

EXPERIMENTAL STUDY

Red sorrel (*Hibiscus Sabdariffa*) prevents the ethanol-induced deficits of Purkinje cells in the cerebellum

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Abstract: *Objectives:* The present study is aimed at investigating the possible protective effects of *H. sabdariffa* on ethanol-elicited deficits of motor coordination and estimated total number of the Purkinje cells of the cerebellums of adolescent male Wistar rats.

Methods: Forty male Wistar rats aged 21 days were divided into five groups. Na/wtr group was given water orally and injected with normal saline intra peritoneally (ip). Eth/wtr group was given water orally and ethanol (ip). Another three experimental groups (Eth/Hsab) were given different dosages of *H. sabdariffa* and ethanol (ip). All groups were treated intermittently for the total period of treatment of two weeks. The motor coordination of rats was tested prior and subsequent to the treatments. The rats were euthanized, and their cerebellums were examined. The total number of Purkinje cells was estimated using physical fractionator method.

Results: Upon revolving drum test, the number of falls of rats increased following ethanol treatment. There was no significant difference between the total number of falls prior and subsequent to treatment in all Eth/Hsab groups. The estimated total number of Purkinje cells in Eth/Hsab groups was higher than in Eth/wtr group.

Conclusion: *H. sabdariffa* may prevent the ethanol-induced deficits of motor coordination and estimated total number of Purkinje cells of the cerebellums in adolescent rats (Tab. 3, Fig. 1, Ref. 42). Text in PDF www.elis.sk.
Key words: alcohol, antioxidant, rotarod.

Introduction

Epidemiological studies have revealed that males drink more ethanol than females (1). Particularly adolescents show drinking behavioural patterns which cause profound public health concern (2). Immediate or late effects of tragic fatal injuries (such as traffic accidents, falls, drowning, etc.) due to early age ethanol consumptions are commonly observed. Male adolescents also more frequently drink to intoxication compared to their female counterparts (1), and hence are more vulnerable to detrimental consequences of ethanol. Such consequences may arise from the ethanol-induced dysfunction of the central nervous system, including the cerebellum. Chronic ethanol intoxication may bring about motor coordination and balance disturbance (3, 4), structural (5–9) and biochemical alterations in the cerebellum of both human subjects and animal models. Pascual et al (10) reported that intermittent intra-peritoneal injection of ethanol in adolescent rats that mimicked intermittent binge ethanol consumption frequently occurs amongst adolescents, significantly increased the apoptosis of brain cells, including the cerebellar neurons. In addition, this

intermittent ethanol administration caused long-lasting behavioural dysfunctions, including the deficits of motor coordination as examined in rotarod tests.

It is believed that the deleterious effects of ethanol on tissues result from by-products of ethanol metabolism, including acetaldehyde, acetate, and reactive oxygen species (ROS). These harmful compounds are mainly produced through oxidative pathways which involve aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase enzymes. In the brain, oxidation of ethanol by CYP2E1 produces ROS including hydroxyethyl, superoxide anion, and hydroxyl radicals, which eventually leads to the damage of nervous tissue (11, 12).

Hibiscus sabdariffa L. (Malvaceae) which is popularly known as roselle or red sorrel has been used as both folk beverages and traditional medicine in many regions of the world. The plants are widely grown in Central and West Africa, South Asia, South East Asia, Central America, and other regions (13, 14). People use the flower calyces of *H. sabdariffa* as a remedy for various types of disorders (13, 15). Indeed, several studies indicated that *H. sabdariffa* L. might potentially have beneficial effects in the treatment of hypertension (16), fever, liver disorders (13, 17), hepatic inflammation (18), diabetic nephropathy (19, 20), trypanosomiasis (21), obesity (22), diarrhoea (23), abscess (15), etc. Such effects might be exerted through different molecular pathways, one of which is through anti-oxidant defence mechanism (17). To our knowledge, not many studies have been done to explore the effects of *H. sabdariffa* to ethanol induced-oxidative damage on

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nervous system. The present study aims at investigating the possible protective effects of *H. sabdariffa* against ethanol-induced cell loss, particularly that of Purkinje cells of the cerebellum, and motor coordination deficits, using adolescent male Wistar rats as models.

Materials and methods

Animals

Forty male Wistar rats aged 21 days were used in this study. Rats are considered 'adolescent' approximately at the age of 30–42 days (24). The experimental procedures of the present study were carried out mostly during this adolescent period as described in a previous study (10). The rats were obtained from the Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada. The experimental procedure and animal handling was approved by Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada (approval number: KE/FK/283/EC).

The rats were divided into five groups. Na/wtr (negative control) group was given water orally and injected with normal saline (NaCl 0.9 %) intra peritoneally. Eth/wtr (positive control) group was given water orally and ethanol 3 g/kg body weight (ip) (10). Eth/Hsab 0.75, Eth/Hsab 1.5, and Eth/Hsab 3 groups were orally given *H. sabdariffa* solutions with different concentration (0.75 g; 1.5 g; and 3 g/200 ml of water, respectively). These groups were treated with the same amount and route of administration of ethanol as the Eth/wtr group. *Hibiscus* solutions were given half an hour prior to the injection of ethanol. All groups were treated according to their prescribed regimen every other two days for two weeks (10) following a three-day period of adaptation. Hence the treatments were given on post-natal days 25, 26, 29, 30, 33, 34, 37 and 38 (10). This pattern of treatment imitates the intermittent type of binge drinking which typically occurs in adolescents (25).

H. sabdariffa solutions were prepared fresh on the day of treatment by boiling three cups of 200 ml of water containing 0.75 g, 1.5 g, and 3 g calyces of *Hibiscus* tea, respectively, for 10 minutes. The dosage and preparation of *H. sabdariffa* solutions were determined according to commonly recommended use of the tea for a person (i.e. 1.5 g of *H. sabdariffa* calyces dissolved in a cup of water) (26). Solutions with a half (0.75 g) and a double (3 g) amount of this standard dosage were also prepared in order to examine the effects of *H. sabdariffa* at lower and higher dosages. The volumes of *Hibiscus* tea given to the rats were calculated proportionally to their body weights, with an assumption that 200 ml of *Hibiscus* tea is sufficient for a person with a body weight of 70 kg. All rats were fed with rat pellets (corn starch 1875 kcal, rice starch 375 kcal, egg white 400 kcal, sucrose 240 kcal, corn oil 630 kcal, agar starch 100 kcal, vitamin B 12, and minerals) and allowed to obtain water *ad libitum*.

Revolving drum test

The protocol of the revolving drum test referred to those described in previous studies (27, 28) with a slight modification. The experimental apparatus consisted of parallel cylinders with a diameter of 7 cm and running surfaces with the width of 8.5 cm each cylinder. Two side-walls were attached to the boundary of

each cylinder, with a diameter of 50 cm. The width of running surfaces allowed the rats to maintain the balance of their bodies; while at the same time it did not permit the rats to turn their body around easily to face the opposite direction. A pile of clothes was placed below the revolving drum apparatus, thus when rats fell off the cylinders they landed softly on these clothes.

The rat was placed on top of the running surface of the stationary revolving drum. It was allowed to stay there for 1 minute to familiarise with the tool, after which the rat was taken off the running surface. The revolving drum was turned on to rotate at 16 rotations per minute. The rat was returned to the top of the running surface of the drum, facing the side opposite to the direction of rotation of the revolving drum. The rat has to walk forward in order to maintain its position on the revolving drum. A stopwatch was started at the time when the rat was put on top of the running surface. The number of falls of the rat during three consecutive minutes was recorded. The stopwatch was stopped every time the rat fell, and started again once the rat was returned to the running surface. This procedure was carried out twice, i.e. prior (on post-natal day 24) and subsequent (on post-natal day 40) to the treatment.

Tissue preparation

All rats were euthanized on the next day (post-natal day 41) following the motor coordination test. They were anaesthetised with Ketalar (50 mg/ml ketamine hydrochloride; 0.1 mg/ml phemeral chloride; PT Kalbe Farma, Jakarta, Indonesia). Once the hind limb withdrawal reflexes of the rats disappeared, trans-cardiac perfusion was carried out through the left ventricle. Isotonic saline was infused for the first few minutes, followed with a fixative consisting of approximately 250 ml of 4 % formaldehyde. The cerebella of the rats were removed, weighed, and immersed in the same fixative mixture for further 96 hours at 5 °C in refrigerator.

The Estimation of the Total Number of the Cerebellar Purkinje Cells

The cerebellar tissues of rats were sliced according to the multi-stage fractionator technique as detailed elsewhere (29, 30). At stage 1, each cerebellar tissue was cut parasagittally into slices with a thickness of approximately 2 mm. A number between 1 and 2 was chosen by lottery. If number 1 was selected, every first slice was sampled for further fractionation. The sampling fraction of this first stage was 2 ($f_1 = 2$). At stage 2, the selected slices were cut into smaller slices with a thickness of about 2 mm. A number between 1 and 2 was again determined by lottery. The sampling fraction of this second stage was 2 ($f_2 = 2$). The above mentioned procedure was repeated at stage 3, while the sampling fraction of this stage was 2 ($f_3 = 2$).

The selected slices from stage 3 were then dehydrated in graded concentration of ethanol solution, cleared with toluene, and infiltrated with parafin. The tissue was subsequently embedded in parafin block. The tissue was cut using Sartorius Werke microtome with the section thickness of 6 µm. One of every 20 sections ($f_4 = 20$) was chosen by lottery, mounted on slides, and stained with toluidine blue. The sections were examined under Meiji Mega Mipa 400/0 light microscope with a x10 magnification of the ob-

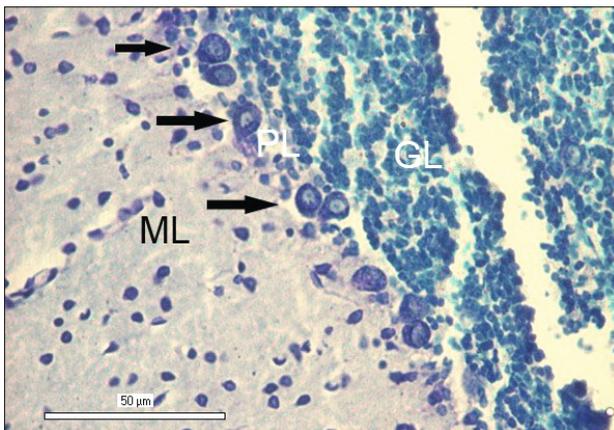


Fig. 1. The micrograph of the cerebellum with its three layers, including molecular layer (ML), Purkinje cell layer (PL), and granule cell layer (GL). The counting of the Purkinje cells was based on the presence of nucleoli (as exemplified by arrows).

jective lens. The Purkinje cells (n) were counted from these sections. The examination of the sections was carried out while the examiner was not aware of which groups the sections belonged to. Only Purkinje cells with nucleoli clearly visible under the microscope were counted (Fig. 1). The total number of Purkinje cells for each cerebellum (N) were subsequently calculated using the following formula (29, 30):

$$N = f_1 \times f_2 \times f_3 \times f_4 \times n$$

In order to increase the reliability of the results of Purkinje cells counting another examiner repeated the counting on several samples.

Statistical analyses

The raw data of the number of falls during revolving drum tests were transformed (31) using the formula:

$$X(t) = \text{square root}(x + 0.5)$$

x = number of falls recorded in each trial and X(t) = transformed value.

This transformation was necessary to avoid mathematical complications arising out of some values being zero (28).

The data of the number of falls in revolving drum tests, the body weights and the total number estimate of Purkinje cells were analysed using analysis of variance (ANOVA) procedure. Independent sample t-test was performed to determine the difference of the total number estimate of Purkinje cells between treatment groups. Independent sample t-tests assessed the equality of the means. Initially, the variances associated with the variable were assumed equal. The independent sample t-tests verified this. Paired t-test was carried out to determine the difference in the number of falls in revolving drum tests prior and subsequent to treatments within groups. Statistical analyses were performed using the Statistical Package for Social Sciences version 18.0 (SPSS Science, Chicago, IL).

Results

Table 1 presents data on the total number of falls in the revolving drum tests during the 3-minute test period before and after treatment, with a one-way ANOVA of these data. The analysis of variance showed that in Eth/wtr group there was a significantly higher total number of falls after treatment than before treatment. In contrast, there was no significant difference between the total number of falls prior and subsequent to treatment in all Eth/Hsab and Na/wtr groups.

The mean body weights of various treatment groups are shown in Table 2. Analysis of variance shows no difference in the body weights between groups before the treatment. However, there were significant reductions in body weights within the groups following the treatment.

Table 3 shows the weight of cerebellum and estimates of Purkinje cell number for all treatment groups. The cerebellar weight of Eth/wtr group was significantly lower than those of Na/wtr and all Eth/Hsab groups ($p < 0.05$). A comparison of estimates of the number of Purkinje cells in the three Eth/Hsab subgroups to both the Na/wtr and Eth/wtr groups - as reference estimates - using one-way ANOVA ("crude analyses") revealed that the number of Purkinje cells in these three treated groups was higher than that of Na/wtr and Eth/wtr groups. The adjusted model of the effects of *H. Sabdariffa* of various dosages on the Purkinje cells number (statistical analyses adjusted to cerebellar weights) also confirmed that there were significantly higher estimate of the number of Purkinje cells in the Eth/Hsab subgroups relative to both Na/wtr and Eth/wtr groups.

Tab. 1. Mean (\pm SEM) of the total number of falls using the revolving drum test in various treatment group.

Groups	Mean \pm SEM of the total number of falls		p*
	before treatment	after treatment	
Na/wtr	1.03 \pm 0.27	1.08 \pm 0.33	0.598
Eth/wtr	2.27 \pm 0.88	2.75 \pm 0.66	0.000
Eth/Hsab 0.75	1.59 \pm 0.47	1.61 \pm 0.42	1.000
Eth/Hsab 1.50	1.12 \pm 0.37	1.01 \pm 0.34	0.516
Eth/Hsab 3.00	1.14 \pm 0.70	1.03 \pm 0.47	0.351

Na/wtr: NaCl 0.9% (intraperitoneal/ip) + water (oral); Eth/wtr: ethanol (ip) + water (oral); Eth/Hsab 0.75: ethanol (ip) + *H. sabdariffa* 0.75 g/200 ml (oral); Eth/Hsab 1.50: ethanol (ip) + *H. sabdariffa* 1.50 g/200 ml (oral); Eth/Hsab 3.00: ethanol (ip) + *H. sabdariffa* 3.00 g/200 ml (oral) * p indicates whether total number of falls is different within the groups before and after treatment (one-way ANOVA)

Tab. 2. Mean (\pm SEM) of body weights (gram) of rats in various treatment groups.

Group	Number of rats	Body weight before treatment (gram)	Body weight after treatment (gram)*
Na/wtr	8	50.0 \pm 3.8	48.1 \pm 3.7
Eth/wtr	8	57.0 \pm 7.1	46.9 \pm 5.9
Eth/Hsab 0.75	8	37.9 \pm 4.6	35.6 \pm 4.2
Eth/Hsab 1.50	8	45.0 \pm 6.5	42.5 \pm 5.3
Eth/Hsab 3.00	8	55.0 \pm 3.8	46.3 \pm 5.2

Na/wtr: NaCl 0.9% (intraperitoneal/ip) + water (oral); Eth/wtr: ethanol (ip) + water (oral); Eth/Hsab 0.75: ethanol (ip) + *H. sabdariffa* 0.75 g/200 ml (oral); Eth/Hsab 1.50: ethanol (ip) + *H. sabdariffa* 1.50 g/200 ml (oral); Eth/Hsab 3.00: ethanol (ip) + *H. sabdariffa* 3.00 g/200 ml (oral) * $p < 0.001$ (One-way ANOVA indicates that there are differences in body weights prior and subsequent to treatment within groups)

Tab. 3. Mean (\pm SEM) cerebellar weight and Purkinje cell measures for the various treatment groups.

Groups	Cerebellar weight (mg)	Purkinje cell number (10^3)	p value		p value	
			Crude*	Adjusted†	Crude*	Adjusted†
Na/wtr	231.9 \pm 12.4	235.1 \pm 8.0	Ref	Ref	-	-
Eth/wtr	219.3 \pm 12.1	210.6 \pm 29.9	0.042	0.025	Ref	Ref
Eth/Hsab 0.75	231.2 \pm 9.0	256.9 \pm 5.3	0.000	0.000	0.001	0.000
Eth/Hsab 1.50	232.7 \pm 6.9	276.9 \pm 15.0	0.000	0.000	0.000	0.000
Eth/Hsab 3.00	235.8 \pm 12.9	421.8 \pm 33.8	0.000	0.000	0.000	0.000

Na/wtr: NaCl 0.9 % (intraperitoneal/ip) + water (oral); Eth/wtr: ethanol (ip) + water (oral); Eth/Hsab 0.75: ethanol (ip) + *H. sabdariffa* 0.75 g/200 ml (oral); Eth/Hsab 1.50: ethanol (ip) + *H. sabdariffa* 1.50 g/200 ml (oral); Eth/Hsab 3.00: ethanol (ip) + *H. sabdariffa* 3.00 g/200 ml (oral)* p indicate that Purkinje cells numbers are different between groups (one-way ANOVA); †p adjusted to cerebellar weight

Discussion

In the present study it has been found that intermittent administration of ethanol on rats throughout adolescent period caused deficits of the number of Purkinje cells of the cerebellum and motor coordination as examined in the revolving drum tests. These deficits were prevented by the administration of *H. sabdariffa*. The rats treated with ethanol and *H. sabdariffa* consistently showed higher estimated total number of Purkinje cells and better motor coordination performance compared to rats treated with ethanol only.

Studies on human subjects have reported pathological alterations of cerebellar structures, including a decrease in the number of Purkinje cells (7, 32), and decrease in the volume and density of Purkinje cells (5) due to chronic ethanol intoxication. Similarly, studies using animal models have shown various maladaptive responses of cerebellum to ethanol, such as increase in the densities of degenerating bodies in the Purkinje cells dendrites (6), alteration of terminal dendritic segments of Purkinje cells (8), and increase in apoptotic death of granule cells (9). It has been suggested that the mechanisms implicated in the ethanol-induced neurotoxicity of cerebellum may include excessive excitatory signalling, thiamine deficiency, glial cells dysfunctional alterations, neurotrophic factors changes, apoptosis, oxidative stress, and impaired ATP production due to mitochondrial damage (33), with oxidative stress being the major cause of neurodegeneration (34). Oxidative stress-induced neuronal death due to ethanol intoxication is mainly caused by the increase in NF- κ B proinflammatory transcription, the downstream pathway of which includes reactive oxygen species producing enzymes, and a decrease in CREB transcription. Hence, it is assumed that anti-oxidant agents that block both the decrease in proinflammatory cytokines and increase in CREB transcriptions may suppress neural degeneration due to ethanol (35).

A number of studies on the effects of *H. sabdariffa* on various disorders converge to a conclusion that *H. sabdariffa* may exert its beneficial effects through its anti-oxidant effects (13). Considerable literature reported that *H. sabdariffa* is composed of various phytochemical constituents, including phenolic compounds, anthocyanins, flavonoids, protocatechuic acid, vitamin C and carotenoid (19, 36). *H. sabdariffa* contains polyphenols, anthocyanins (including delphinidin-3-sambubioside, cyanidin-3-sambubioside, and delphinidin-3-glucose), and flavonoids, all of which may act as anti-oxidants (14, 36). The ascorbic acid content of *H. sabdariffa* (13) is higher than that of orange or mango (14). Ascorbic acid

or vitamin C is known to be important reactive oxygen species scavengers in the non-enzymatic pathway of anti-oxidant defence mechanism (37–39).

A recent review highlighted anthocyanins, a subgroup of flavonoids (which belongs to polyphenols) consisting of more than 500 natural pigments in parts of many plants, as one possibly excellent candidate of neuroprotective agent against ethanol toxicity (34). Many investigators reported that anthocyanins improved the oxidant status of brain following ethanol exposure, as indicated by lipid peroxidation, glutathione, and anti-oxidant enzymes levels. Among types of anthocyanins which cover glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and penutidin; cyanidin is considered to be most plentiful in parts of many plants and has potent anti-oxidant capacities. Cyanidin-3-glucoside (C3G), an example of cyanidin, with its metabolites, mainly protocatechuic acid (PCA) and aglycon cyanidin (Cy), are shown to be able to prevent H₂O₂-elicited ROS formation, anti-oxidant enzymes attenuation, and apoptotic progression in human neuronal-like cells SH-SY5Y (40). It is also thought that anthocyanins prevent the ethanol neurotoxicity via their anti-inflammatory activities (34). However, since the C3G content was found to be minor in *H. sabdariffa* (41), it remains undetermined whether major anthocyanins content in *H. sabdariffa* such as cyanidin-3-sambubioside can be as effective against ethanol as C3G in their anti-oxidant and anti-inflammatory actions. In rats model of hepatic inflammation, it has been shown that *H. sabdariffa* polyphenol extract prevented NF- κ B translocation via its suppression on JNK and p38 MAPK protein activation, and hence inhibited the expression of COX-2 and iNOS proinflammatory proteins (18). Nevertheless, whether or not *H. sabdariffa* may actually modulate anti-oxidant enzymes, scavenge ROS, reduce oxidative damage, or modify transcriptional factors such as NF- κ B and CREB, in the ethanol-intoxicated rats' cerebella, warrants further studies.

The results of the statistical analyses have shown that the number of Purkinje cells and the weight of cerebellum in the three groups treated with ethanol and *H. sabdariffa* were higher than in both negative (Na/wtr) and positive (Eth/wtr) controls. As it is well-known that Purkinje cells proliferate only during pre-natal life, this raises a question as to whether *H. sabdariffa* actually stimulates abnormal proliferation of Purkinje cells (42). However, morphological examinations of the specimens of the present study did not confirm the presence of any immature, atypical, or pleiomorphic forms of cells which usually characterize malignant

growth of cells. In addition, *H. sabdariffa* is generally considered to have anti-cancer effects (13); hence it seems unlikely that it causes abnormal proliferation of Purkinje cells. The precise cause of this finding, however, is uncertain at present.

In summary, our study demonstrated that intermittent intraperitoneal ethanol administration brought about the deficits of both the motor coordination and the estimates of the total number of cerebellar Purkinje cells in male pubescent Wistar rats; and *H. sabdariffa* reversed this deterioration. Although suggestions are put forward that *H. sabdariffa* may exert its potentially beneficial effects through its anti-oxidant and anti-inflammatory mechanism, it remains uncertain as to through which molecular cascade it actually works. Thus, further studies are required to reveal such mechanism.

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