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The Effect of Different Extracts of Beetroots as Antioxidant and Anti-Anaemia On Phenylhydrazine-Induced Rats

Original Paper

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Abstract **Aim:** evaluate antioxidant and anti-anaemia activity of dichloromethane, hydroethanolic, and alkaloids-free hydroethanolic extracts of beetroot (*Beta vulgaris* (L.) subsp. *vulgaris*) on phenylhydrazine-induced rats. **Methods:** Male rats were divided into five groups: normal control group, negative control group, dichloromethane extract group, hydroethanolic extract group, and alkaloids-free hydroethanolic extract group. All groups were induced with phenylhydrazine (30 mg.Kg^{-1} BW) for three days, except for the normal control group. After induction, each treatment group received each extract (200 mg.Kg^{-1} BW) for 21 days. The haematology parameters (haemoglobin levels, the number of erythrocytes, and haematocrit levels) were measured using Haematology Analyzer, and the antioxidant activity was measured through MDA level parameters in rats. Data were analysed using one-way ANOVA and then continued with the Tukey test. **Results:** The results showed that the hydroethanolic extract of beetroot increased the percentage of erythrocytes (33.5%), haemoglobin (25%), and haematocrit (24.4%) to the negative control group, which was comparable to the normal control group ($p > 0.05$). In addition, the best antioxidant activity was shown in the hydroethanolic extract of beetroot, which is comparable to the normal group ($p > 0.05$). **Conclusion:** The beetroot hydroethanolic crude extract could be potentially produced in a natural pharmaceutical product as a beneficial resource within anti-anaemia and antioxidant activities.

Keywords *Anti-anaemia – Antioxidant – Beta vulgaris – Beetroot – Phenylhydrazine*

INTRODUCTION

Anaemia is a condition when the number of red blood cells or haemoglobin concentration in the body is lower than normal. Anaemia is a problem that can occur at any age. WHO estimates that 42% of children less than five years of age and 40% of pregnant women worldwide are anaemic. In Indonesia, anaemia's prevalence in women of reproductive age is 27.85% and in children under five years is 36.78% (World Health Organization, 2020). The main cause of anaemia is lack of nutrition (inadequate supply of iron), drug toxicity, blood loss, genetic, or pathological diseases (Chaddha & Mittal, 2016).

Anaemia due to iron deficiency has a significant impact on human health. However, this rarely directly causes death. Erythrocytes require large amounts of iron to produce heme and haemoglobin. Iron deficiency results in decreased formation of red blood cells, which causes anaemia. Based on the research by Jaiswal et al. (2014), the ethanol extract of beetroot contains high iron, folic acid, and vitamin C. These contents are presumed related to the extract hematinic effect on phenylhydrazine-induced rats. Beetroot also contains iron, magnesium, potassium, manganese, copper, sodium, calcium, zinc (Odoh & Okoro, 2013), and bioactive compounds such as betalains, alkaloids, flavonoids, terpenoids, steroids, glycosides, and saponins (Hadipour et al., 2020). These contents have benefits in improving the haematopoiesis process.

Flavonoids are polyphenolic secondary metabolic compounds. These compounds have a protective effect against membranes lipo-peroxidative damage caused by free radicals (Nijveldt et al., 2001). Flavonoid-rich extracts effectively overcome the effects of phenylhydrazine induced malondialdehyde (MDA) damage to red blood cells membrane glycerol back-bone and peroxidation of phospholipids (Ologundudu et al., 2009; Chaddha & Mittal,

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2016). On the other hand, tannins were centred on tannic acid, and other hydrolysable tannins are responsible for reductions in feed intake related to the antinutritional effects. It could bind with protein and red blood cells (Chung et al., 1998), affecting haemoglobin levels. Tannins could also inhibit iron absorption. Therefore, most pregnant women who consume the source of tannins like tea go through anaemia (Shah et al., 2020), and it is recommended that tannin-rich foods are not consumed together with meals (Santos-Buelga & Scalbert, 2000). Alkaloids from *Datura stramonium sp.* can reduce levels of erythrocytes, haemoglobin, and haematocrit by interfering with the process of erythropoiesis and causing the process of destruction of blood cells (Benouadah et al., 2016). Quinoline group alkaloids are used as antimalarial drugs by providing a binding effect to heme, a product related to haemoglobin, making the heme-quinoline conjugate toxic and interferes with haemoglobin activity (Heinrich et al., 2012).

Extracting solvent is one of the factors that influence the extraction results. The solvent's polarity is an aspect that underlies the selection of the extracting solvent (Tiwari et al., 2011). The hydroalcoholic extract contains phenolics, flavonoids, alkaloids, carbohydrates, glycosides, and tannins in beetroot (Ahmad et al., 2013). Alkaloids in plant material can be removed by acidification using citric acid. The residue extracted with 70% ethanol still contains flavonoid compounds as in the crude extract (Widiyanti et al., 2016). Meanwhile, tannins are slightly soluble in dichloromethane. This study aims to evaluate the effects of anti-anaemia and antioxidants in different extracts include dichloromethane extract (DEB), hydroethanolic extract (HEB), and alkaloids-free hydroethanolic extract (AFHEB) of beetroots. The difference in chemical composition contained in each extract obtained is expected to indicate differences in anti-anaemia and antioxidant activities. Thus, this study can be used as a reference to optimize the development of beetroots extract as a supplement to overcome anaemia conditions.

MATERIALS AND METHODS

Collection of Plant Material

Fresh beetroots were obtained from Indonesian Peasant Stores. The plant grows on a farm in Lembang, West Java, Indonesia. It was harvested at the age of 2 to 3 months beet plants. The plant was authenticated in Herbarium Bogoriense, Biology Research Center, Indonesian Institute of Sciences, Cibinong, Indonesia.

Preparation of The Extracts

The plant material was air-dried at room temperature and then grounded to powder. Afterward, the dried powder of beetroot was divided into two parts. The first part, namely releasing-alkaloid beetroot powder. The dried powder (820.0 g) was added with a weak citric acid solution to the formed water-soluble alkaloids salt (Widiyanti et al., 2016). Releasing-alkaloid beetroot powder was then extracted using hydroethanolic (ethanol: water, 70:30) for 3´24 h in a macerator. The filtrate obtained was labelled as an alkaloids-free hydroethanolic extract of beetroot (AFHEB)). Whereas the second part, beetroot powder (820.0 g) was extracted separately using hydroethanolic or dichloromethane in the same way as the previous procedure. The filtrates were then labelled as hydroethanolic extract (HEB) and dichloromethane extract (DEB) of beetroots, respectively. Each filtrate was evaporated using a vacuum rotary evaporator N-1200 BS series (EYELA, Shanghai, China) at 50°C. The percentage of yield of each extract was calculated. Each extract's physicochemical characteristics, such as water content, total-ash content, and loss on drying, were performed according to the Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008) and WHO guidelines (World Health Organization, 2011).

Phytochemical screening of the extracts

Secondary metabolites such as alkaloid, phenolic, flavonoid, triterpenoids, steroids, and saponin in the extracts were identified qualitatively. The chemical reagents used were Dragendorff, Mayer, and Bouchardat reagents for alkaloids detection; $FeCl_3$ reagent for phenolics detection, $AlCl_3$ reagent for flavonoids detection; Liebermann-Burchard reagent for triterpenoids/steroids detection and gelatine reagent for tannins detection (Hanani, 2015; Harborne, 1987; Ministry of Health Republic of Indonesia, 2008).

Determination of Total Flavonoids Content (TFC)

Total flavonoid content was evaluated using the colorimetric method described by Chang et al. (2002). Briefly, one mL of extract (10000 ppm in methanol) was added with 3 mL of methanol, 0.2 mL of $AlCl_3$, and 0.2 mL of sodium acetate 1 M and distilled water up to 10 mL. The solution was mixed and then incubated for 30 minutes. The absorbance was measured at 415 nm against methanol blank with a Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). Quercetin was used as the standard with concentration ranging from 5–19 ppm for the construction of a calibration curve, and the concentrations are expressed as quercetin equivalents (mg QE.g^{-1} extract). The test was performed in triplicate, and the results were expressed as mean ± SD.

Determination of Total Iron (Fe) Content

Extract (0.5 g) was put into the vessel, then added HNO_3 and H_2O_2 30%. Then the destruction process is carried out and allowed to stand at room temperature. After this, the solution was transferred to a 50.0 mL flask and distilled water was

added to the boundary mark. The solution was put into a test tube, and the test solution is measured using ICP-OES. This test was conducted at the Regional Health Laboratory, DKI Jakarta, Indonesia.

Preparation of Animals

The Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the experimental design, with ethical approval number: KET-552/UN2.F1/ETIK/PPM.00.02/2019. The design used includes Randomized Design. Twenty-five male rats aged 2–3 months, 150–250 g, were obtained from Research Animal Breeder, Bekasi, Indonesia. The animals are divided into five groups, where each group consisted of four animals. Before treatment, the animals were acclimatized for seven days. At this stage, the animals were given standard drinks and feed.

Experimental Design

Animals are grouped into (1) Normal Control Group: No treatment; (2) Negative Control Group: received Na-CMC 0.5%; (3) Extract Group I: received DEB; (4) Extract Group II: received HEB; (5) Extract Group III: received AFHEB, at a dose of 200 mg.Kg^{-1} BW. All groups (except the normal control group) were induced with phenylhydrazine hydrochloride (Fisher Scientific Company, New Jersey, USA) (30 mg.Kg^{-1} BW i.p once a day for three days) to induce anaemic conditions, then were given the test substance once a day orally for 21 days. On the 22nd d the animals were injected with ketamine intramuscularly at a dose of 40 mg.Kg^{-1} BW. The blood sample was taken through the orbital sinus and collected in the EDTA tube.

Anti-anaemia Activity Assay

The haematology examinations (haemoglobin levels, the number of erythrocytes, and haematocrit levels) of blood serum were performed at Primate Research Center, Bogor Agricultural University, Bogor, Indonesia using Automated Haematology Analyzer MEK-6450K (Nihon Kohden, Tomioka, Japan).

Antioxidant Activity Assay

One mL blood serum was put in the test tube, 0.5 mL TCA 20% was added to it, then centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected. One mL of supernatant was added with 1 mL of TBA 0.67% into a tube, then placed in a water bath at a temperature of 95–100ºC for 10 minutes, then cooled with running water. Tetraethoxypropane (TEP) was used as standard with a concentration of 0.6–2.6 nmol.mL^{-1}. The absorbance was measured at 532 nm using a Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan).

Table 1. The Result of Beetroot Extraction.

Type of Extract	Characteristics		
	Percentage of Yield (%, w/w)	Loss of Drying (%, w/w)	Ash Content (%, w/w)
DEB	1.92	2.48	2.16
HEB	34.52	9.89	9.66
AFHEB	24.55	13.27	19.75

Note: DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Table 2. Phytochemical Screening Results of Beetroot Extracts.

Compounds	DEB	HEB	AFHEB
Alkaloids	+	+	-
Flavonoids	+	+	+
Tannin	-	+	+
Phenolic	+	+	+
Saponin	-	+	+
Steroids	-	-	-
Triterpenoids	-	-	-

Note: (+) = detected; (-) = not detected, DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Data Analysis

The data were presented in mean and standard deviation. Kruskal Wallis and Mann Whitney were used to evaluate statistical significance.

RESULTS

Characteristics of the beetroot extracts include a percentage of yield, loss of drying, and ash content shown in **Table 1.** Based on **Table 1**, HEB contains more chemical compounds than other extracts (AFHEB > DEB).

Phytochemical screening aims to identify the presence of chemical substances in the sample qualitatively. Based on the results in **Table 2**, it can be concluded that phenolic, flavonoids, tannins, alkaloids, and saponin compounds are present in extracts. Meanwhile, saponins compounds are only found in HEB. HEB contains high levels of flavonoids and iron (Fe) compared to other extracts (AFHEB > DEB) (**Table 3**).

Based on **Table 4,** the erythrocyte, haemoglobin, and haematocrit levels of beetroot extract groups showed a significant difference compared to the negative control group ($p < 0.05$). HEB group showed the best result, where all the blood parameters comparable to normal control group. Beetroot extract also showed activity as an antioxidant,

shown by reduced MDA levels (**Figure 1**). The best antioxidant activity also showed by HEB, comparable to the normal control ($p = 0.352 > 0.05$).

DISCUSSION

The selection of the suitable organic solvent affects the success in solid-liquid extraction. It is usually done based on the dielectric constant (ε) characteristic of the extracting solvent, which is closely related to its polarity index (Katritzky et al., 2004). Water has a dielectric constant of 78.30, while ethanol is 24.30 (Khoddami et al., 2013) and dichloromethane is 9.1 (Saeker et al., 2006). The polarity index values for water, ethanol, and dichloromethane were 9.1, 5.2, and 3.7, respectively (Snyder, 1974). It shows that water is more polar than the other two solvents (water> ethanol> dichloromethane). The principle of 'like dissolved like' is defined as the condition where a phytochemical substance is dissolved in a similar polarity solvent (Wakeel et al., 2019). In other words, a polar solvent will be able to dissolve a polar substance, and a nonpolar solvent can dissolve a nonpolar substance. Some solvents might have similar polarity index values but could attract compounds of different quantities (Khoddami et al., 2013). For example, in the use of absolute ethanol extracting solvents or ethanol-aqueous (with variations of ethanol concentration in them). This difference can occur due to the differences in each solvent's ability to form chemical bonds with the bioactive metabolites contained in the plant matrix (Huaman-Castilla et al., 2019). Other things that can also affect plant chemical constituents' acquisition are the bioactive compounds' chemical structure, the extraction time, and the temperature used (Khoddami et al., 2013). The difference in the type and polarity of the extracting solvent can cause differences in the quality, quantity, toxicity, bioactivity, and safety of the extract produced (Eloff, 1998).

In this study, the extracted beetroot activity was tested using a variety of solvents as anti-anaemia and antioxidants. A previous study by Jaiswal et al. (2014) reported that ethanol extract of beetroot (200 mg.Kg^{-1} BW) extracted in a Soxhlet apparatus effectively increases the levels of haemoglobin and erythrocytes. Vitamins (such as Vitamin C and folic acid) and minerals (one of them is iron) in beetroots are active ingredients responsible for these activities. Since some of the compounds, such as tannins and alkaloids could interfere with the hematopoietic activity, in this research, we try to optimize the result by evaluating the anti-anaemic activity in dichloromethane extract (DEB), hydroethanolic extract (HEB), and alkaloids-free hydroethanolic extract (AFHEB) of beetroots.

The yields of different extracts of beetroots increased in the following order: DEB < AFHEB < HEB. The highest yield was found in HEB (34.52%) followed by AFHEB (24.55%) > DEB (1.92%). It shows that polar compounds dominate in beetroots. It might also contain non-secondary metabolite polar compounds that also dissolve during the extraction process, such as carbohydrates (which are composed of fibres and sugar) (Neha et al., 2018), proteins, essential and non-essential amino acids, and other compounds (Hadipour et al., 2020). Beetroot contains bioactive compounds such as phenolics (epicatechin, catechin hydrate, vanillic acid, p-coumaric acid, protocatechuic, caffeic acid, syringic acid, proline, dehydro vomifoliol, 4-hydroxybenzoic acid, chlorogenic acid, and ferulic acid, etc.), flavonoids (betagarin, betavulgarin, cochliophilin A, dihydroisorhamnetinas, 2,5-dihydroxy 6,7-methylenedioxyisoflavone, 3,5 dihydroxy-6,7-methylenedioxyflavanone, 5-hydroxy-6,7 methylenedioxyflavone, rutin, quercetin, and 40-hydroxy-5-methoxy-6,7- methylenedioxy flavanone), saponins (betavulgarosides (I, II, III, IV, V, VI, VII, VIII, IX, X), hederagenin, akebonoic acid and gypsogenin), betalains (betacyanin

Table 3. Flavonoids and Iron Contents of Beetroot Extracts.

Type of Extract	Iron (Fe) content (mg.Kg^{-1})	Flavonoids Total Content (mgQE.g^{-1})
DEB	6.52	1.66
HEB	20.43	8.35
AFHEB	11.30	6.07

Note: DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Table 4. The Anti-anaemia Activity of Beetroot Extracts.

Groups	Blood Parameters		
	Erythrocytes (x10^{6}µL)	Haemoglobin (g.dL^{-1})	Haematocrit (%)
Normal Control	7.00 ± 0.78^a	16.78 ± 0.37^a	50.55 ± 1.22^a
Negative Control	4.73 ± 0.95^b	12.48 ± 0.48^b	37.82 ± 1.49^b
DEB	6.31 ± 0.52^a	14.94 ± 0.46^c	45.18 ± 1.48^c
HEB	7.12 ± 0.61^a	16.66 ± 0.39^a	50.04 ± 1.22^a
AFHEB	6.96 ± 0.55^a	15.46 ± 0.31^c	46.54 ± 0.86^c
Normal Level Value	5.0–12.0	11.1–18.0	36.0–52.0

Note: Different letters in the same column show significant differences ($p < 0.05$). DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

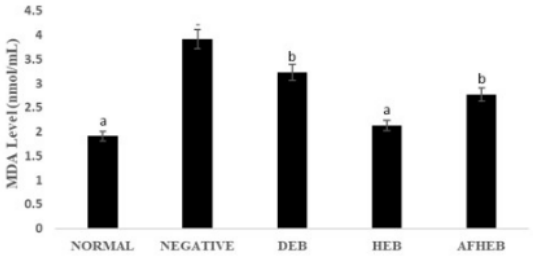

Figure 1. MDA levels after treatment for 21 days.

and betaxanthin), alkaloids (calystegine B1, calystegine B2, calystegine C1, calystegine B3 and ipomine), triterpenoids/ steroids (beta-amyrin acetate, boehmerylacetate and friedelin), volatile compounds (pyridine and 4-picolene), and so on (Hadipour et al., 2020).

Tiwari et al. (2011) explained that 70% ethanol as an extraction solvent was able to dissolve phenolic compounds (include flavonoids) better than absolute ethanol. Ethanol dissolves polyphenolic compounds, tannins, flavonoids (flavonols), triterpenoids, steroids, and alkaloids. In comparison, DEB showed the least number of phytochemicals. Dichloromethane (CH_2Cl_2) is a solvent with a medium polarity level. The polarity of dichloromethane due to its electronegative Cl atoms (Houghton & Raman, 1998). Dichloromethane is specially used for the selective extraction of only terpenoids (Tiwari et al., 2011) but is still able to dissolve alkaloids and aglycones (include flavonoids) (Houghton & Raman, 1998). Dichloromethane has a low ability to dissolve tannin compounds. Therefore, in the phytochemical screening evaluation (Table 1), tannins were not detected in DEB. Meanwhile, the AFHEB contains tannin but no alkaloids. Both total flavonoid and iron levels in the AFHEB are lower than in the HEB (**Table 3**). In this research, the procedure for removing alkaloids from the dried powder of beetroot not only results in the loss of alkaloids but also other compounds including flavonoids, iron or might be the pigment compounds of beetroot. Betalains are 'chromoalkaloids' found in plants in the order Caryophyllales (except in the Caryophyllaceae and Molluginaceae families) (Wink, 2010). Betalains are a natural nitrogen-containing pigment compounds that have two structural groups, viz. the red-violet betacyanins (betanin, prebetanin, isobetanin and neobetanin) and the yellow to orange betaxanthins (vulgaxanthin-1, vulaxanthin-2, and indicaxanthin) (Hadipour et al., 2020). These compounds are indole-derived (Ninfali et al., 2017; de Oliveira et al., 2020) that can be water or alcohol-extracted and stable at pH from 2 to 6 (Azeredo, 2009). Halwani et al. (2018) reported that red pigment compounds (betanine and vulgaxanthin-1) were found in high amounts in water extract and citric acid extract of beetroot in low pH conditions. The presence of this compound can result in a false positive reaction in alkaloid identification using Dragendorff and Mayer precipitated reagents. It is known that these two reagents can also react with nitrogen-containing compounds other than alkaloids (Evans, 2009). We recommend that when identifying alkaloids with these reagents, the test solution must be free from compounds with nitrogen atoms (such as proteins, amino acids, or betalains).

The results showed that phenylhydrazine induction for three days in the negative control group reduced the number of erythrocytes by 32.4%. Phenylhydrazine induces oxyhaemoglobin into methaemoglobin and produces hydrogen peroxide. This hydrogen peroxide causes lipid peroxidation in the red blood cell membrane resulting in lysis of the blood cells, leads to the condition known as haemolytic anaemia (Singh et al., 2014). In this study, the increase in Fe levels and antioxidant activity was in line with the increase in anti-anaemia activity. The DEB and AFHEB showed less potential activities antioxidant (**Figure 1.**) and lower Fe content than the HEB. The same trend was shown in the anti-anaemia activity (**Table 4**). Iron levels influence haemoglobin production in the body. Iron is an essential component in the formation of heme molecules. The body needs nearly 30 mg of iron each day in the erythropoiesis process (the formation of erythrocytes in bone marrow), and only 1–2 mg can be absorbed (Lesjak & Srai, 2019). As the flavonoid-rich plant, administration of beetroot prevents further oxidation on the red blood cells, therefore prevent further damage to the cells (Chaddha & Mittal, 2016). The pigment in beetroot also acts as an antioxidant. Research showed that beetroot betalains could reduce MDA levels in male mice (Clifford et al., 2015). Red pigment betacyanin is also a powerful antioxidant and protects against several types of cancer (Neha et al., 2018). Betacyanins have better antiradical activity in vitro due to their hydroxyl group position than betaxanthin (Azeredo, 2009).

Polyphenols (including phenolic acids, flavonoids, lignans, and stilbenes) are a source of natural antioxidants that can be obtained from medicinal plants. Flavonoids (including flavonols, flavanones, catechins, flavones, anthocyanidins, and isoflavonoids) are found in vegetables and fruits. Flavonoids have also been studied to improve haematological parameters, increase iron levels in the spleen tissue, and ferroportin expression in iron deficiency anaemia (Yazhar et al., 2017). In addition, secondary metabolites such as saponins, phenolics, and glycosides might also be responsible for the acclaimed anti-anaemic potential of plants used in traditional medicine (Gbadamosi et al., 2012).

This research shows that the content of tannins and alkaloids in the beetroot ethanol extract does not affect its activity as anti-anaemia. However, it is crucial to quantitatively measure the presence of tannins and alkaloids in each extract to support definite conclusions regarding their connection to anti-anaemia activity in beetroots.

CONCLUSION

The hydroethanolic extract of beetroot has good anti-anaemia and antioxidant activities. Based on this study, the chemical constituents of the extract plays an important role in its pharmacological activity. The appropriate extraction methods of beetroot can produce an extract as a source of raw materials with maximum chemical content to achieve the intended pharmacological activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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