºC until time of use.

Examination of extract quality

The examination of extract quality was including phytochemical screening. Phytochemical screening was done by using Fansworth method to determine the containment of alkaloids, flavonoids, tannins, Quinones, phenolics, saponins, steroids, triterpenoids, monoterpenoids and sesquiterpenoids in the ethanol extract of zedoaria rhizomes[9].

Test organism

The fungus that used in this research was *Aspergillus niger* ATCC 32611. This fungus was obtained from the health department in Bandung, Indonesia.

Culture media and inoculum preparation

The culture media that used were Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) from Oxoid. The inocolum preparation was done by taking one Ose of *A. niger* colony from the agar tube. Then it put into a reaction tube containing sterile physiological NaCl and shaken it up untill homogeneous. The turbidity of fungal suspension was measured using Spectrophotometer at 600 nm to the equivalent of 0.3 Mc Farland solution.

Antifungal activity of extract

The antifungal activity of the test extracts was determined by the agar diffusion technique. The volume of 20 ml Mueller-Hinton Agar was poured into a sterilized petri dish, then 50 µL fungal suspensions with 0.3 McFarland in turbidity were added. The mixture of fungal suspension and agar was homogenized until it became solid. The media is then perforated to make holes for storing the extract. Different concentrations of extracts, i.e., 5%, 10% and 25% were added into each hole. Taking care not to allow spillage of the solution to the surface of the agar medium. Three replicates of each concentration were maintained. The plates were allowed to stand on the laboratory bench for 1 h to allow for proper diffusion of the extract into the media. The plates were incubated upside down at room temperature for 96 h and the diameter of holes in each concentration was measured every day. Plates were observed for zones of inhibition. The antifungal activities of the extracts

were determined by measuring the diameter of the inhibition zone around the well that was filled with the extract.

Fractionation method

The thick extracts of zedoaria were fractionated using liquid-liquid extraction method with a funnel. The solvent used is n-hexane as a polar solvent, ethyl acetate as a semi-polar solvent, and water as polar solvents. 5 g of condensed extract were dissolved in 10 ml ethanol, then it put into a separating funnel. After that, about 100 ml of distilled water was added and shaken until all mixed. 300 ml of n-hexane was added and the funnel was shaken vigorously for 30 min, then allowed to stand. A layer of n-hexane was collected in a bottle, whereas the water layer that has been separated from the layer of n-hexane was added again with a new n-hexane and continued with the same process as much as 3 times. 100 ml of ethyl acetate was added to the remaining water layer separation of n-hexane, then shaken vigorously for 30 min. After which it was allowed to stand for 24 h. Then the ethyl acetate layer was removed and stored in a bottle. The same process was repeated 3 times. The solvent of fractions was then evaporated using a rotary evaporator. Each of condensed fraction was weighed and the yield was calculated.

Antifungal activity of fractions

This step was aimed to determine the fraction that gives the greatest antifungal activity against *A. niger*. The antifungal activity of the fractions was determined using the same technique with the extract.

Minimum Inhibitory Concentration Determination

Minimum Inhibitory Concentration (MIC) MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube[10,11]. In this method, the broth dilution technique was utilized where the plant extract was prepared to the highest concentration in sterile distilled water and serially diluted (twofold) to a working concentration using Sabouraud broth and later inoculated with 0.2 ml suspension of fungal strains. In the process of determining the MIC, is made of the sample solution with varying concentrations of 5%, 2.5%, 1%, 0.75%, 0.5%, 0.25% and 0.1%. After 18 hours of incubation at 37º C, the test tubes were observed for turbidity. The least where no turbidity was observed was determined and noted as the minimum inhibitory concentration (MIC) value[12].

Minimum Fungicidal Concentration (MFC)

The MFC is defined as the lowest concentration where no fungal growth is observed. This was determined by the broth dilution resulting from the MIC tubes by sub-culturing to antifungal free agar[10,11]. In this technique, the contents of the test tubes resulting from MIC was streaked using a sterile wire loop on agar plate free of fungi and incubated as 37º C for 18 hours. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MFC[12].

Antifungal of comparative analysis

Comparative tests was carried out using various concentrations of nystatin, as follows: 0.01%; 0.005%; 0.0025%; and 0.001%. Nystatin was tested on the same way on the activity of extracts and fractions above. Comparative tests of a sample can be done by making a standard curve of a graph or a comparison substance, in which the logarithm of the concentration described in the x-axis and the diameter of the barriers described in the y-axis.

Result and Discussion

The antifungal activity test results showed that the greater the concentration of extract used, was directly proportional to the increase in the diameter of the inhibition zone. The result can be seen in table 1.

Table 1: The antifungal activity of Zedoaria rhizomes ethanol extract

Time of incubation (h)	Diameter of Zone Inhibition of various concentrations (mm)		
	5% w/v	**10% w/v**	**25% w/v**
24	0.000±0.0000	0.000±0.0000	0.000±0.0000
48	12.325±0.1479	12.550±0.1118	13.050±0.1802
72	12.475±0.0829	12.700±0.0707	13.200±0.0707
96	12.500±0.0707	12.825±0.1479	13.225±0.0829

The antifungal activity of the extracts related to the components of secondary metabolites in the extract. Based on the results of phytochemical screening, it is known that the chemical components of the ethanol extract of zedoaria rhizomes contain flavonoids, tannins, monoterpenoid and sesquiterpenoids, steroids, and triterpenoid. In another study, the ethanol extract leaves of *Hardwickia binata* showed the high range of activity against *A. niger* because of the presence of saponins, steroids, flavonoids, coumarins and tannins[13]. The tannins isolated from the medicinal plants possess remarkable toxic activity against bacteria and fungi and may assume pharmacological importance[14]. In another study, revealed that flavonoids with antifungal activity have been isolated from *Amboyna wood* was found to be active against *A. niger*[15]. Anthocyanidine (flavonoid) isolated from *Bryophyllum pinnatum* was found to be active against the plant pathogen *A. niger* and the clinical fungus Candida albicans[16]. These data revealed that the presence of various bioactive secondary metabolites which might be responsible for their medicinal attributes, such as antifungal, especially flavonoids and tannins.

The antifungal activity of fractions was conducted to determine the fraction that gave the greatest antifungal activity. The fractions were tested at concentrations of 10% in DMSO as solvent. The diameter of inhibition zone of fractions can be seen in Table 2.

Table 2: Antifungal activity of the fractions

Fractions	Diameter of Zone Inhibition (mm)
n-hexana	14.093±0.0094
Ethyl acetate	0.0000±0.0000
water	0.0000±0.0000

From the data above, n-hexane fraction exhibited the most active fraction for antifungal activity against *A. niger*. Antifungal candidate of n-hexane fraction can be seen from its MFC value. The smaller of the MFC value, the more active of antifungal potent of the fraction. The MFC value of n- hexana fraction was ranged at 0.25-0.50%w/v, can be seen in table 3. From the results it is known that *A. niger* was susceptible to both the n-hexana fraction and rhizome extracts.

Table 3: The MFC value of n-hexana fraction

Concentration (%w/v)	Colony growth
5,00	-
2,50	-
1,00	-
0,75	-
0,50	-
0,25	+
0,10	+

Notes: (+) colony presence; (-) colony absence

When the antifungal activity of the n-hexane fraction against *A. niger* compared with nystatin, an appealing value of 1: 7,22 x 10-7 was obtained. This figure clearly indicates that nystatin as a synthetic antifungal agent is more effective and rapidly kills *A. niger* than the integration of secondary metabolites

present in the n-hexane fraction. However, nystatin, which is a class of polien antibiotics, has a disadvantage in its use, is associated with its side effects when used for systemic aspergillosis infection, so its only used to treat for local infections. Polyenes possess a lower but non-negligible affinity for cholesterol, the human counterpart of ergosterol. This slight affinity for cholesterol explains the high toxicity associated with antifungals and is responsible for several side effects[17]. For this reason, only Amfoterisin B is given systemically, while nystatin and natamycin are only used locally or orally. These two last molecules fortunately possess a very limited systemic activity, since their absorption trough gastrointestinal mucosa is almost nonexistent[18, 19].

Conclusion

In the current research, the ethanolic extract of *C. zedoaria* rhizome and n-hexana fraction were found to be active on *A. niger* in comparing to ethyl acetate an water fraction.

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