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Toxic compounds of *Curcuma aeruginosa* causes necrosis of mice hepatocytes

Eka Pramytha Hestianah*, Idha Kusumawati**, Lucia Tri Suwanti***,
and Sri Subekti****

ABSTRACT

*Department of Veterinary
Anatomy, Veterinary Faculty,
Airlangga University
(Universitas Airlangga)

**Department of
Phytochemistry and
Pharmacognosy,
Pharmacy Faculty
Airlangga University

***Department of
Parasitology,
Veterinary Faculty,
Airlangga University
(Universitas Airlangga)

Correspondence

Dr. Eka Pramytha Hestianah,
drh, M.Kes
Department of Veterinary
Anatomy, Veterinary Faculty,
Airlangga University
(Universitas Airlangga)
Kampus C Unair,
Jl. Mulyorejo, Surabaya 60115
Phone: +6231-5992785
Email : ekamirta@gmail.com

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BACKGROUND

People have been using *Curcuma aeruginosa* rhizome as a traditional herbal medicine as appetite stimulant, without realizing its side effects. Herbal plants contain tens to hundreds of compounds, some of which are toxic. The aim of this research was to determine which toxic compound of *Curcuma aeruginosa* rhizome has an impact on apoptosis and PARP-1 expression of hepatocytes in male mice.

METHODS

Eighty eight male *Balb C* mice were divided into 10 groups treated respectively with *Curcuma aeruginosa* rhizome chloroform extract, methanol extract, essential oil, infusion, and press juice, at dosages of 0.004g/kgBW and 0.06g/kgBW, and 1 control group. The treatment was given orally once a day for 10 days and on the 11th day, the research animals were sacrificed, and their liver taken for histopathologic slide preparation with *Apopteq Detection Kit*, and immunofluorescence. Compounds in *Curcuma aeruginosa* rhizome were analyzed with gas chromatography/mass spectrometry. The data obtained were analyzed by one-way ANOVA, and Partial Least Squares to determine which compounds had an impact on murine hepatocytes.

RESULTS

The result of one way ANOVA showed that the chloroform groups at dosages of 0.004g/kgBW and 0.06g/kgBW showed the highest apoptosis of mice's hepatocytes ($p < 0.05$). There were significant differences in PARP-1 expression between control and treatment groups. The highest PARP-1 expression was in the essential oil group at a dosage of 0.06g/kg BW ($p < 0.05$).

CONCLUSION

Curcuma aeruginosa rhizome given to mice orally causes necrosis of mice's hepatocytes.

Key words : Apoptosis, PARP-1 expression, hepatocyte, mice

Senyawa toksik ekstrak temu ireng menyebabkan nekrosis dari hepatosit mencit

ABSTRAK

LATAR BELAKANG

Temu ireng merupakan tumbuhan obat yang banyak digunakan sebagai obat tradisional, seperti penambah nafsu makan, tanpa menyadari efek samping yang ditimbulkannya. Tumbuhan obat mengandung banyak bahan-bahan yang dapat bersifat toksik. Penelitian ini bertujuan untuk menentukan senyawa toksik dari rimpang temu ireng yang berpengaruh terhadap terjadinya apoptosis, dan ekspresi PARP-1 hepatosit pada mencit jantan.

METODE

Mencit jantan Balb C sebanyak 88 ekor dibagi menjadi 10 kelompok perlakuan terpapar ekstrak kloroform, ekstrak metanol, minyak atsiri, infusa, perasan rimpang temu ireng dengan dosis 0,004g dan 0,06 g/kgBB pada masing-masing kelompok dan 1 kelompok kontrol. Perlakuan diberikan sekali sehari selama 10 hari dan hari ke 11 mencit dikorbankan, diambil organ hepar, dibuat sediaan histopatologi dengan Apopteq Detection Kit, dan imunofloresen. Senyawa dalam rimpang temu ireng dianalisis dengan GC/MS. Data yang diperoleh dianalisis dengan ANOVA one-way, dan Partial Least Squares (PLS) untuk menentukan senyawa yang berpengaruh.

HASIL

Hasil analisis ANOVA one way menunjukkan rata-rata apoptosis hepatosit tertinggi pada kelompok yang diberikan chloroform dengan dosis 0,004 g and 0,06 g/kgBB ($p < 0,05$). Terdapat perbedaan bermakna ekspresi PARP-1 hepatosit antara kelompok kontrol dan kelompok perlakuan. Ekspresi PARP-1 tertinggi pada kelompok minyak atsiri dosis 0,06 g/kgBB ($p < 0,05$)

KESIMPULAN

Rimpang temu ireng yang diberikan per oral pada mencit menyebabkan terjadinya nekrosis hepatosit mencit.

Kata kunci : Apoptosis, ekspresi PARP-1, hepatosit, mencit

INTRODUCTION

Herbal plants are widely used to promote and rehabilitate health, and to prevent and cure disease.⁽¹⁾ One of the herbal plants that has often been used is *temu ireng* (*Curcuma aeruginosa* Roxb). It has been reported that the compounds contained in chloroform and methanol extracts of *Curcuma aeruginosa* rhizome can cause uterine relaxation, while in case of alopecia, *Curcuma aeruginosa* rhizome hexane extract of can stimulate hair growth.^(2,3)

People think that traditional medicines/ herbal plants such as *Curcuma aeruginosa*

rhizome may be safely consumed, as they do not have negative effects on the liver or kidneys. People believe and see *Curcuma aeruginosa* rhizome as worm killer, appetite stimulant, phlegm cleaner, reliever of sore throat, and others. These uses of *Curcuma aeruginosa* rhizome are supported by research conducted in Indonesia or overseas, but most studies are still limited to the pharmacology and phytopharmacy of herbal plants based on their use by some people with functions that are empirically tested.⁽⁴⁻⁶⁾

The opinion that herbal plants are totally safe and free from side effects is wrong. Herbal

plants contain tens to hundreds of compounds, some of which are toxic. Two types of toxic effect have been reported, firstly, that caused by intrinsic factors of the herbal plants themselves such as excessive dosage, allergic reaction e.g. vomiting, inflammation, diarrhea. Secondly, that caused by external factors such as mistakes or failure of medicine preparation, mistakes in identifying the herbal plants, and contamination.⁽⁷⁾

The World Health Organization (WHO) states that inconsistent compound content of herbals and toxic compounds contained in herbal plants can cause toxic effects.⁽⁸⁾ Toxic compounds in traditionally consumed medicines can cause nephrotoxicity, damage to epithelial cells of the small intestine, and also damage to hepatocytes.⁽⁹⁻¹¹⁾ The present research study was conducted because there has not been any clinical research on the toxic compounds in *Curcuma aeruginosa*.

Compounds in a herbal plant can be separated in a common way, by bioassay-guided fractionation, which needs modern separation techniques such as chromatography combined with mass spectrography, followed by nuclear magnetic resonance (NMR) to elucidate structure. The technique requires special skills, is very difficult and time-consuming, and needs abundant materials.⁽¹²⁻¹⁴⁾ For that purpose, this research uses chemometrics Partial Least Squares (PLS) by correlating chromatogram profiles of compounds contained in *Curcuma aeruginosa* rhizome with toxic effects observed from apoptosis and expression of poly (ADP-ribose) polymerases-1 (PARP-1). The research aimed to find toxic marker compounds in *Curcuma aeruginosa* rhizome which impact on apoptosis and PARP-1 expression of male murine hepatocytes.

METHODS

Research design

This experimental study was carried out at the laboratory of Pathology, Veterinary Medical

Faculty, the laboratory of Pharmacognosy and Phytochemistry, Pharmacy Faculty, Airlangga University (*Universitas Airlangga*), and the laboratory of Biological Central Science, Brawijaya University. The study was conducted from March to August 2010.

Animals

Experimental animals were two-month old male Balb C mice, weighing from 25g to 30g, obtained from *Pusat Veterinaria Farma*, Surabaya. The animals were given food and water ad libitum.

Intervention

Eighty eight male mice were divided into 10 groups, respectively treated with *Curcuma aeruginosa* rhizome chloroform extract, methanol extract, *Curcuma aeruginosa* rhizome essential oil, infusion, and press juice; and one control group. The treated groups were given 2 types of oral dosage of *Curcuma aeruginosa* rhizome extract at 0.004g/kgBW and 0.06g/kgBW, based on ethnomedicine. The control group was given sodium carboxyl methyl cellulose (CMCNa) + distilled water. The treatment was given orally once a day for 10 days. On the 11th day, the experimental animals were sacrificed.

Plant sample

Curcuma aeruginosa rhizomes were collected from Jombang, and identified at *Balai Konservasi Tumbuhan Kebon Raya Purwodadi*, Pasuruan.

Preparation of extract

Five kilograms of *Curcuma aeruginosa* rhizome was sliced into pieces of 0.3-0.5 cm and distilled for the essential oil. In addition, 500 g of dry powdered *Curcuma aeruginosa* rhizome was extracted four times by maceration at room temperature with 5 liters of chloroform, followed by four times maceration with methanol.

For preparation of the crude drug infusion, the rhizomes were boiled in a pan at a

temperature of 90°C for 15 minutes, then cooled and strained. *Curcuma aeruginosa* press juice was obtained by grating fresh *Curcuma aeruginosa* rhizomes and pressing with clean flannel cloth. The *Curcuma aeruginosa* rhizome infusion and squeezed juice were then freeze-dried.

Measurement of apoptosis and PARP-1 expression

Data on the percentage of apoptotic hepatocytes in the histopathologic slides processed with the Apoptosis Detection Kit, were collected from 1500 hepatocytes, examined in several high-power fields.^(15,16)

PARP-1 data were collected by checking preparations with immunofluorescence analysis using the Olympus FV1000/FV10-ASW model confocal laser scanning microscope, at EX (peak excitation wave length) of 495 nm, and EM (peak emission wave length) of 519 nm, based on the intensity of the light emitted by the hepatocytes. The light intensity was displayed in graph form, then 3 highest peaks were taken from the graphs to get the average score and use it as data.

Metabolite profiling data were obtained by identifying compounds from the *Curcuma aeruginosa* extract, based on fragmentation and matching of mass spectra obtained from every form of extract and compared to the US National Institute of Standards and Technology (NIST) mass spectral database.

Data analysis

The data obtained were analyzed with one-way ANOVA at a significance level of $p < 0.05$ and chemometrics Partial Least Squares (PLS) to determine which compounds exert influence on hepatocytes.

Ethical clearance

Ethical approval for this study was obtained from the research ethics committee, Veterinary Medicine Faculty, Airlangga University, Surabaya.

RESULTS

The data on histopathological apoptosis and PARP-1 expression in hepatocytes of the male mice receiving *Curcuma aeruginosa* rhizome extract, can be seen in Table 1. The results of one-way ANOVA analysis on hepatocyte apoptosis showed no significant difference between the control group and the methanol group at a dosage of 0.004 g/kgBW; and between the essential oil groups at dosages of 0.004 g/kgBW and 0.06 g/kgBW ($p > 0.05$). Duncan post hoc analysis showed that the highest proportion of apoptosis of mice's hepatocytes was in the chloroform groups at dosages of 0.004 g/kgBW and 0.06 g/kgBW ($p < 0.05$). While the results of one-way ANOVA on PARP-1 expression showed significant differences in all treatment groups, the essential oil group at a dosage of 0.06 g/kgBW showed the highest mean PARP-1 expression of $3369.77 \pm 194.16\%$ ($p < 0.05$).

Compounds contained in the five types of *Curcuma aeruginosa* rhizome extract (chloroform extract, methanol extract, essential oil, infusion, pressed *Curcuma aeruginosa* rhizome) juice, obtained by means of gas chromatography–mass spectrometry (GC/MS) and tested by means of Partial Least Squares (PLS), can be seen in Figure 1, where the names of the compounds are not shown. From the results of PLS analysis, three compounds were obtained that were correlated with murine hepatocyte apoptosis and PARP-1 expression. These were 9-methyltetrahydrocyclo [7.3.1.0(2.7).1(7.11)] tetradecane; epicurzerone; and cis-1,3-dimethyl-2-methylene cyclohexane.

DISCUSSION

Table 1 shows that the five types of *Curcuma aeruginosa* rhizome extract given to the experimental animals did not show significant differences in apoptosis between the control group and the essential oil groups at dosages of 0.004 g/kgBW and 0.06 g/kgBW. This indicates

Table 1. Mean percentages of hepatocyte apoptosis and PARP-1 expression by five types of *Curcuma aeruginosa* rhizome extract

	Extract type										
	Control	Chloroform		Methanol		Essential oil		Infusion		Squeezed juice	
	0	A	B	A	B	A	B	A	B	A	B
Apoptosis (%)	0.30 ± 0.13 ^a	1.49 ± 0.22 ^d	1.33 ± 0.24 ^d	0.29 ± 0.04 ^a	1.13 ± 0.13 ^c	0.31 ± 0.14 ^a	0.32 ± 0.22 ^a	0.77 ± 0.13 ^b	0.96 ± 0.12 ^c	0.72 ± 0.15 ^b	1.05 ± 1.14 ^c
PARP-1 (%)	1280.14 ± 232.20 ^a	2324.78 ± 198.19 ^b	3166.47 ± 146.17 ^{fg}	2588.38 ± 181.55 ^c	2840.42 ± 170.20 ^d	3187.24 ± 169.84 ^{fg}	3369.77 ± 194.16 ^g	3028.98 ± 216.50 ^{d,ef}	2854.60 ± 229.62 ^{d,e}	3076.89 ± 306.29 ^{e,f}	3141.48 ± 279.76 ^{fg}

All dosages given as g/kg body weight. Means with differing superscript within rows were significantly different at the $p < 0.05$ based on one way ANOVA analysis and Duncan post hoc multiple comparisons
Note: A=0.004 g/kgBB; B=0.06 g/kgBB

that *Curcuma aeruginosa* rhizome causes necrosis of the hepatocytes, as can be seen from the mean PARP-1 expression score of 3187.24 and 3369.77%. The highest hepatocyte apoptotic activity occurred at a mean of 1.33% with the chloroform extract of *Curcuma aeruginosa* rhizome. Apoptosis is the physiologic process of programmed cell death occurring in all organs, but apoptotic overactivity indicates cell death or necrosis.⁽¹⁷⁾

Partial Least Square analysis (Figure 1) test results showed the presence of apoptosis (A), where compound number 61, 9-methyltetracyclo[7.3.1.0(2.7).1(7.11)]tetradecane, had the strongest correlation, as seen from the short distance between the point representing the compound and the point representing hepatocyte apoptotic activity. The correlation of compound 61 resulting from GC/MS analysis with that resulting from PLS showed that there

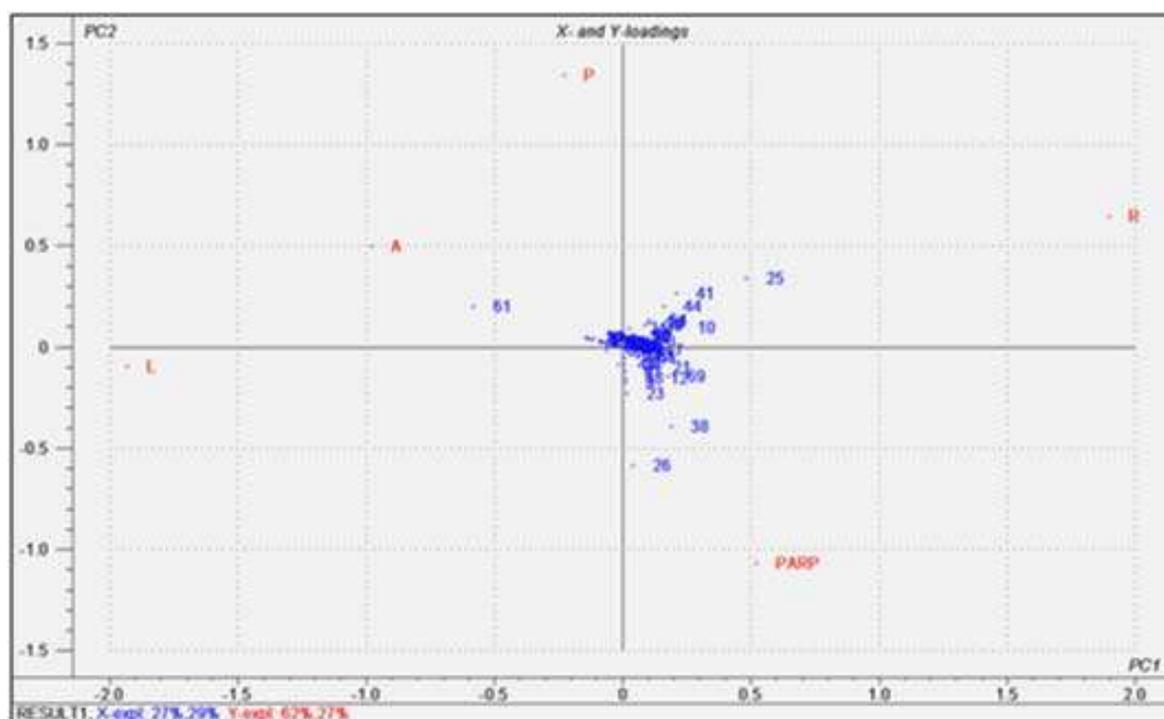


Figure 1. PLS test result of compounds contained in *Curcuma aeruginosa* rhizome
Note: A = apoptosis; PARP = PARP-1 expression

was a positive role of 9-methyl tetracyclo [7.3.1.0(2.7).1(7.11)] tetradecane on hepatocyte apoptotic activity. This means that large amounts of the compound contained in *Curcuma aeruginosa* rhizome will be associated with high apoptotic activity. Apoptotic overactivity indicates abnormal cellular death, as shown by the significant differences in PARP-1 expression between control and treated groups (Table 1). Poly (ADP-ribose) polymerase (PARP-1) is an enzyme group that can repair DNA damage, but if PARP-1 is too active, it can lead to cell death.^(18,19) Therefore it is necessary to carry out research on compounds which play a role in hepatocyte apoptosis based on PARP-1 expression.

Traditional medicines have toxic effects on specific organs. Moreover, there is little information about the chronic toxic effects due to prolonged use and the dosis consumed, which can cause acute or chronic intoxication.^(20,21) *Curcuma aeruginosa* rhizome is often used as an appetite stimulant for children without considering the dosage. The results of a previous study by Hestianah et al.⁽²²⁾ showed that longer administration of *Curcuma aeruginosa* rhizome will worsen hepatocyte necrosis, while the compounds responsible for cell death are unknown. An in vitro study showed that *Curcuma aeruginosa* rhizome increases the percentage of cell death in Baby Hamster Kidney (BHK-21) fibroblast cell cultures.⁽²³⁾

One influencing factor in obtaining the chromatogram profile that can show the chemical content of an extract is the method and solvent used in extraction, so different solvents were used in this research. The solvents used were chloroform, methanol and water for extraction by maceration, while steam distillation was used to obtain the essential oil. In addition, manual pressing was used to obtain the extract without added solvent. These five types of extracts were meant to provide totally different chemical compositions, to obtain different toxic effects so that the correlation between chemical content and toxic effect could be obtained.

Because the bioactive compounds of *Curcuma aeruginosa* rhizome are unknown, it is difficult to determine the quality of the product. However, the compounds contained in *Curcuma aeruginosa* rhizome can be known due to developments in chemical analysis. A clear profile chromatogram which is the profile of the chemical composition of *Curcuma aeruginosa* rhizome extract can be known by means of gas chromatography combined with mass spectroscopy (GC-MS) using a chemometric approach. The structure of a chemical compound can be known from the NIST database of mass spectra of chemical compounds.⁽²⁴⁾

The use of 5 different types of *Curcuma aeruginosa* rhizome extracts was proved to be able to induce PARP-1 expression of murine hepatocytes. Post hoc Duncan test results showed that the group of mice exposed to *Curcuma aeruginosa* rhizome essential oil at a dosage of 0.06g/kgBW had the highest hepatocyte PARP-1 expression, with a mean of 3369.771. Overexpression of PARP-1 in this research indicated that the cells suffered from necrosis.⁽¹⁸⁾

From PLS analysis, there were several compounds which correlated with PARP-1 expression activity, but those with the strongest correlation were compounds numbers 26 and 38, i.e. cis-1,3-dimethyl-2-methylene cyclohexane and epicurzerenone. The strength of correlation with PARP-1 expression activity can be seen from the short distance between the point representing the compound and the point representing hepatocyte PARP-1 expression activity. Shorter distances indicate stronger correlation between both activity points.

Correlation between the compounds in *Curcuma aeruginosa* rhizome as a result of GC/MS analysis with those from PLS showed that there was a positive role of compounds number 26 (cis-1,3-dimethyl-2-methylene cyclohexane) and number 38 (epicurzerenone) in hepatocyte PARP-1 expression activity. The term "positive" means that the compound point and PARP-1 expression activity point (PARP) are located in

the same quadrant. It also means that a larger amount of cis-1,3-dimethyl-2-methylene cyclohexane and epicurzerenone contained in *Curcuma aeruginosa* rhizome extract would be also followed by higher PARP-1 expression activity.

According to GC/MS analysis, cis-1,3-dimethyl-2-methylene cyclohexane was obtained from the squeezed juice of *Curcuma aeruginosa* rhizome, with a relative percentage area of PARP-1 expression of 29.61%. On the other hand, epicurzerenone was obtained from the methanol extract, essential oil, and press juice, with relative percentage areas of respectively 6.59%, 27.78%, and 8.48%. Oneway ANOVA analysis showed that mice exposed to essential oil at 0.06g/kgBW had the highest PARP-1 expression, due to the relatively high epicurzerenone percentage area in *Curcuma aeruginosa* rhizome essential oil (27.78%). The relative percentage area of PARP-1 expression of 29.61% for cis-1,3-dimethyl-2-methylene cyclohexane was larger than that for epicurzerenone, but the former compound did not show as much PARP-1 expression activity as epicurzerenone. This might have been caused by the interaction of some compounds in *Curcuma aeruginosa* rhizome which had a positive correlation with PARP-1 expression. The interaction could presumably decrease the effect of cis-1,3-dimethyl-2-methylene cyclohexane on hepatocyte PARP-1 expression activity.

The univariate analysis results showed that there were differences in hepatocyte PARP-1 expression due to the different extract dosages (0.004g/kgBW and 0.06g/kgBW). This was related to the epicurzerenone compound contained in *Curcuma aeruginosa* rhizome which had a positive role and strongly correlated with hepatocyte PARP-1 expression activity. Therefore increased dosages of essential oil of *Curcuma aeruginosa* rhizome would be followed by increased PARP-1 expression activity. The same would also apply to cis-1,3-dimethyl-2-methylene cyclohexane compounds contained in the press juice of *Curcuma aeruginosa* rhizome.

The *Curcuma aeruginosa* rhizome extract contains toxic compounds, which when given to mice are absorbed in the small intestine and from there to the liver. The compounds penetrate the hepatocyte cell membrane and reduce the activities of the cells, resulting in structural changes and rupture of organelles. PARP-1 has a role in the life and death of cells under various conditions of stress. PARP-1 activity requires that nicotinamide adenine dinucleotide (NAD⁺) be hydrolyzed to ADP-ribose, leading to reduction of NAD⁺ and depletion of ATP. Mitochondria are organelles for the production of ATP for the cells, so that damage to mitochondria will influence cell survival.^(18,19) Lack of ATP causes damage to the hepatocyte that is called necrosis.

The finding of toxic compounds in *Curcuma aeruginosa* rhizome in this study will give the society information about the dangers of using this rhizome and can be used as a guide in consuming *Curcuma aeruginosa* rhizome. Finally, this study was carried out to inform that *Curcuma aeruginosa* rhizome is safe when used as a traditional herb. A limitation of this study was in the collection of *Curcuma aeruginosa* rhizomes that grow well in the same area and can be harvested at the same age in sufficient quantities to conduct the research.

CONCLUSION

Curcuma aeruginosa rhizomes containing the toxic marker compounds of 9-methyltetracyclo[7.3.1.0(2.7).1(7.11) tetradecane; epicurzerenone; and cis-1,3-dimethyl-2-methylene cyclohexane are correlated with mice hepatocyte PARP-1 expression.

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