PHARMACY: Jurnal Farmasi Indonesia
(*Pharmaceutical Journal of Indonesia*)
VOL 20 (02) 2023: 106-113

Received 20-10-2022 Accepted 21-08-2023 Available online 15-09-2023

GC-MS Profile of Antioxidant Secondary Metabolites of Red Ginger from Batu Malang with Hepatoprotector Effect on Wistar Rats Exposed to Ethanol

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ABSTRACT

The liver is the organ most susceptible to the adverse effects of alcohol use. Decreased superoxide dismutase can be used as an indicator of liver cell damage due to excessive alcohol consumption. Red ginger (*Zingiber officinale* var. *Rubrum*) is known to have a hepatoprotective effect through antioxidant and anti-inflammatory mechanisms. This study was to prove the effect of red ginger extract from Batu Malang, Indonesia to restore normal levels of superoxide dismutase in the liver of Wistar rats induced by ethanol. Gas Chromatography-Mass Spectroscopy (GC-MS) metabolite profiling analysis of the red ginger ethanol extract was also carried out to ensure the presence of antioxidant compounds. Male Wistar rats were divided into 5 groups with 6 rats each, including negative control (C-) which was only given standard feed, positive control (C+) which was only given 1.8 mL/200g body weight of 40% ethanol orally, and treatment group which were given 40% ethanol as much as 1.8 mL/200 g body weight and red ginger extract at a dose of 250 (T1), 500 (T2), and 750 (T3) mg/kg body weight. Liver organs were taken to examine superoxide dismutase levels after 14 days of treatment in all groups. This research shows that ethanol with a concentration of 40% can reduce superoxide dismutase levels in the liver of rats significantly ($p<0.05$). All treatment groups that were given red ginger extract had higher superoxide dismutase levels than the positive control (C+) group ($p <0.05$), but the optimal dose to increase superoxide dismutase levels in Wistar rats induced by oral administration of 40% ethanol is 250 mg/kg body weight. Based on the results of metabolite profiling using GC-MS, found three dominant compounds thought to act as antioxidants, including Zingiberene, Gingerol, and 6-shogaol. Furthermore, further research is needed to evaluate the safety of using red ginger extract.

Keywords: Ethanol induction, GC-MS profiling, hepatoprotective, red ginger extract, superoxide dismutase

Introduction

Death and disability from alcohol use are increasing worldwide. In 2018, the Centers for Disease Control and Prevention (CDC) reported that the death from alcohol use in the United States was 37,329 people. This condition is caused by liver disorders due to alcohol use or commonly referred to as Alcoholic Liver Disease (ALD) (World Health Organization 2018). In Indonesia, based on the results of the 2017 demographic and population survey, the largest alcohol consumption was carried out by men aged 20-24 years (Kementerian Kesehatan RI 2018).

Drinking ethanol in excess in the long term can cause oxidative stress in the liver (Mandrekar et al., 2016). Acetaldehyde, a toxic metabolite from the alcohol metabolism process combined with Lipopolysaccharide (LPS) can cause liver inflammation (Liu 2014; Arab et al. 2019). Oxidative stress due to alcohol consumption can be prevented by Superoxide Dismutase (SOD), which is an endogenous antioxidant in the body. Superoxide dismutase works by catalyzing reactive oxygen species into non-reactive forms, so they do not become toxic to the tissues (Zhang et al. 2017; Ighodaro and Akinloye 2018). However, in pathological conditions, the toxicity of acetaldehyde and LPS that accumulates in the liver can cause a decrease in the amount and activity of SOD (Meng et al., 2018).

Red ginger (*Zingiber officinale* var. *Rubrum*) contains active flavonoids and polyphenols which are considered safer than corticosteroids for current ALD therapy. The hepatoprotective effect of red ginger stems from its potential as an antioxidant and anti-inflammatory (Haniadka et al. 2013). Red ginger compounds such as 6-gingerol, shogaol, and zingerone are reported to be able to act as exogenous antioxidants and anti-inflammatory agents to prevent excessive ROS formation (Ceni et al., 2014; Ibusuki et al., 2017).

Research on the effects of ginger as an antioxidant includes the effect of 6-gingerol to increase SOD levels in the livers of rats damaged by the hepatocarcinogen diethylnitrosamine (Alsahli et al. 2021). In addition, ginger oleoresin can also increase SOD levels in the kidneys of rats under stress (Wresdiyati et al., 2007). Ginger has also

been shown to increase Malondialdehyde (MDA) levels in the reproductive organs of rats damaged by alcohol administration (Akbari et al., 2017). However, the effect of red ginger (*Zingiber officinale var. rubrum*) extract on SOD levels in rat liver damaged by oral administration of alcohol has not been carried out.

This study is to prove the hepatoprotective ability of red ginger extract to increase SOD levels in the liver of rats given oral alcohol. Ethanol is used to induce liver damage in rats because it is the most consumed type of alcohol according to the World Health Organization (WHO). Meanwhile, the concentration of ethanol that has been reported to significantly reduce liver SOD levels is 20 - 40% (Liang et al. 2021). This study also carried out a metabolite profiling analysis of the red ginger extract used, to ensure the presence of gingerol and other antioxidant compounds. The red ginger used in this study comes from Batu Malang, Indonesia. From Nishidono et al. (2020), it is known that differences in ginger varieties can cause differences in the types and levels of active compounds in them, which can further affect their antioxidant activity. Gas Chromatography-Mass Spectrometry (GC-MS method) was used to profile the active metabolite of red ginger extract (Ashraf et al., 2017; Mohamed et al., 2016).

Research Method

Materials

The crude drugs used in this study came from red ginger grown in the Materia Medica Batu area, Technical Implementation Unit of the Health Service, Batu City Malang, Indonesia. Preparation of the red ginger extract and the treatment of Wistar rats were carried out at the Laboratory of Pharmacology, Brawijaya University Indonesia. Experimental animals in this study used 2-3 months old male Wistar rats, with body weights ranging from 165-220 grams. The study was approved with the issuance of ethical clearance by the Ethics Commission of Universitas Brawijaya with the number 004-KEP-UB-2021.

Preparation of the red ginger extract

A total of 50 grams of red ginger crude drugs was extracted by maceration using 250 mL of 96% ethanol solution (Technical Solution). Maceration was carried out in two stages, by immersing the crude drugs in ethanol and stirring using a magnetic stirrer (Thermo Scientific). The maceration filtrate which has been separated from the crude drugs precipitate is then dried using a rotary evaporator (IKA RV10®). The result of this process is a thick extract of red ginger (Rahmadani et al., 2015).

Treatment of red ginger extract and ethanol on Wistar rats

The five treatment groups each consisted of 6 Wistar rats. The treatment groups were negative control group (C-) which was only given standard feed, positive control group (C+) which was only given 40% ethanol (Merck), treatment group 1 (T1) given 40% ethanol and red ginger extract 250 mg/kg body weight, treatment group 2 (T2) given 40% ethanol and red ginger extract 500 mg/kg body weight, and treatment group 3 (T3) given 40% ethanol and red ginger extract 750 mg/kg body weight.

Red ginger according to each treatment dose was dissolved in 2 mL of aquadest, then given orally to rats using a nasogastric tube. In this study, the extract was administered 60 minutes before the administration of ethanol, to prove the hepatoprotective effect of red ginger extract (Afrianti et al., 2014). The amount of ethanol given to the treatment group was calculated based on the amount of daily consumption in humans, which was 100 mL per day (World Health Organization 2020). Meanwhile, the conversion factor for giving alcohol to rats is 0.018, then the maximum total volume of ethanol that can be given to rats per day was 1.8 mL (Laurence and Bacharach 1964). All treatments in each group were carried out for 14 days.

Liver organ harvesting procedures

On the 15th day, after all treatments were given, the rats were anesthetized with 0.3 ml of ketamine (Ivanes®, Ikapharmindo) at a dose of 20 mg/mL intramuscularly, and killed using the cervical vertebral dislocation method. Furthermore, the rats were dissected for liver organ harvesting which was then followed by examination of SOD levels.

Examination of liver superoxide dismutase in Wistar rats

A total of 100.0 mg of liver from all treatment groups were homogenized using 1.0 mL of Phosphate Buffer Saline (PBS, Merck) pH 7.4. Furthermore, the liver-PBS solution was centrifuged (Thermo Scientific) at 40 °C, at a speed of 4000 rpm for 15 minutes. A total of 500 µL liver-PBS supernatant from each treatment group was added with 200 µL 100 mM EDTA (Merck), 100 µL 25 mM Nitroblue Tetrazolium (NBT, Serva) (except for C- group), 100 µL 25 mM Xanthine (Sigma Aldrich), 100 µL 1 unit Xanthine Oxidase (Nacalai), and vortex (Thermoline) until homogeneous. The homogeneous solution was incubated (Mayon Scientific) at 37°C for 30 minutes, and then centrifuged at 4000 rpm for 5 minutes. The results of the centrifugation were filtered, and the supernatant was added with aquadest to a volume of 3.0 mL. The sample was then read for absorbance at a wavelength of 580 nm (Gilson). The results of the examination of liver SOD levels from the spectrophotometer readings are expressed in units of U/mg protein (Katerji et al., 2019).

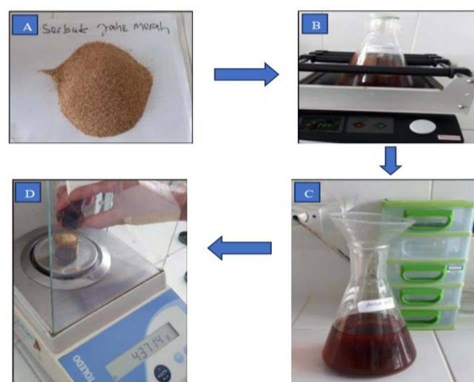

Figure 1. Red ginger extraction process with 96% ethanol; (A) red ginger crude drugs, (B) maceration process of crude drugs with 96% ethanol, (C) macerated filtrate, and (D) red ginger extract

Data analysis of SOD levels after treatment

The data from the calculation of SOD levels from each treatment group were not normally distributed (Shapiro Wilk test, $p<0.05$) and were homogeneous (homogeneity test, $p>0.05$). Furthermore, the differences in each treatment group were tested with the Kruskal-Wallis test and followed by the Mann-Whitney test.

GC-MS metabolite profiling analysis of red ginger extract

Metabolite profiling analysis of ginger extract was performed using a GC-MS system (Agilent 7890B). Ten microliter of ginger extract (10 mg/mL) was injected into the HP–5MS UI column (30 m x 0.25 mm x 0.25 μm) (Agilent Technologies AG19091S-433UI). The initial temperature column was held at 50 °C for 2 min, and raised by 5 °C/min up to 280 °C. The flow rate of helium as a carrier gas was constant at 1 ml/min. The total compound separation time was 54.80 minutes, and the m/z scan was in the range of 30.00 - 650.00. Analysis of peak determination of red ginger secondary metabolite performed with the NIST 14 GC-MS Library database (Ashraf et al. 2017).

Results and Discussion

Crude drugs used in this study was light brown in color with a fine size (sifted through 60 mesh). After the maceration process with 96% ethanol, a thick dark brown extract with a characteristic spicy ginger odor was obtained (Figure 1). The yield of the extract was 8.57 grams (17.1%). Red ginger extract was then diluted to adjust the dose of treatment in Wistar rats.

Exposure to 40% ethanol in rats for 14 days has been shown to reduce SOD levels in the liver. This study resulted in the lowest mean SOD level in the positive control group, with a mean ± SD value of 42.8 ± 2.28 U/mg. The levels of SOD were significantly lower when compared to the liver SOD levels of the negative control group which a had value with a mean ± SD of 63.8 ± 3.84 U/mg ($p<0.05$) (Table 1). This research results are in accordance with previous studies which showed that administration of ethanol for 14 days could induce liver damage, such as changes in histopathological features (fatty hepatocytes, necrosis, and degeneration), increased levels of MDA, and also decreased levels of glutathione (GSH) and SOD (Xing et al. 2015).

Ethanol can cause the formation of acetaldehyde and Lipopolysaccharide (LPS) which are hepatotoxic (Ceni et al. 2014; Louvet and Mathurin 2015). Accumulation of acetaldehyde and LPS can produce free radical substances such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl anion ($OH^{\bullet-}$). They can bind to proteins, unsaturated fatty acids, and DNA which can further damage hepatocytes through lipid peroxidation (Osna et al., 2017). The liver is responsible for metabolizing 90% of the absorbed ethanol (Liu 2014; Li et al. 2015). Superoxide dismutase is an endogenous antioxidant in the liver, which works by catalyzing free radical substances into non-reactive forms (Zhang et al. 2017; Ighodaro and Akinloye 2018). However, if the liver is exposed to toxic substances continuously, there will be an increase in free radicals and it requires more SOD to make it non-reactive (Meng et al. 2018).

The normal liver SOD value itself can be different, depending on the type of organ sample, the unit of measurement used, and the type of experimental animal used. For example, from a study conducted by Akbari et al. (2019), the normal liver SOD level in that study was 50 ± 5 U/mg Protein. Meanwhile, according to Wang et al. (2019), the normal liver SOD level was 75 ± 25 U/mg protein. Red ginger extract was shown to significantly increase liver SOD levels in rats treated with 40% ethanol when compared to the positive control group ($p<0.05$) (table 2). The highest increase in SOD levels occurred in the T2 group, which was given red ginger extract at a dose of 500 mg/kg body weight, although it was not significantly different from the group given 250 mg/kg body weight red ginger extract (T1).

For example, research conducted by Bhandari et al. (2003) proved that ginger extract at a dose of 200 mg/kg body weight could reduce levels of Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP), and tissue lipid peroxidase in rats exposed to liquor. In addition, ginger extract can also escalate the antioxidant capacity and glutathione peroxidase activity in the brain and liver of mice exposed to alcohol (Shati and Elsaid 2009), or increase the levels of Glutathione S-transferase (GST), GSH, and SOD of mice exposed to alcohol (Nwozo et al., 2014).

Table 1. Liver superoxide dismutase (SOD) levels in Wistar rats after 14 days of treatment in all treatment groups.

Treatment Group	N	Mean ± SD (U/mg)	Minimum	Maximum
C-	4	63,8 ± 3,84	58,11	66,58
C+	4	42,8 ± 2,28	39,73	45,05
T1	4	59,2 ± 2,63	55,23	60,72
T2	4	59,8 ± 4,83	54,23	66,04
T3	4	53,2 ± 0,67	52,34	53,96

*Note; C- (negative control, rats were given standard fed), C+ (positive control, rats were given 40% ethanol), T1 (rats were given 40% ethanol and red ginger extract at a dose of 250 mg/kg body weight), T2 (rats were given 40% ethanol and red ginger extract at a dose of 500 mg/kg body weight), and T3 (rats were given 40% ethanol and red ginger extract at a dose of 750 mg/kg body weight).

Table 2. The results of the Mann-Whitney test on the liver superoxide dismutase levels of Wistar rats after 14 days of treatment

Treatment group	C-	C+	T1	T2	T3
C-	-	0,021*	0,149	0,386	0,021*
C+	0,021*	-	0,021*	0,021*	0,021*
T1	0,149	0,021*	-	0,564	0,021*
T2	0,386	0,021*	0,564	-	0,021*
T3	0,021*	0,021*	0,021*	0,021*	-

Note: *) significant if $p<0,05$

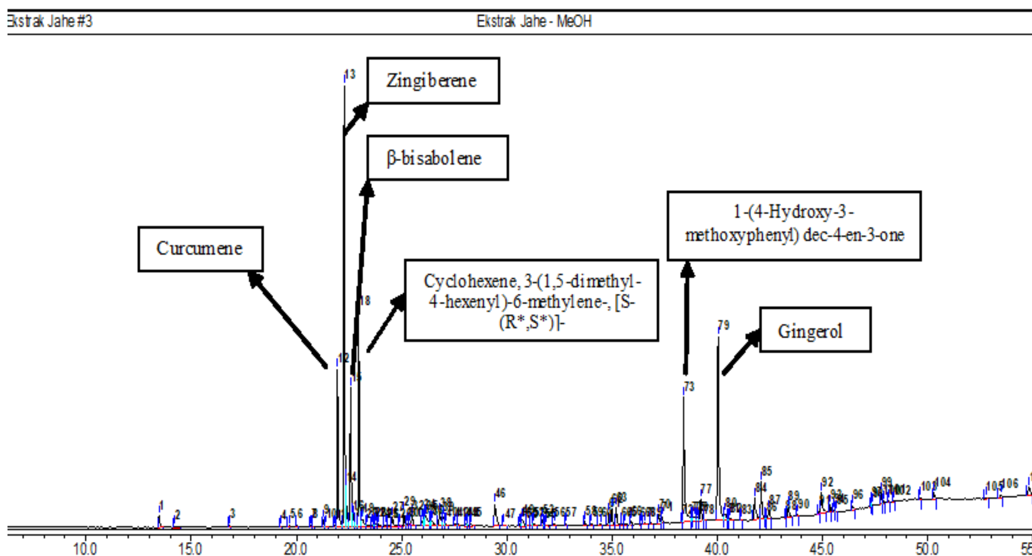

Figure 2. GC-MS chromatogram of red ginger extract using HP–5MS UI column (30 m x 0.25 mm x 0.25 μm) and using helium with a fixed flow rate of 1 ml/min

This study proves the liver protective effect of red ginger extract. Extract doses of 250 and 500 mg/kg body weight have antioxidant and anti-inflammatory, as shown by a significant increase in liver SOD levels in rats exposed to alcohol when compared with positive control group. This is in tune with previous studies regarding the effect of ginger extract as hepatoprotective in alcohol abuse.

The T3 showed significantly increased SOD levels compared to the positive control group, but significantly lower SOD levels when compared to the negative control group, T1, and T2. The decrease in the effect of red ginger extract with the largest dose is not yet known. The administration of white ginger extract with a toxic dose of 2000mg/kg body weight was still able to significantly increase liver SOD levels when compared to the positive control group (Abdulaziz Bardi et al. 2013). Likewise, giving ginger extract at a dose of 1000mg/kg body weight was able to significantly increase liver SOD levels in ethanol-induced rats compared to the positive control group (Akbari et al. 2019).

Based on the results of secondary metabolite profiling of red ginger extract using GC-MS, 6 large peaks were seen indicating the presence of 6 main metabolites. Zingiberene (peak 13; 19.55% area) was the largest metabolite, followed by gingerol (peak 79; 16.14% area) and 6-shogaol/1-(4-Hydroxy-3-methoxyphenyl) dec-4-en-3-one (peak 73; 11.06% area) (Figure 2).

Table 3. GC-MS metabolite profile of red ginger extracts with known antioxidant potential activity

Peak Number	Retention Time (min)	Compound Name	Chemical Formula	Molecular Weight	Similarity Index	Area* (%)	Antioxidant Activity
1	13.46	endo-Borneol	$C_{10}H_{18}O$	154.25	929	0.60	Increases SOD and GSH liver in diabetic rats (Madhuri and Naik 2017)
12	21.94	Curcumene	$C_{15}H_{22}$	202.33	913	7.65	DPPH radicals scavenging (Račková et al. 2013)
13, 14	22.26; 22.34	Zingiberene	$C_{15}H_{24}$	204.35	914	19.55	Reduce total oxidative stress in rat cortical neurons (Togar et al. 2015)
15	22.57	ß-Bisabolene	$C_{15}H_{24}$	204.35	883	7.79	DPPH scavenger activity (Kazemi and Rostami 2015)
18	22.95	beta-Sesquiphellandrene	$C_{15}H_{24}$	204.35	907	9.72	DPPH radicals scavenging (Zhao, Zhang et al., 2010)
33	25.99	beta eudesmol (2-Naphthalene methanol, decahydro-a,a,4a-trimethyl-8-methylene-, [2R-(2a,4aa,8aß)]-	$C_{15}H_{26}O$	222.36	801	0.28	Suppressing a detoxifying enzyme (Srijiwangsa et al., 2018)
34, 35	26.06; 26,14	Zingerone	$C_{11}H_{14}O_3$	194.23	767	1.84	Inhibit ROS and lipid peroxidation (Ahmad et al. 2015)
53	31.66	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	792	0.28	Scavenging effect on DPPH radicals (Pinto et al. 2017)
61, 62	34.84; 34.97	8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	854	1.41	Scavenging effect on DPPH radicals (Pinto et al. 2017)
70, 71, 74, 75, 76, 77 79, 80	37.20; 37.36; 38.78; 38.92; 39.04; 39.20; 40.03; 40.37	Gingerol	$C_{17}H_{26}O_4$	294.38	888	16.14	Reduce H_2O_2 and MDA; increasing antioxidant of SOD and GSH (Abolaji et al. 2017); and DPPH radicals scavenging (Račková et al. 2013)
82, 89	40.60; 43.36	21 25-dihydroxy-vitamin d3	$C_{27}H_{44}O_3$	416.64	709	0.95	Reduced oxidative stress in diabetic rat kidneys (Wang et al. 2020)
73, 84, 92	38.40; 41.77; 44.93	6-shogaol	$C_{17}H_{24}O_3$	276.37	934	11.06	Increase the GSH/GSSG ratio; lowering the level of ROS (Chen et al. 2014)

Note: Only shown metabolite that has peak area > 0.20% and has known antioxidant activity, bold are the six main metabolites**.**

Research for secondary metabolite profiling of red ginger ethanol extract has been carried out by Nur et al. (2020), however, showed that there were differences in the types and concentrations of

compounds contained in the ethanol extract of red ginger. Zingiberene was the main secondary metabolite compound in both studies, but in this study, the concentration of Zingiberene was lower than those reported by Nur et al. (2020), 19.55% vs. 31.43%. In this study, gingerol and 6-shogaol compounds could be found, while in Nur et al. (2020) was not detected. The existence of different types of secondary metabolites from the ethanol extract of red ginger can be caused by differences in the location and growing environment of the red ginger plant. A study states that gingerol and 6-shogaol levels are strongly influenced by pH and soil texture, as well as the condition of soil organic or mineral compounds (Setyawati et al. 2021). For example, it was found that the gingerol content was higher in red ginger plants grown in soil with a pH of 5.21 and with a low percentage of organic carbon and nitrogen (less than 1%) (Yusron 2009).

The content of gingerol and 6-shogaol is very important in red ginger because they were reported to increase superoxide dismutase activity. In addition, gingerol and 6-shogaol have anti-inflammatory properties that are useful as free radical scavengers (Febriani et al., 2018; Mao et al., 2019). The 6-shogaol is known to have better activity than gingerol, in terms of against superoxide radicals, DPPH radicals, and hydroxyl radicals (Dugasani et al. 2010). Meanwhile, zingiberene compounds have antioxidant activity by reducing total oxidative stress in cortical neurons exposed to H_2O_2 (Togar et al., 2015). Other compounds that have potential antioxidant activity are described in Table 3.

This study has limitations in not measuring parameters to determine the condition of the liver, such as liver function tests (ALT and AST). Further research is needed to carry out metabolite profiling and bioassay-guided isolation from red ginger extract to determine active compounds that have antioxidant activity. Although there is potential antioxidant activity of red ginger, its safety needs to be evaluated. In this study, the use of red ginger extract at all doses of 250 – 750 mg/kg BW did not cause the death of the Wistar rats. However, evaluation still needs to be carried out to assess the safety of using red ginger extract in humans. Therefore, it is necessary to conduct a toxicity test related to the administration of red ginger extract.

Conclusion

From this study, it can be concluded that red ginger extract from Batu Malang, Indonesia has hepatoprotective activity by increasing the level of SOD which is reduced due to the toxicity of 40% ethanol. However, increasing the dose of the extract did not increase the hepatoprotective activity. The most effective dose of red ginger extract was 250 mg/kg body weight because this dose was able to increase levels of SOD equivalent to the negative control group. Based on the results of metabolite profiling using GC-MS, three dominant compounds are known to have antioxidant activity, including zingiberene, gingerol, and 6-shogaol.

Acknowledgment

We would like to express our gratitude to the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya Malang, which has helped the research process.

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