Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(9S):147-151

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Evaluation acute toxicity and antibacterial activity of *Penicillium sp* endophitic fungus extract of kunyit putih (*Curcuma zedoaria*) in mice (*Mus musculus* L.)

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ABSTRACT

Evaluation acute toxicity and antibacterial activity of Penicillium sp endophitic fungus extract of kunyit putih (Curcuma zedoaria) in mice (Mus musculus L.) had been done. Acute toxicity at concentration until 2000 mg/kg BW and anticahterial test was done used S. dysentriae bacteria by giving 6 treatments contain treatment I (normal control), treatment II (negative control), treatment III (dose I 125 mg/kg BW), treatment IV (dose 250 mg/kg BW), treatment V (dose 500 mg/kgBB), and treatment VI (positive control ciprofloxacin dose 10 mg/kg BW). Observation was done based on amount of S. dycentriae bacteria in faeces taken once in 2 days along 12 days. Bacteria population data on the mice faeces analyzed by statistical analysis. At acute toxicity no mortality was observed at 2000 mg/kg BW for 24 h observation. Observed for toxic symptoms such as behavioural change, locomotion convulsion was not found. The experiment antibachterial activity result that treatment III to VI show the population of Shigella which is not different. Effect treatment time to population of bacteria shows in the 10 and 12 the bacteria amount fall below infection dose (2,40 x 10^5 and 6,76 x 10^4) respectively, thus can be concluded that the extract had acted as a cure.

Keywords: Endophytic fungus, *Penicillium sp*, *Curcuma zedoaria,* acute toxicity, antibacterial

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INTRODUCTION

The searching of bioactive compound source is continuously observed to overcome various diseases and resistent antibiotics. Development in sciences and technology, the researchers start to looking for new source of bioactive compounds, one of them is by using of endophytic microbe which is contained in the plant [1-2]. Endophytic microbe which is contained in the plant's tissue could produce the secondary metabolite with its main cells [3-5]. Every high level plant can contain some endophytic microbe and can be used as isolate source of endophytic microbe, one of them is traditional plant *Curcuma zeodaria* [6].

This plant was used for curing stomach diseases, toothache, blood stagnation, leucoderma, tuberculosis, enlargement of spleen, and for promoting menstruation intraditional medicine in Asia [7]. In the previous experiment, three fungus have been isolated from *C. zedoaria* and identified that *Penicillium sp*, *Fusarium sp*, and *Aspergillus sp*. Two secondary metabolite compounds had been isolated from endophytic fungus *Penicillium sp* in *Curcuma zedoaria* and identified as Di-(2-ethylhexyl) phthalate (1) and one new compound as 5 (4'-ethoxy-2'-hydroxy-5'-methyl-2',3'-dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on) (2) [8]. The antibacterial activity of this two compounds had been tested as *invitro* used bacteria test *S. dycentriae* and *E. Coli* and indicated active as anti bachterial with MIC 250 µg/mL respectively [9]. For further development of the research In this paper we

reported evaluation acute toxicity and testing of the antibacterial activity with *invivo* method from the extract endophytic fungus *Penicillium sp* containing active antibacterial compounds using mice (*Mus musculus* L.)

EXPERIMENTAL SECTION

Material for *invivo* assay: culture stock bachterial *S. disentryae*, culture stock endophitic fungus *Penicillium sp*, EtOAC, Sodium Carboxy Methyl Cellulose (SCMC), *Nutrien Agar* (NA), Salmonella Shigella Agar (SSA), Potato Dextrose Agar medium (PDA), Potato Dextrose Broth (PDB), ciprofloxacin were of E-Merck grade, aquades, pellet diet and Swiss Webster mice.

The apparatus in the research were counter colony, autoclave, incubator, water bath, microscope petri-plates, magnetic hotplate flasks, and generally apparatus in organic and microbiology

Preparation

Cultures of endophytic fungus

The pure colony endophytic fungus placed on petri-plates containing potato dextrose agar medium (PDA) (200 g potato, 20 g dextrose, and 15 g agar in 1 L of H_2O, supplemented with 100 mg/L of chloramphenicol to suppress bacterial growth). The plates were incubated at 25±2° C until fungus growth appeared. The plant segments were observed once a day for the growth of endophytic fungus. Furthermore the plate segments were immediately transferred into new PDA plates and then subcultured until need fungus amount were obtained [10].

Cultivation and extraction of pure fungal strain

The purified fungus (a small park) was transferred under sterile conditions to the PDB medium. For chemical investigations, the fungal strains were static cultivated into 15 flasks (1L each) containing 400 mL of PDB medium for 3 weeks at room temperature. Fungus in the 3 weeks cultures were vacuum-filtered and the filtrate fractionated thrice by liquid-liquid partition with ethyl acetate (1:1). Then the solvent phase was evaporated under reduced pressure using rotary vacuum evaporator at 40 ^{o}C to produce the ethyl acetate fraction of liquid cultures [11-12]. The ethyl acetate extract were suspended in 1% SCMC to preparate suitable forms of the dosages.

Selection and maintenance of animals

Swiss webster mice weighing between 20 to 30 g of either sex were used. Animals were housed under standard conditions of temperature, 12h/12h light/dark cycle and fed with standard pellet died and tap water. All the experiments were approved and conducted as per guidelines of Institutional ethical Committee [13].

Acute toxicity study in mice

Acute toxicity test was done by Weil method using mice weighing 20 – 25 g, maintained under controlled conditions of temperature (20-25^{0}C) and humidity (55%) were used for toxicity study as per the internationally accepted protocol drawn under the OECD guidelines 423. The mices were divided into 5 groups, group I dose 0,0 mg/kg BW (normal control), group II dose 250 mg/kg BW, group III (500 mg/kg BW), group IV (1000 mg/kg BW) and group V (2000 mg/kg BW). The over night fasted animals were administered orally and observation until 24 h. Observation continued during 14 days was done and observed for toxic symptoms such as weight body, behavioural change, locomotion convulsion and mortality [14].

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Invivo assay antibachterial activity in mice (*Mus muscullus*)

The mice were divided into 6 groups, each consisting of 4 animals. Group I (normal control) received pellet diet + 1 ml 1% SCMC, Group II (negative control) received pellet diet + 0,1 suspension bachterial + 1 ml 1% SCMC, Group III – V received pellet diet + 0,1 ml suspension bachterial and 1 ml ethyl acetate extract at dose 125, 250 and 500 mg/kg BW respectively with strat day 4th for group with treatment at *S. dysentriae* until day 14, and group VI recived pellet diet + suspension bachterial and standart antibiotic 10 mg/kg BW. Standart antibiotic used ciprofloxacin.

The animals were induced by 0,1 ml bachterial oraly in the beginning. Ethyl acetate extract was given after incubation periode which is the 4 day until 12 day. Every day to observation general condition and disorder indication. Feces sample is collected every 2 days. Feses were weighing into 1 g, then diluted 5 times start from 10^{-1} until 10^{-5}. Furthermore 3 last dillution series were taken and sowed in SSA medium, incubated at room temperature during 24 h, then total of *S. typii* and *S. dysentriae* bachterial in faeces were calculated .The same treatment also used in standard antibiotic [15].

Statistical Analysis

Bachterial Population data in mice feses was statistical analysis ANOVA alfa 0,05, followed by Duncan New Multiple Range Test (DNMRT) test at alfa 0.05

RESULTS AND DISCUSSION

Acute toxicity study

Acute toxicity study of ethyl acetate extract of endophytic fungi *Penicilliu sp* from *Curcuma zeodaria* was determined. Since no mortality was observed at 2000 mg/kg BW for 24 h observation. Observed for toxic symptoms such as body weight, behavioural change, locomotion convulsion and mortality for 14 days. The observation towards animal body weight drastic reduction was not found and generally the body weight increased during 14 days and relatively normal.

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***Invivo* assay antibachterial activity**

Effect of dosage to the amount of bacteria in faeces

Statistical analysis conducted to logarithma of bacteria cell amount (*S. Dysentriae*) using ANOVA (p level < 0.05) showed the antibacteria extract had different activities in hampering bacteria population growth between 3 dose used. In addition, different duration tests also showed distinct activities confirmed by different amount of bacteria cell population. Logarithm of dosage and duration day also provided (p level 0.05).

Further tests on amount of bacteria cell of *S. dysentriae* according to Table 1 which only gave weft (treatment 1) show different that statistically significant compare to other treatments (II-VI), while treatment III to VI show no significant different. All variation of extracts concentration tested showed effectiveness similar to antibiotic used as standard. Treatment II had *S. dysentriae* population that not significant different to treatment III and still showed high population of *Shigella*. Duncan new multiple range test (DNMRT) calculated further in alpha level 5% for various treatment on bacteria amount of *S. dysentriae* showed on Figure 1.

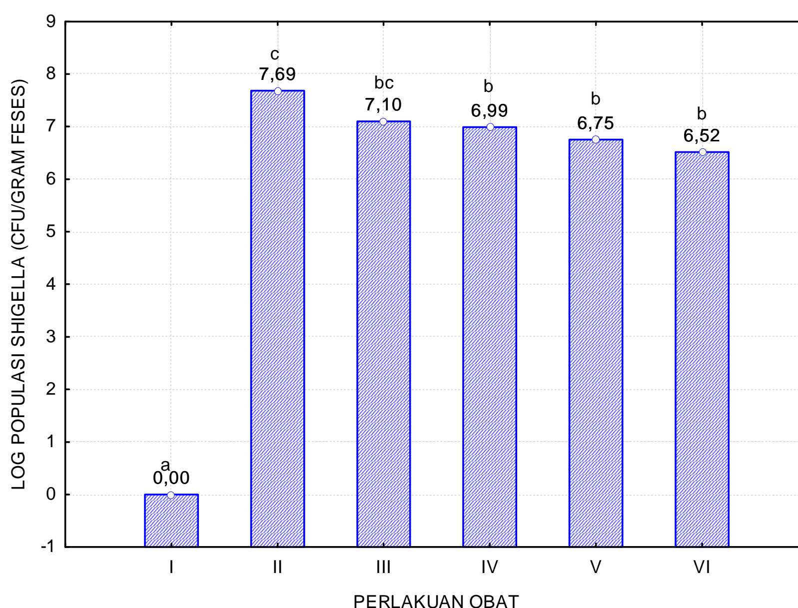

Figure 1. Grafik Connection dose with population *S. dysentriae*

According to data on Table 1 for dose 3 (treatment V) reveal lowest amount of bacteria cell (5.6234×10^6 cfu/g faeces). Treatment III, IV and V showed still high bacteria cell amount above the positive control of treatment VI (ciprofloxacin antibiotic 10 mg/kg BW) though not significantly different. Cell bacteria amount of dose 3 is 1.2589×10^7 represent the highest amount compared to other dose used. Based on this fact it can be concluded that the most effective dosage is treatment IV (dose 2).

Effect of treatment time to *S.dysentriae* population

Treatment time for 10 and 12 days showed no different that statistically significant of *Shigella* population and indicate low population whereas in between day 2, 4, 6 and 8 had no different and indicate still high population. All of treatment conducted show tendency of decreasing bacteria amount the longer time of treatment was given (this could means the decrease of bacteria amount without giving drugs due to antibodi of mice although need longer time to heal). In case of Shigella, day 8 still showing bacteria amount above infection dose (> 5.7×10^5) and at day 10

and 12 showed at Table 2, the bacteria amount fall below infection dose ($2{,}40 \times 10^5$ and $6{,}76 \times 10^4$) respectively, thus can be concluded that the extract had acted as a cure. Result of further DNMR test at alpha level 5% on various time exposures to *S.dysentriae* bacteria amount at given treatment (dose effect) showed on Figure 2.

Table 1. Effect of various treatments to bacteria cell amount of *S. dysentriae* in 12 days of treatment

Treatment	Log of average amount of bacteria cell *S.disentryae* (cfu/g faeces)	Average amount of bacteria cell *S.disentryae* (cfu/g faeces)
I	0	0^{a}
II	7,69	$4{,}8977 \times 10^{7c}$
III	7,10	$1{,}2589 \times 10^{7bc}$
IV	6,99	$9{,}7723 \times 10^{6b}$
V	6,75	$5{,}6234 \times 10^{6b}$
VI	6,52	$3{,}311 \times 10^{6b}$

Note: Numbers follow by same subscript indicate not significant different according to Duncan New Multiple Range Test (DNMRT) 5%.

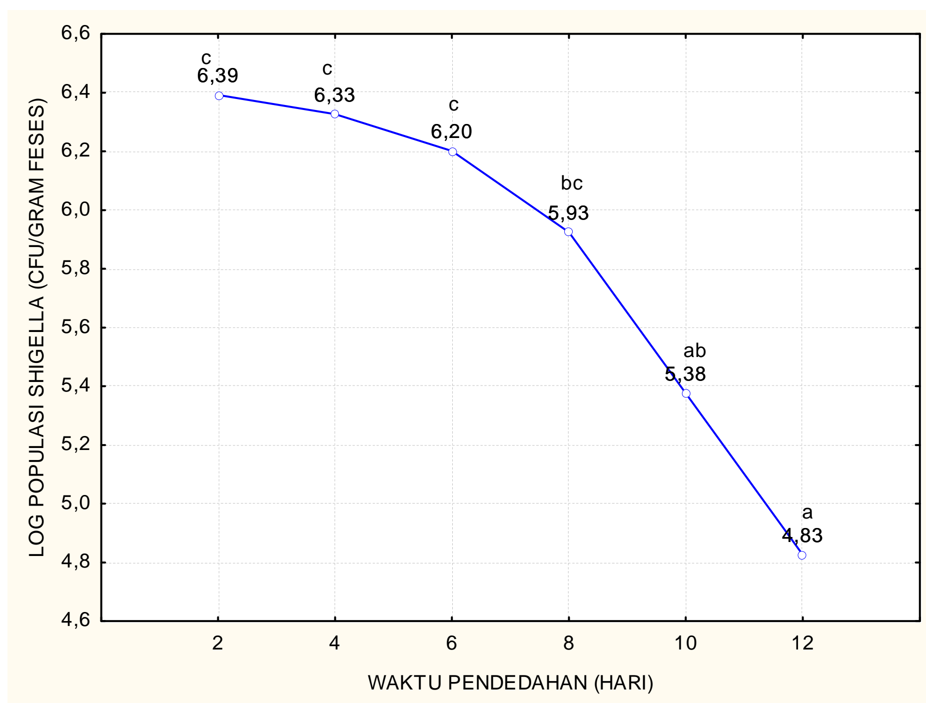

Figure 2. Relationship between exposure time with *Shigella* population

Table 2. The effects of treatment time to *S. dysentriae* population

Day	Log ofaverage number of cell bacteria amount *S. disentryae* (cfu/g faeces)	Average number of cell bacteria amount *S. disentryae* (cfu/g faeces)
0	0	0
2	6,39	$2{,}45 \times 10^{6c}$
4	6,33	$2{,}14 \times 10^{6c}$
6	6,20	$1{,}58 \times 10^{6c}$
8	5,93	$8{,}51 \times 10^{5bc}$
10	5,38	$2{,}40 \times 10^{5ab}$
12	4,83	$6{,}76 \times 10^{4a}$

Note: Numbers follow by same subscript indicate not significant different according to Duncan New Multiple Range Test (DNMRT) 5%.

The effects of dose and incubation time to the population of *S. dysentriae* in faeces

The efects of dose and incubation time to the population of *S. dysentriae* in mice faeces showed on Table 3. It can be seen from the table that as incubation time increase the bacteria amount also increase until certain day then it fall and decrease and reach phase of death. On day 2 trough day 4 *S. dysentriae* bacteria cell amount continue to increase given the dose exceed the infection dose that can caused sickness (10^4 cfu/g). According to [16] incubation time of *S. dysentriae* occurred on day 1 to day 4. On day 8 all treatment conducted show decrease of cell bacteria and the most significant decrease observed on treatment IV, V and treatment VI (positive control). Result obtained from further DNMR test at alpha level 5% on various treatment to the bacteria amount of *S. dysentriae* showing no significant different between treatment III-V to treatment VI (positive control). Average number of bacteria cell showed a decrease on day 10 while number of bacteria cell at treatment III-V still higher then positive control with

lower number showed by treatment V. *S. dysentriae* experienced life period shorter then Salmonella. It life time range from 2 to 10 days and will undergo phase of death starting from day 8 [17]. On treatment II day 10, *S. dysentriae* exhibit decrease of bacteria cell amount. Symptom caused by Shigellosis occurred after incubation time. At this time mice was healing itself due to phase of death of *Shigella.*

Tabel 3. Acount cell *Shigella dysentriae* bacteria(cfu/mL) at Treatment time 12 days

Treatment	Number of cell bacteria amount *Shigella dysentriae* x 10^5 cfu/gr faeces Once in 2 days						
	0	2	4	6	8	10	12
I	0	0	0	0	0	0	0
II	0	5,2	6,0	320	830	165	157
III	0	5,7	8,2	232	460	81	32
IV	0	4,3	5,7	123	390	43	8,4
V	0	4,6	6,4	106	283	15	1,65
VI	0	5,5	6,5	81	272	12	1,20

Acknowledgements
The authors are very grateful to the Directorate General of Higher Education who has supported this research with 2015 research grant.

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