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RESEARCH ARTICLE

Antioxidant and anti hyperalgesia activity of ethanol extract and fraction from red ginger (*Zingiber officinale* var. Rubrum) in early painful diabetic neuropathy mice

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Abstract

Background: Gingerol and shogaol are two major bio-components of Zingiber officinale var. Rubrum (red ginger) has been widely reported as an antioxidant crucial in painful diabetic neuropathy (PDN). **Objective:** Determines the activity of the ethanolic extract of red ginger rhizome and its fraction by in vitro antioxidant and the in vivo study in PDN mice. **Method:** Red ginger was extracted using ethanol and fractionated with n-hexane, chloroform, ethyl acetate, and n-butanol. The antioxidant was tested using DPPH and CUPRAC. Diabetes was induced using alloxan 225 mg/kg BW ip in male BALB/c mice. After 14 days, mice were randomly divided into normal, diabetic, ethanol extract, chloroform fraction, ethyl acetate fraction on the same dose of 400 mg/kg BW, metformin (200 mg/kg BW), and gabapentin (100 mg/kg BW). Treatments were given orally, once daily, for 21 days. Latency time and blood glucose levels were measured every week. Histology of the spinal cord was analysed using Hematoxylin-eosin staining. **Result:** The ethyl acetate fraction had the best antioxidant activity using DPPH (IC50 13.93 ± 0.06) and CUPRAC (IC50 4.07 ± 0.06). This fraction showed the most potent ability to decrease BGL (69.78 ± 18.36%), same as metformin, and hyperalgesia (59.34 ± 7.90%) better than gabapentin. This treatment repaired the spinal cord by reducing the number of inflammatory cells and neuron degeneration in PDN mice. **Conclusion:** The EERG of 400 mg/kg BW significantly affects arthritis-induced hyperalgesia.

Introduction

One of the chronic diseases that cause the world's highest mortality is diabetes mellitus (IDF, 2019). Prolonged hyperglycemia in a diabetic patient can change biochemical metabolism in the body and cause nerve function impairment, called diabetic neuropathy. Patient with diabetic neuropathy usually complains about pain early (Aslam *et al.,* 2014). Without appropriate treatment, this condition will affect patients' quality of life. The lack of drug effectiveness, such as opioids, antidepressants, and anticonvulsants, is the major problem in treating painful diabetic neuropathy (PDN) (Kaur *et al.,* 2011). Hence, developing a new lead compound as a drug in PDN is critical.

Currently, the bio-natural compound in plants is growing, especially in Indonesia. One of the potential plants is Zingiber officinale, also known as ginger, one of the Zingiberaceae family. In Indonesia, there are three varieties of ginger, i.e., Zingiber officinale var. Amarum, Zingiber officinale var. Roscoe and Zingiber officinale var. Rubrum (Azizah *et al.,* 2019). Ginger is widely known to have many active compounds such as phenolic (gingerols, schools, and parasols) and terpenes (β-bisabolene, α-curcumin, zingiberene, α-farnesene, and β-sesquiphellandrene) (Kizhakkayil &

Sasikumar, 2011; Mao *et al.,* 2019*)*—the highest level of gingerol, one of the phenolic compounds in Zingiber officinale var. Rubrum (red ginger) was reported (Azizah *et al.,* 2019). This result followed another research study, which found that phenolic and flavonoid compounds in red ginger are higher than in different varieties (Oboh *et al.,* 2012a, 2012b).

Red ginger was previously reported as antidiabetic (Rackova *et al.,* 2013) and an antioxidant agent (Oboh *et al.,* 2012b*).* 6-Shogaol, as a biomarker in red ginger, was also found to significantly reduce blood glucose levels in PDN mice (Fajrin *et al.,* 2019). 6-Shogaol successfully decreased the number of transient receptor protein vanilloid 1 (TRPV1) and N-methyl-ᴅ-aspartate receptor subunit 2B (NMDAR2B) in mice's spinal cord (Fajrin *et al.,* 2020). The latest study revealed that both become the specific target for some drugs to treat PDN (Singh *et al.,* 2014). Additionally, 6-gingerol activity can reduce plasma glucose and insulin levels in mice with high-fat diet-induced obesity (Sampath *et al.,* 2017). Another finding revealed many bioactive compounds in red ginger as antiinflammation and neuroprotector (Mao *et al.,* 2019).

Antioxidant activity is known to be essential in diabetic pathophysiology. Chronic hyperglycemia leads to flooding of oxidative stress (Kaur *et al.,* 2011). The report of extract and fraction of red ginger activity in diabetic neuropathy has not yet been confirmed. Our study aimed to test in vitro the antioxidant activity of the extract and fraction of red ginger and to analyse their activity in improving spinal cord histology in diabetic neuropathy mice.

Methods

Materials

Red ginger rhizomes were collected from Pecifarm farmers in Kencong, Jember (East Java, Indonesia). Indonesian Institute of Sciences, U.P.T. Balai Konservasi Tumbuhan Kebun Raya Purwodadi, Indonesia, has identified them (No. 1458. IPH.6/HM/X/2015). Gentamicin and metformin as a controlled drug were obtained from Indofarma. All the reagents in this experiment were bought from Merck. All in vivo procedures were approved by the Ethical Clearance Committee of the Faculty of Medicine, Jember University (No: 774/H25.1.11/KE/2016).

Extraction and fractionation

Red ginger rhizomes were ground into a powder and macerated using ethanol 96% (1:4) for two to 24 hours. This process was repeated until the maserat was in the clear phase solution. The extract was concentrated using a rotary evaporator at 40 °C to get the thick extract.

Graded fractionation was obtained from ethanol extract using n-hexane, chloroform, 1-butanol, and ethyl acetate as solvents. Red ginger extract was diluted with 75 mL water before fractionation. The fractionation process of each solvent was prepared using a 2:1 comparison between solvent and extract. The extraction and fractionation results were weighed for the calculation of yield.

Phytochemical screening

The identification of phytochemical compounds in the extract and fraction of red ginger was carried out using silica gel G60F254 of thin-layer chromatography. As many as 10 mg of samples were diluted into 1 mL of ethanol and bottled into the stationary phase. The identified compounds included alkaloids, flavonoids, saponins, steroids, terpenoids, tannins, and polyphenols.

DPPH assay

The antioxidant activity was determined using the DPPH method described earlier with modification (Kedare & Singh, 2011). As much as 0.3 mL of each sample (extract, fraction, and vitamin C) was mixed with 1.2 mL DPPH solution 0.1 mM and incubated in the dark at room temperature for 60 minutes. The mixture's absorbance was measured at 515 nm, with vitamin C as the positive control. The scavenging ability of the sample to DPPH radical was determined according to the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Acontrol and Asample were the absorbances of the reference and sample obtained from the UV-visible spectrophotometer.

Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric-reducing capacity of the fractions was determined by the CUPRAC method (Apak et al., 2007). One millilitre of copper (II) chloride solution (0.01 M prepared from CuCl2.H2O), 1 mL of ammonium acetate buffer at pH 7.0, 1 mL of neocaproin solution (0.0075 M), and 0.6 mL of aquadest were mixed to 0.5 mL of plant extract or standard of different concentrations solution. The final volume of the mixture was adjusted to 4.1 mL by adding 0.6 mL of distilled water. The resulting mixture was incubated for 30 minutes at room temperature, and the absorbance of the solution was measured at 450 nm. The antioxidant activity was

determined as CUPRAC capacity (%) = (1-As) x 100, where 'As' represents the sample absorbance.

Preparation of experimental animal

Thirty-five male mice strain Balb/C (20-30 g, six to seven weeks) were prepared by the animal laboratory of the Faculty of Pharmacy, University of Jember. All mice were habituated for seven days before being used as models. On day zero, each mouse was injected intraperitoneally with 225 mg/kg body weight (B.W.) of alloxan (Sigma-Aldrich, A7413) for inducing diabetes. The normal group was injected with normal saline with an injection volume equivalent to that of the diabetic group. A mouse was considered diabetic neuropathy when its blood glucose level was higher than 200 mg/dL until two weeks after induction, followed by hyperalgesia (Zangiabadi *et al.,* 2014)**.**

Treatments

After two weeks of induction, thirty-five mice were divided into seven treatment groups. Each group consisted of four to five mice. Group I as a normal control (sodium CMC 1% 1 mL/kg BW). Group II as a diabetic control (sodium CMC 1% 1 mL/kg BW), Group III as antidiabetic control (metformin 200 mg/kg BW), Group IV as antihyperalgesic control (gabapentin 100 mg/kg BW), Groups V-VII test groups (ethanol extract 400 mg/kg BW; ethyl acetate fraction 400 mg/kg BW; chloroform fraction 400 mg/kg BW). All treatments were administrated orally once daily for 21 days. The experimental animal scheme is illustrated in the figure below.

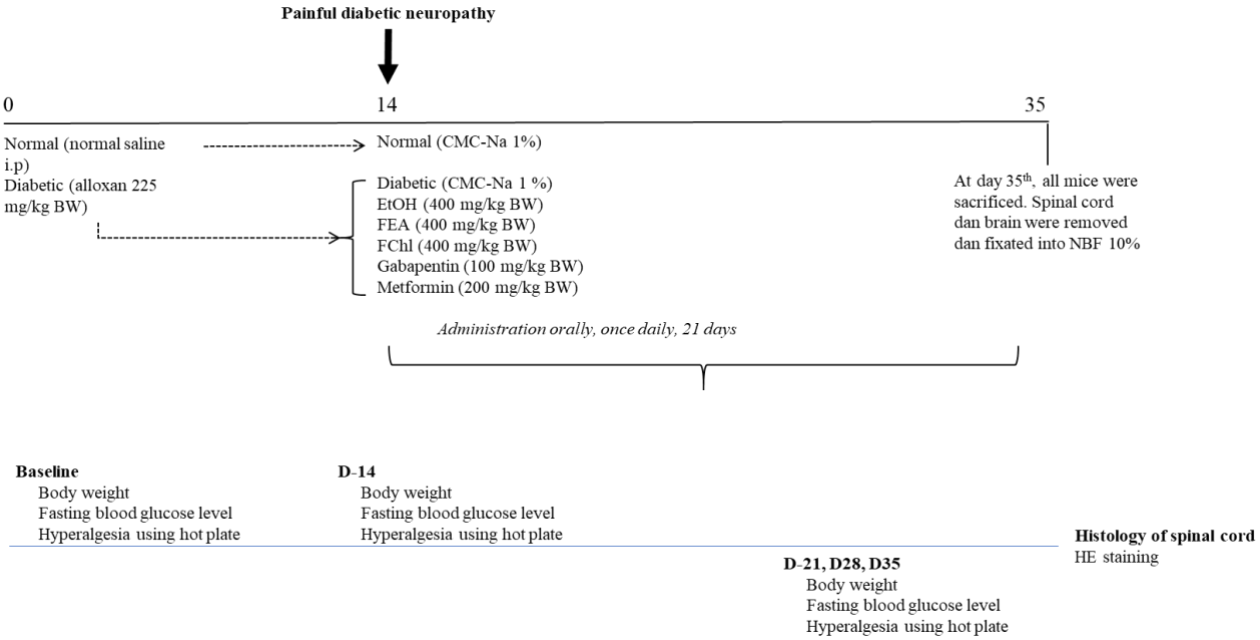


Figure 1: Experimental scheme in mice

Blood glucose and hyperalgesia measurement

Fasting blood glucose levels and latency time toward thermal stimulus were determined every week for five weeks. Blood samples were taken from the retro-orbital plexus and then centrifuged at 2000 rpm for ten minutes to separate the serum. The decreasing blood glucose level was calculated using the glucose oxidase-peroxidase method with the procedure written in the kit (GOD-FS, Diagnostic Systems GmbH, Holzheim, Germany).

The hyperalgesia behaviour was measured using a test on a hot plate (Ugo Basile, 35100, Italy). All mice were adapted for one hour before the experiment. Each mouse was placed individually on the hot plate set at 50°C. The time needed for each mouse to show pain response was considered latency time. Pain responses were shown as licking, jumping, and rearing. This experiment's cut time was thirty seconds to prevent nerve damage (Fajrin *et al.,* 2019).

Histopathology of the spinal cord of mice

At the end of the study (day 35), mice were sacrificed by cervical dislocation. The spinal cord was isolated and fixated in neutral buffer formalin ten percent before

staining. The spinal cord was stained using Hematoxylin-Eosin (HE), and the morphology of the dorsal horn of the spinal cord was observed using a light microscope with 1000x magnification.

Data analysis

All data were shown as mean (with the standard error of mean/S.E.M). Data were statistically analysed using an independent t-test, and one-way ANOVA was continued with Tukey post hoc. The significant difference was defined if $p < 0.05$ using a confidence level of 95%. Graphical and statistical analysis was determined using GraphPrism 5.

Results

Extraction and fractionation

After extraction and fractionation, all samples were calculated as the yield percentage. Table I shows that the n-hexane fraction has the highest yield percentage compared to the other fractions, as seen in Table I. It indicates more compounds of red ginger contained in the non-polar phase. Residue has the highest yield among all the fractions because red ginger extract has many sugar and polysaccharides but lacks any vital bio-compound.

Table I: Extraction and fractionation results

Extract/fraction	Yield (%)
Ethanol extract	2.56
N-hexane	24.38
Chloroform	20.77
Ethyl acetate	2.45
N-butanol	17.46
Residue	32.85

Phytochemical Screening

Almost all extracts and fractions were identified to contain alkaloids, flavonoids, saponin, terpenoids, steroids, and polyphenols, except residue, which does not include any of them. Table II shows that all the extracts and fractions of red ginger do not contain tannin. Even if the extract and fraction have the bioactive compound's same composition, they might differ in concentration.

Table II: Phytochemical screening of extract and fraction from red ginger

Samples	Alkaloid	Flavonoid	Saponin, terpenoid, steroid	Polyphenol	Tannin
Ethanol extract	+	+	+	+	-
N-hexane fraction	+	+	+	+	-
Chloroform fraction	+	+	+	+	-
Ethyl acetate fraction	+	+	+	+	-
N-butanol fraction	-	-	-	-	-
Residue	-	-	-	-	-

Antioxidant activity

Antioxidant assays of red ginger show that the ethyl acetate fraction has the smallest IC50 than ethanol extract and other fractions using two methods, DPPH and CUPRAC, as seen in Table III. This value indicates that the ethyl acetate fraction has the most potent antioxidant activity among all extracts and fractions.

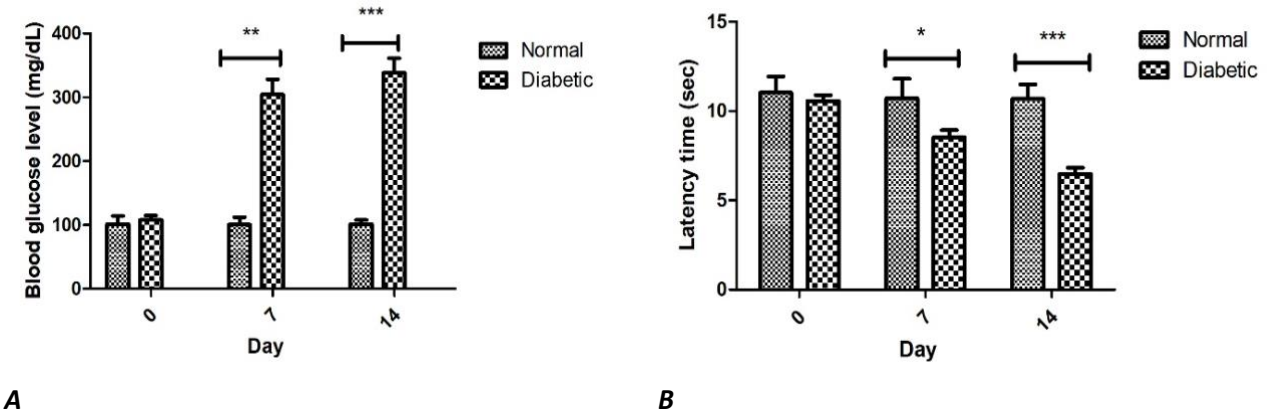
Table III: Antioxidant activity of extract of red ginger and its fraction using DPPH and CUPRAC method

Sample	DPPH assay IC_{50} (ppm)	CUPRAC assay EC_{50} (ppm)
Ethanol extract	18.42 ± 0.47^{a}	5.37 ± 0.21^{a}
N-hexane fraction	24.63 ± 0.55^{b}	6.91 ± 0.22^{b}
Chloroform fraction	17.71 ± 0.23^{a}	4.68 ± 0.07^{c}
Ethyl acetate fraction	13.93 ± 0.06^{c}	4.07 ± 0.06^{d}
N-butanol fraction	34.45 ± 0.96^{d}	16.62 ± 0.49^{e}
Residue	95.91 ± 0.97^{e}	49.99 ± 0.24^{f}
Vitamin C	3.34 ± 0.01^{f}	3.96 ± 0.02^{d}

Data are presented as the mean ± standard of deviation (SD). The analysis was conducted one way, and ANOVA was continued with Tukey. The confidence interval is 95%. The different superscript letters indicate significant group differences ($p < 0.05$).

The effect of alloxan injection on blood glucose levels and hyperalgesia in PDN mice

Before injection, the fasting blood glucose level between the normal group (101.42 ± 12.71 mg/dL) and the diabetic group (107.93 ± 6.76 mg/dL) was not significantly different ($p > 0.05$). Alloxan injection successfully increased the blood glucose level of mice in the diabetic group (307.10 ± 25.33 mg/dL) on day seven compared to the normal group (100.63 ± 11.52 mg/dL, $p < 0.05$). This condition stayed until day 14, with the fasting blood glucose level in the diabetic group still higher (338.17 ± 23.10 mg/dL) and significant compared with the normal group (95.60 ± 5.49 mg/dL, $p < 0.05$). As explained in many previous studies, alloxan has a potent effect of causing beta pancreas cell damage and causing insulin impairment to allow glucose to enter the cell. After alloxan uptake by the pancreas' insulin-secreting beta cells reaches its maximum, its toxicity via ROS increases, leading to the beta cells damage (Ighodaro *et al.,* 2017). This condition was followed by a higher fasting blood glucose level, as seen in Figure 2A.



A ***B***

*Shows a significant difference between the normal and diabetic groups ($p < 0.05$).
**Shows a significant difference between the normal and diabetic groups ($p < 0.01$).
***Shows a significant difference between the normal and diabetic groups ($p < 0.001$). The statistical differences were tested using an independent t-test. The confidence interval was 95%.

Figure 2: Fasting blood glucose level (A) and latency time (B) between normal (n = 5) and diabetic groups (n = 30) before treatment (baseline/day- 0, 7, and 14)

As well as fasting blood glucose level, latency time after thermal stimulus between the normal group (11.04 ± 0.90 sec) and diabetic group (10.56 ± 0.32 sec) was similar at the beginning of the study ($p > 0.05$). It showed that the condition of all mice was the same (Figure 2B). After alloxan injection, the diabetic group's latency time continuously decreased on day seven and day 14 to 8.55 ± 0.39 sec and 6.49 ± 0.34 sec. This value was significantly different ($p < 0.05$) compared to the normal group on day seven (10.72 ± 1.11 sec) and day 14 (10.70 ± 0.80 sec).

The effect of ethanol extract of red ginger and its fraction in body weight of neuropathy diabetic mice

The diabetic condition caused a decrease in body weight in the diabetic group every week. Unlike the diabetic group, the red ginger extract and fraction treatment showed various effects on body weight. Some treatments, except normal control and gabapentin, caused decreased body weight at the end of the study (Fig. 3). The explanation of this phenomenon was unclear. Even though 6-shogaol is responsible for the spicy taste and might reduce their appetite, many related factors might be responsible for body weight.

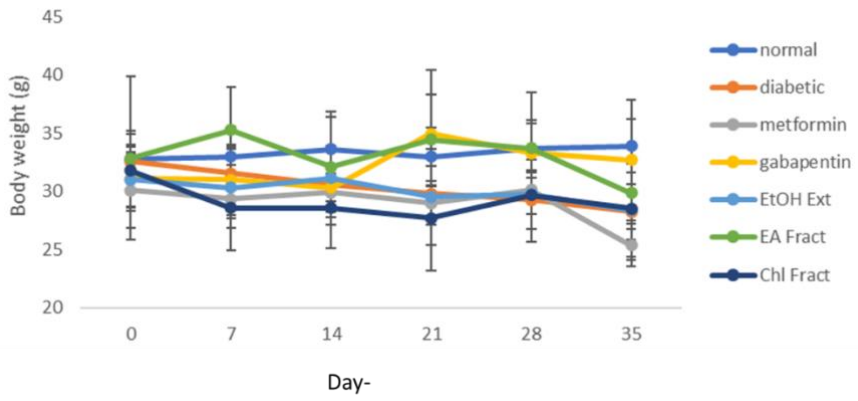
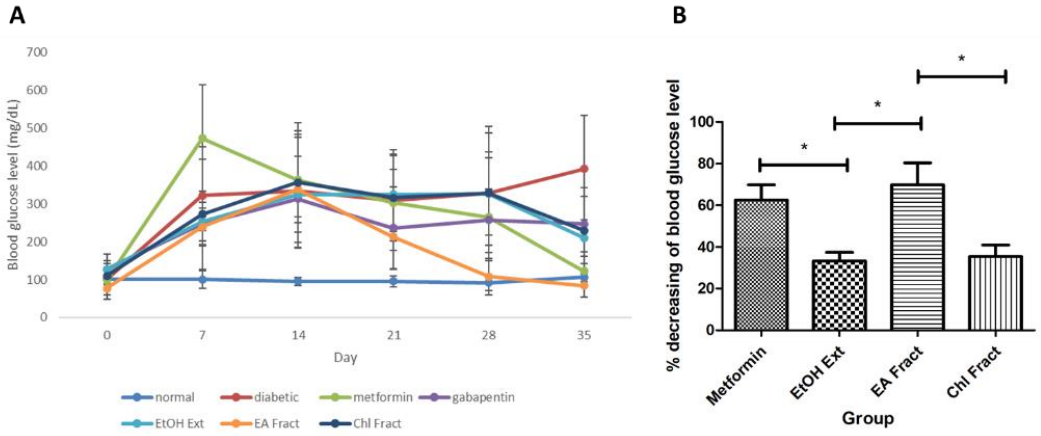


Figure 3: The body weight of each group before alloxan injection (day zero), after alloxan injection (days seven and 14), and after treatment (days 21, 28, and 35).

The activity of ethanol extract of red ginger and its fraction in reducing blood glucose level in neuropathy diabetic mice

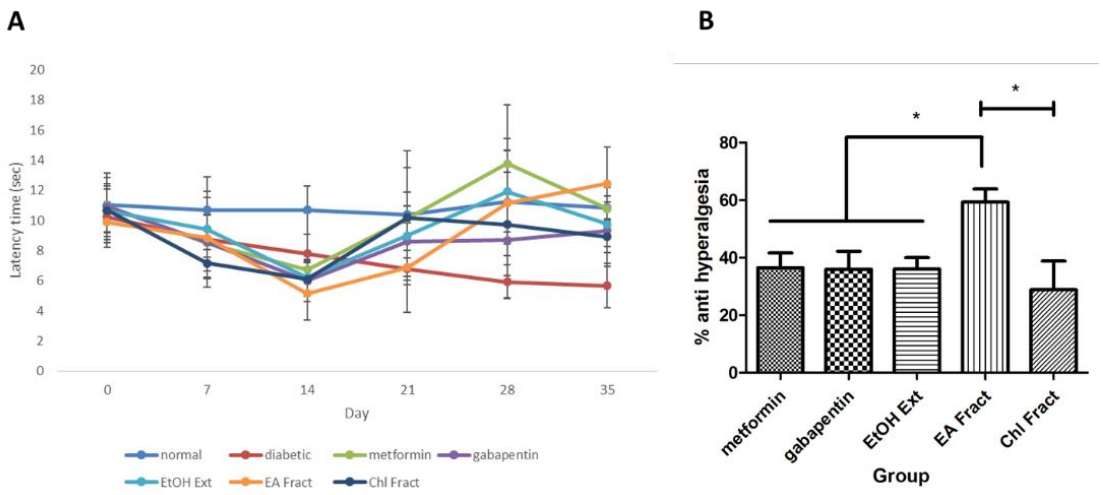
Administration of red ginger extract and its fraction showed decreased fasting blood glucose concentration at different levels after 21 days of daily treatment. Among treatment groups, ethyl acetate fraction administration had higher blood glucose levels than ethanolic extract and chloroform fraction in the same doses. The activity of ethyl acetate fraction was seen as similar to metformin as drug control. Both groups reached blood glucose levels close to baseline (Fig. 4A). Based on the percentage decrease in blood glucose levels, ethyl acetate fraction had the highest potency to decrease blood glucose in diabetic mice, as much as 69.78 ± 18.36%. The ethyl acetate fraction activity was similar to metformin as drug control (62.56 ± 14.49%, $p > 0.05$, Fig. 4B).



*Shows a significant difference between groups ($p < 0.05$). The statistical differences were tested using a one-way ANOVA. The confidence interval was 95%.

Figure 4: A) Fasting blood glucose levels of each group before alloxan injection (day zero), after alloxan injection (days seven and 14), and after treatment (days 21, 28, and 35). B) The percentage of decreasing blood glucose levels between groups

It reduced blood glucose levels correlated with hyperalgesia, which was determined as latency time toward the thermal stimulus. Latency time between treatment groups showed a positive tendency as diminished hyperalgesia (Fig. 5A). The ethyl acetate fraction could prolong latency time toward thermal stimulation (59.34 ± 7.90%) better than gabapentin (35.94 ± 12.56%) as a drug for neuropathy diabetic treatment (Fig. 5B, $p < 0.05$). However, the anti-hyperalgesia activity of metformin (36.50 ± 10.29%), ethanol extract (36.06 ± 12.56%), and chloroform fraction (31.73 ± 22.34%) were not significantly different with gabapentin (Fig. 5B, $p > 0.05$).

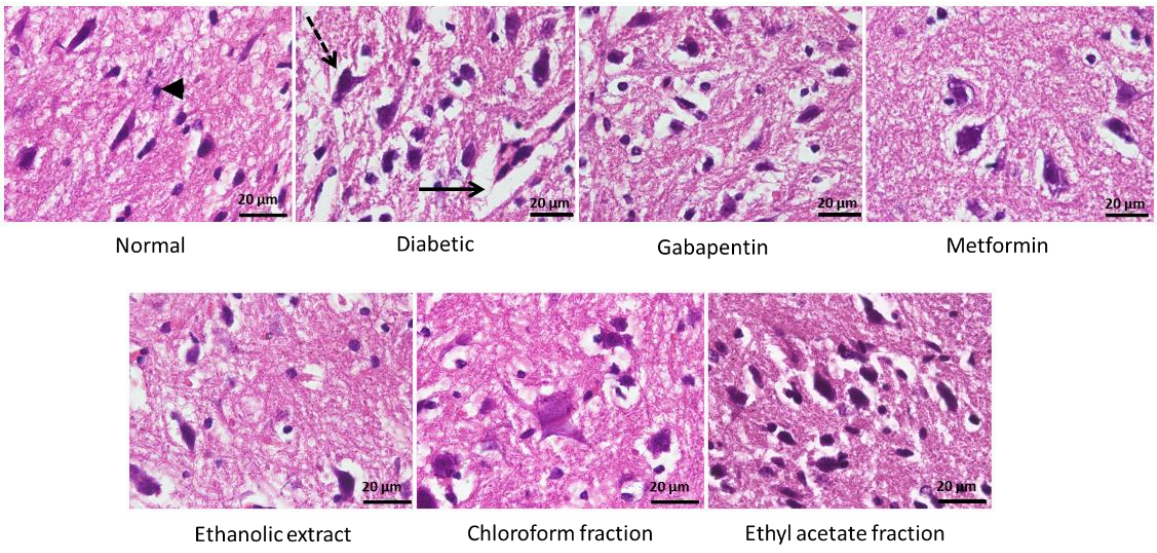


*Shows a significant difference between groups ($p < 0.05$). The statistical differences were tested using a one-way ANOVA. The confidence interval was 95%.

Figure 5: A) Latency time of each group before alloxan injection (day zero), after alloxan injection (days seven and 14), and after treatment (days 21, 28, and 35). B) The percentage anti hyperalgesia between groups

The morphology of the dorsal horn of spinal cord mice after treatment using ethanol extract of red ginger and its fraction

Ethyl acetate fraction administration improved the dorsal horn area's morphology in mice's spinal cord with neuropathy diabetes. Descriptively, the treatment reduces nucleolus protrusion, the number of inflammatory cells, and neuron degeneration compared to the diabetic group (Fig. 6). The treatments also show a descriptive increase in the number of astrocyte cells. Some red ginger compounds have been studied for antioxidant and neuroprotective effects. It might be related to their action in improving the morphology of the spinal cord of mice. The detailed mechanism associated with this action was still unclear. Even though it might be because of the antioxidant activity, more studies are needed to clarify.



The black arrow shows neuron degeneration, the dotted black arrow shows nucleolus protrusion and the the black arrow shows the astrocyte.

Figure 6: Histology of mice spinal cord after treatment using hematoxylin-eosin staining in 1000x magnification

Discussion

The increasing blood glucose levels after considering diabetes affected mice's nerve function and showed hyperalgesia behaviour. Oxidative stress is believed to be the primary key to nerve damage and is associated with hyperalgesia in the early stage of neuropathy, called painful diabetic neuropathy. Glucose accumulation was known to easily across the Schwann cell through glucose transporter 3 (GLUT3). Increased glucose levels will accumulate pyruvate formation via several pathways and cause the TCA cycle to become high, stimulating the metabolism of anaerobes and producing lactate products (Pang *et al.,* 2020). This lactate will allow entry into the axon via the monocarboxylate transporter (MCT). Glucose can also enter the axon directly via GLUT3 at the node Ranvier. The high lactate influx causes TCA dysfunction and oxidative phosphorylation, causing injury to the mitochondria, mitochondria rupture, and ROS establishment. The accumulation of acylcarnitine will trigger extracellular calcium entry into the axons. Intracellular calcium into the mitochondria will alter the mitochondrial charge and induce apoptosis, which is responsible for nerve dysfunction in DN (Feldman *et al.,* 2019).

Increased formation of ROS on PDN activates Transient Vanilloid Protein Receptor 1 (TRPV1), which is spread on peripheral nerve fibers as in the skin, dorsal root ganglia (DRG) as well as central nerve fibers such as the spinal cord (Pabbidi *et al.,* 2008). Activation of these receptors causes the entry of Ca2+ in the peripheral nerve fibers while depolarisation occurs (Luongo *et al., 2012*). Subsequently, ROS activates the NR2B subunit N-methyl-D-aspartate (NMDA) receptor of the spinal cord. This condition results in hyperalgesia and allodynia (Zhuo, 2013). NMDA receptor expression was also increased in the spinal cord in diabetic neuropathy (Bai *et al.,* 2014; Fajrin et al., 2020).

The red ginger extract and fraction had the same phytochemical substances as alkaloid, flavonoid, saponin, terpenoid, steroid, and polyphenol. Moreover, red ginger has many bioactive compounds, such as shogaol and gingerol, which are responsible for their activity. However, the in vivo test showed different results. The main reason is the differences in the type and amount of chemical compounds extracted. Our previous finding showed that ethanol ginger extract contained 6-shogaol (Fajrin *et al.,* 2020). However, this research is limited in that we did not determine the 6-shogaol concentration in the extract and its fraction of red ginger. The latest study (Haroen *et al.,* 2024) showed that ethyl acetate fraction contained triterpenoid and saponin. The ethyl acetate fraction showed higher total phenolic and flavonoid compounds than the methanol extract.

Based on our results, ethyl acetate fraction activity showed the same action as metformin, an oral antidiabetic drug, to reduce blood glucose levels in diabetic mice. Decreased blood glucose levels were significantly associated with pain relief in the PDN state. However, our results showed that the ethyl acetate fraction was better than metformin in minimising hyperalgesia. This phenomenon is probably due to the ethyl acetate fraction's pain-reducing activity because of its ability to lower blood glucose levels and antioxidant activities. Interestingly, a previous study found that 6-shogaol in ginger could reduce TRPV1 and NMDAR2B expression in the spinal cord of mice with painful diabetic neuropathy (Fajrin *et al.,* 2020). The reduced number of NMDAR2B was related to improving pain behaviour in PDN.

Conclusion

The ethyl acetate fraction of red ginger at 400 mg/kg BW decreased blood glucose levels as much as metformin. However, its effect on reducing hyperalgesia was better than metformin's. It was shown that the ethyl acetate fraction improved hyperalgesia in painful diabetic neuropathy not only because it maintained blood glucose levels.

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