

Antioxidant and Antifungal Activity of Jeringau (*Acorus calamus L.*) Extract in Some Organic Solvents in Vitro

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Abstract

Candidiasis is one disease that has experienced by 75% of women in Indonesia caused by fungal infection, *Candida albicans*. Candidiasis can cause serious complications such as infertility. One of the best known ingredients to cure infertility problems is jeringau rhizome (*Acorus calamus L.*). This research is the first step to process for standardization and scientification of jeringau rizophome as one of the basic ingredients of Madura traditional medicine, jamu "Subur Kandungan". It was aimed to investigate antioxidant and antifungal activity of jeringau rizophome in some organic solvents. Samples were extracted by maceration method using ethanol, chloroform, and n-hexane. Antioxidant activity assay was determined using DPPH method. Ascorbic acid was used as control. Antifungal activity test on *Candida albicans* was done by using kirby bauer method to measure diameter of inhibition zone and microdilution plate method to determine MIC and MFC. The highest antioxidant activity was revealed by ethanol followed by chloroform and n-hexane, while the highest antifungal activity was obtained by ethanol followed by n-hexane and chloroform. The MIC value of ethanol, chloroform and n-hexane were founded at concentration of 0.39% and the MFC at a concentration of 0.78%.

1. INTRODUCTION

Infertility is one of the reproductive problems experienced by several couples in the world. In Indonesia, infertility rate is

categorized high ranged from 12-15% (Yusnita, 2012). There are various factors that cause infertility in woman, one of them is vaginal discharge or candidiasis. *Candida Vaginitis* is

one of the most common infections in women of reproductive age. About 75% of adult women experience at least one episode of vaginitis by candida during their lifetime. Unfortunately, about 40 - 50% of women who experience the first episode are likely to have a recurrence and 5% may exhibit a "recurrent" form characterized by at least three or more episodes of infection per year (Mulu, 2015).

Abnormal vaginal discharge is commonly caused by *Candida albicans* infection. Recently, drug therapy and keep the feminine hygiene are the ways to treat candidiasis. In the last decade there has been a tremendous surge in public acceptance and interest in natural therapies in both developed and developing countries. These herbal remedies are available not only in drug stores, but now also in grocery stores and supermarkets. It is estimated that up to four billion people (representing 80% of the world population) who live in developing countries rely on herbal medicinal products as the main source of health care. Traditional medicine practices that involve the use of herbs are seen as an integral part of the culture in the community (Ekor, 2013).

Indonesia's biodiversity is ranked 3rd after Brazil and Zaire. It is home to 30,000 out of 40,000 medicinal herbal plants in the world. Medicinal plants are known in Indonesia as "Biofarmaka Plant"; defined as plants which are useful for traditional medicine. It is the ingredients of leaf, fruit, tuber or root. Throughout the centuries, Indonesia's indigenous people developed their curing illnesses and keeping their health. In general, there are about 30,000 species of medicinal plants owned by Indonesia, and potentially to develop herbal products which have equal quality with modern medicines (Yusuf, 2017).

One of the plants having potency as herbal medicine is jeringau (*A. calamus*). Jeringau extracts has biological activity against microbial such as *Salmonella typhosa*, *Candida albicans*, virus, and nematode as well as insect and pathogen vector that harm human, animal, and plant (Hartati, 2012). This activity is

related to the active compounds contained in jeringau such as terpenes, alcohols, aldehydes, and phenols (carvacrol, eugenol, thymol, cinamaldehyde, cinnamic acid, and perylaldehyde) (Hartati, 2012). This plant also contains an essential oil called calamus oil. The use of calamus oil is not limited to foods and beverages, but also to deodorant, soap, beauty cream, and etc.

Pathogenic microbes, free radicals can also cause infertility, thus antioxidant compounds are needed in neutralizing these free radicals. In previous study, active compounds that can be used as antioxidants and also contained in ethanol extract of jeringau are alkaloids and triterpenoids (Muchtaromah, et al., 2017). Hence this research aims to investigate the best solvent of Jeringau extract that produced the highest antioxidant and antifungi activities based on the polarity.

2. MATERIALS AND METHODS

Sample Preparation

Simplicia of Jeringau, ready for harvested, was obtained from UPT. Materia Medica Batu Indonesia, while *C. albicans* 41-SV isolate was purchased from Microbiology Laboratory of Medical Faculty of Brawijaya University. Plant identification was confirmed by using taxonomical book (FLORA) by (Steenis, 2008). Thermogravimetri method was used to determine the water content of simplicia before extraction process (Legowo and Nurwantoro, 2007).

Extraction Procedure

Extraction method used in this study was maceration using some organic solvents such as ethanol p.a (BRATACO Chemical) as polar solvent, chloroform p.a (BRATACO Chemical) as semipolar solvent and n-hexane p.a. (BRATACO Chemical) as nonpolar solvent. Total of 100 g of Jeringau simplicia was put into 500 ml erlenmeyer and added by 400 ml solvent. The mixture was stirred until

homogeneous and then macerated for 24 hours on room temperature. The homogenate was shaken vigorously on rotary shaker in 120 rpm for hour and repeated 3 times. Macerated mixture was further filtered on buchner vacuum. Filtrate was concentrated by rotary vacuum evaporator on boiling point 78.4 oC. The procedure referred to the previously procedure described by Muchtaromah, et al. (2016) on *Centella asiatica leaves*.

Antioxidant Activity Test

The free radical scavenging activity of Jeringau extract was evaluated by free radical 2,2-difenil-1-pikrilhidrazil (DPPH) method. Percentage of inhibition was measured by following formula (Molyneux, 2004). The DPPH solution was prepared in ethanol, chloroform and n-hexane and subsequently added to several concentration (25 ppm, 50 ppm, 100 ppm, 200 ppm, dan 400 ppm). Ascorbic acid was used as standard.

$$\% \text{ of Inhibition: } \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}} \times 100\%$$

The absorbance changes were measured at 517 nm. IC₅₀ is a number that shows the concentration of extract that can inhibit radical activity by 50% (Molyneux 2004). The IC₅₀ values were calculated using linear regression analysis and used to indicate antioxidant activity. Classification of antioxidant activity according to Jun et.al (2003) was categorized as strong (IC₅₀<50 ppm), active (IC₅₀ 50-100 ppm), moderate (IC₅₀ 101-250 ppm), weak (IC₅₀ 250-500 ppm) and not active (IC₅₀>500 ppm).

Antifungal Activity Assay

The antifungal activity of Jeringau extract assay was consisted of three stage: diameter of inhibition zone, minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC).

Diameter of inhibition zone was measured by the diffusion technique (Kirby Bauer method) with concentration of 100%. Total of

0.1 g jeringau extract using variation of solvent (ethanol, chloroform and n-hexane) was diluted until total volume 100 µl. Three sterile disc papers (6 mm) were inserted into the extract solution of jeringau and saturated for 30 minutes. Then the disc papers were put into an agar plate that had been spread with *C. albicans*. Categorization of inhibitory zone by Pan, et al., (2009) was classified as strong (> 6 mm), moderate (3-6 mm) and weak (0-3 mm).

Determination of MIC was performed using microplate dilution method described by (Andrews, 2001). Dilution stratification was then performed to produce the final concentration (50%, 25%, 12.5%, 6.25, 3.13%, 1.56%, 0.78%, and 0.39%). Then 100 µL each concentration of samples was inserted in a 96-well plate and added with a 100 µL suspension of *C. albicans* that adjusted to equal with McFarland 0.5 standard before. Sterile sabouraud dextrose broth was used as control of sterility and microbial inoculum as growth control. MIC was defined as the lowest concentration of sample that inhibited the visible growth of a microorganism after overnight incubation at 30 °C for 18 h to 20 h. *C. albicans* growth was observed visually by comparing turbidity of the sample and controls. MFC was the smallest concentration that could kill microbes, characterized by *C. albicans* could not grow on the plate, which indicated the microbes had died at this concentration. Confirmation of MIC and MFC values were done by streak plate from the antifungal test results in solid dilution.

Data analysis

Data % of antioxidant activity is represented by percentage of inhibition analyzed using Linear Regression test by SPSS 16.0. Data of inhibitory zone, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are presented descriptively in the form of figures and tables.

3. RESULTS

Moisture in the material greatly affects the quality and storage of a food thus the determination of the moisture content of a material is essential for proper processing and handling. The result of calculation of water content of dry simplicia jeringau rhizome equal to 19.0%.

Antioxidant activity of Jeringau extract with various solvents

Antioxidant activity is the ability of a compound or extract to inhibit oxidation reaction which can be expressed by %

inhibition or scavenging. The Percentage of inhibition data of Jeringau extracts are presented in Table 1. The 400 ppm extract showed the best antioxidant activity, where among them, the ethanol extract was the highest (88,43 %), followed by chloroform and n-hexane extract (51,19 and 21,65%), respectively. Ascorbic acid has the highest percentage of inhibition compare to those three extracts cause ascorbic acid is a strong antioxidant that has been clinically tested thus often used as a standard in a research.

Table 2. Data % Antioxidant activity of Jeringau extract in some solvents compared to ascorbic acid (control)

(x) Concentration (ppm)	(y) Antioxidant Activity (% inhibition)			
	Etanol extract	Kloroform extract	n-heksana extract	Ascorbic acid (Control)
25	7,73	4,03	1,17	39,85
50	15,80	8,87	4,18	93,02
100	38,29	23,59	3,22	92,91
200	61,40	40,19	8,12	92,18
400	88,43	54,19	21,65	91,20

The percentage of inhibition of Jeringau extract in ethanol, chloroform, n-hexane and control of each concentration to DPPH is presented in curves as follows (Fig. 1).

The result revealed that ethanol extract was obtained IC₅₀ at 100 - 200 ppm and categorized as moderate. Chloroform was obtained IC₅₀ at 300 - 400 ppm and classified as weak. N-hexane extract was reached IC₅₀ > 400 ppm that means it had no antioxidant activity compared to ascorbic acid that got IC₅₀ at 25-50 ppm (Strong).

Furthermore Figure 1 showed that up to a concentration of 400 ppm, free radical capture activity from extracting had not been constant for all extracts, as in n-hexane and vitamin C. Ascorbic acid at concentration of 100 ppm decreased in activity as it reaches the optimum concentration limits.

Percentage of antioxidant activity then analyzed by using linear regression equation so

that obtained coefficient of determination (R₂) and inhibition concentration (IC₅₀). The following values of R₂ and IC₅₀ are shown in Table 3.

Ethanol had the lowest IC₅₀, 137.7 mg/L. This means that with the addition of antioxidants from ethanol extract of 137.7 mg/L, will capture free radicals as much as 50% of the total free radicals. This suggests that the smaller the IC₅₀ value of a compound the higher the antioxidant activity. IC₅₀ value of these solvents respectively were 137,7 mg/L for ethanol (moderate), 315,8 mg/L for chloroform(weak) and 1011 mg/L for n-hexane (weak) or no antioxidant activity. Vitamin C as a control has a very strong antioxidant power (27.71 mg/L) compared to extracts of jeringau.

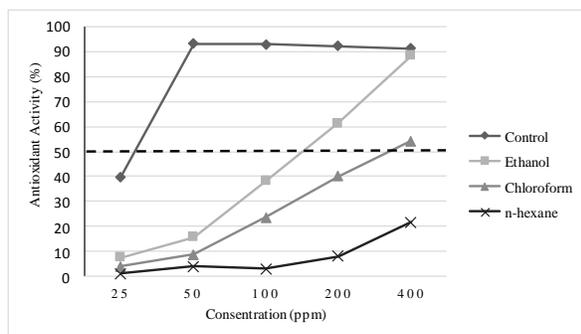


Figure 1. Percentage of antioxidant activity of jeringau extract with various solvents compared to ascorbic acid as a control

All of various solvents had high coefficient of determination (R^2) that means there is strong correlation between the addition of extract concentration (x) and IC_{50} value (y). The highest coefficient of determination was obtained by ethanol i.e 0.9938 followed by chloroform (0.9862) and n-hexane (0.9643).

Table 3. Results of coefficient of determination (R^2) and IC_{50} of Jeringau extracts compared to Control

No.	Sample	R^2 value	IC_{50} (mg/L)	Catagorized
1.	Ethanol	0.9938	137.70	moderate
2.	Chloroform	0.9862	315.80	weak
3.	N-heksana	0.9643	1011.00	very weak/in active
4.	Vitamin C (C+)	0.9172	27.71	strong

Antifungal activity of Jeringau extract with various solvents on *C. albicans*

All of various solvents had inhibitory activity against *C. albicans* growth. This inhibitory activity is pointed by a clear zone around the paper disc. The highest inhibitory activity was obtained by nystatin as positive control (17,68 mm) followed by ethanol (3,72 mm), n-hexane (3,32 mm) and chloroform (2,22 mm) (Table 4). Antifungal activity of the

solvents were interpreted according criteria proposed by Pan, et al., (2009) Nystatin was classified as strong since it has inhibitory zone > 6mm. Subsequently ethanol and n-hexane was grouped as moderate caused of its inhibitory zone was 3-6 mm and chloroform was categorized as weak caused by its inhibitory zone 0-3 mm (Table 2).

Table 4. Inhibition zone of jeringau extract with various solvents and control

No.	Sample	Inhibition zone (mm)	Categorized
1.	Ethanol	3.72	moderate
2.	Chloroform	2.22	weak
3.	N-heksana	3.32	moderate
4.	Nystatin (C+)	17.68	strong

Streak plate test was performed to determine minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) value of various solvent. Concentration of 0,39% was minimum inhibitory concentration that could inhibit *C. albicans*

growth whereas concentration of 0,78% was minimum fungicidal concentration that could remove the fungi for all various solvent (Table 5).

Table 5 MIC and MFC value of Jeringau extracts with various solvent

Concentration of test sample	Total Coloni Account (CFU/mL)			MIC - MFC		
	Ethanol extract	Chloroform extract	n-heksana extract	Ethanol extract	Chloroform extract	n-heksana extract
Microbe control	123 x 10 ⁹	123 x 10 ⁹	123 x 10 ⁹			
0,39%	55 x 10 ⁶	40 x 10 ⁶	186 x 10 ⁵	MIC	MIC	MIC
0,78%	0	0	0	MFC	MFC	MFC
1,56%	0	0	0			
3,13%	0	0	0			
6,25%	0	0	0			
12,50%	0	0	0			
25.00%	0	0	0			
50.00%	0	0	0			
100.00%	0	0	0			

4. DISCUSSION

Moisture of Jeringau simplicia in this research was 19%. The National Agency of Drug and Food Control of Republic of Indonesia or NADFC states that the water content for dry matter should range from 10-12% to ensure the quality of the material during storage (Ma'mun, et al., 2006). The potential of Jeringau as an antibacterial allows it to have long storage durability despite its high water content. However, it should not be stored for long periods or immediately used in the extraction process

Extract concentration of various solvents has positive correlation with antioxidant activity (Figure 1). The linear regression curve showed that the higher the concentration of the extract, the higher the antioxidant activity. This means that free radicals scavenging are still effective to the concentration of 400 ppm and have not turned into prooxidants. However, the addition of concentrations may have the opposite effect, the higher the concentration the lower the antioxidant activity. This is proven by vitamin C as a positive control, it showed a significant decrease in antioxidant activity at 100 ppm due to have reached the optimal concentration (Olugbami, et al., 2015)

Barua, et al., (2014) reported that radical scavenging activity (IC₅₀ values) of *A. calamus* on DPPH assay in ethanol, hydro-ethanol and

aqueous extracts were 54.820; 74.248 and 93.066 µg/mL, respectively. As lower IC₅₀ values indicate higher scavenging activity, thus, it was seen that the ethanol extract exhibited higher scavenging potential followed by the hydro-ethanol extract and the aqueous extract.

Ethanol solvent indicated the lowest IC₅₀ value in this research (Table 3). The ethanol extract has the highest antioxidant activity may caused by the polar active substance of several antioxidant compounds contained within. The previous research proved that rhizome of jeringau extracted with ethanol has a group of antioxidant compounds such as alkaloids and triterpenoids (Muchtaromah, et al., 2017) where it can function as an antioxidant by donating hydrogen atoms in DPPH.

Alkaloids and triterpenoids are polar compounds which are known to have more hydroxyl group (-OH) than alkyl group (-CH). This hydroxyl group (-OH) donates its hydrogen atom thus the DPPH radical becomes stable. Total hydroxyl groups contained in the material greatly affect its activity as an antioxidant. DPPH reacts with antioxidants then results 1,1-diphenyl-2-picrilhidrazine (in reduced form) and antioxidant radicals (Nickavar, et al., 2006).

N-hexana showed the lowest of antioxidant activity among other extracts. (Table 2 and 3). This result is caused by its

characteristic as nonpolar solvent which will bind with nonpolar compound in Jeringau simplicia. In accordance with Nickavar, et al., (2006), n-hexana has alkyl group that do not have the ability to provide hydrogen or electron donors thus can't be a free radical scavengers.

Antifungal Activity of Jeringau Extract againts *C. albicans*

According to Pan et al., (2009) classification, the highest inhibitory activity was reached by nystatin as positive control (17,68 mm) followed by ethanol (3,72 mm), n-hexane (3,32 mm) and chloroform (2,22 mm). Jeringau extract with ethanol and n-hexana solvents were categorized as moderate antifungal in this research.

Rita, et al., (2017) reported that the essential oil concentration of *A. calamus* influence the growth and biomass inhibition of *C. albicans*. Minimum Inhibitory Concentration (MIC) of essential oil toward *C. albicans* is 1% with the inhibition of 7.83 mm. Prayitno study (2015) revealed that the methanol extract of red Jeringau rhizome has activity against *Malassezia furfur* growth with minimum inhibitory concentration and effective dose at a concentration of 60%, while Wulandari, et al. (2015) reported that methanol extract from red Jeringau rhizome has antibacterial activity against *S. flexneri* growth with effective concentration of 100%.

The ability of jeringau extract as antifungal may caused by alkaloids contained within. Alkaloids is basic substance (Harborne, 1987) with pH >7 (Rahayu, 2009). This basicity may suppress *C. Albicans* growth caused usually it grow in pH 4.5 – 6.5. Moreover Jeringau rhizome is known to contain flavonoids, steroids, saponins, and terpenoids (Saman, et al., 2013). The biological activity of flavonoid compounds can damage the cell walls of *C. Albicans* that consisting of lipids and amino acids. The cell walls will react with the alcohol group on the flavonoid compound thus it will be damaged and the compound can enter into

the nucleus of the fungal cell that will lead the cell lysis. Moreover the lipophilicity of flavonoid may damage the cell wall of *C. Albicans* (Melderer, 2002; Cowan, 1999).

The species of *Euglena* sp. and *Phacus* sp. dominance in those periods (Table 1). The results of previous studies showed those species can be found in water temperature high (Çelik & Ongun, 2007) summer, winter (Thakur et al., 2013), water transparency, temperature, TN, TP and DO low and high (Tian et al., 2013). Euglenophyta influenced by the end of the dry season (Sulastri, 2011), summer, autumn (Sevindik, 2010), winter and spring (Wu et al., 2013), nutrient enrichment (Çelik & Ongun, 2007), increase the temperature to 39 °C (Shanthala et al., 2009). Furthermore, the high of nitrate and low of DO correlation with the existence of Euglenophyta (Sulastri, 2011).

Concentration of 0,39% was minimum inhibitory concentration that can inhibit *C. albicans* growth. Concentration of 0,78% was minimum fungicidal concentration whereas can remove fungi for all various solvents. Susanti (2016) reported an antimicrobial activity test of Jeringau extract with a concentration of 4% (control of extract), 2%, 1%, 0.5%, and 0% (control of fungi) inhibit *C. albicans* growth with minimum inhibition concentration (MIC) of 0.5%. The result of this study yielded a lower MIC (0.39 %) than Susanti's study (0.5%)

5. CONCLUSION

Rhizome of Jeringau has antioxidant and antifungal activity against *C. Albicans*. Ethanol is the most promising solvent for extracting the active compound of Rhizome Jeringau which functions as antioxidant and antifungal, proven to produce the highest antioxidant and antifungal activity than chloroform and n-hexana.

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