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The Aquaeous Extract of Root Nodules *Vigna radiata* (rnVr) which Inoculated by Rhizobium as an Orally Available Anemia Therapeutic Candidate

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Abstract

The extract of root nodules *Vigna radiata* (rnVr) which inoculated by Rhizobium is considered beneficial as an orally available anemia therapeutic candidate, because it contain the leghemoglobin. The positive control mice (group I) were fed with the high nutrient pellet.The twelve mice (*Mus musculus*) was treated with the "aking rice pellet" that representing the low nutrient food for 21 days until they suffered anemia. Then, the anemia mice were treated orally with rnVr in different concentration groups:II. 0% III.33%; IV.67% and V.100%, respectively and fed with the "aking rice pellet". After 14 days, the blood mice were collected from orbital sinus. The hemoglobin (Hb) concentration were analyzed by spectrophotometry and blood plasma profile protein were analyzed with electrophoresis (SDS-PAGE). All anemia mice that treated with rnVr showed the increasing of Hb and group that treated with 100% extract of rnVr could reach a normal Hb value, raising from 9.85 to 12.68 g/dL. There were observed the proteins which have molecule weight 36.5 and 35.7 kDa that indicated the existing erythropoietin. The increasing haemoglobin concentration and erythropoietin suggested if extract of rnVr could increasing red blood production and potential as an orally available anemia therapeutic candidate.

Keyword: root nodule; *Vigna radiata*; Haemoglobin; protein profile; erythropoetin.

Introduction

The World Health Organization has proposed that if the prevalence of anaemia appropriate interventions based on food diversification and food fortification, iron supplementation. The recommendation to recovery severe acute malnutrition is therapeutic diets along with medical care.

The "aking rice" is the term of food that consumed by some Indonesian people, especially in the poor family. Aking rice is the recycle of leavings rice. These leavings rice is dried and steam once again, so that the nutrient of this food is lower than rice. Previous study suggested if the mice that fed aking rice for 21 days, the hemoglobin decreased from 12,9-13,5 gr/dL to 8,4-8,7gr/dL which indicate suffered anemia (Rahmawati, 2008).

Leghemoglobin is an oxygen carrier and a hemoprotein found in the nitrogen-fixing root nodules of leguminous plants. It is produced by legumes in response to the roots being infected by nitrogen-fixing bacteria, so-called rhizobia, as part of the symbiotic interaction between plant and bacterium: roots uninfected with Rhizobium do not synthesize leghemoglobin. Leghemoglobin has close chemical and structural similarities to hemoglobin, and like hemoglobin, is red in colour. The protein was believed to be a product of both plant and the bacterium (O'Brian *et al*. 1987) in which the apoprotein is produced by the plant and the heme (an iron atom bound in a porphyrin ring) is produced by the bacterium. (Santana *et al*. 1998).

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The leghemoglobin from nodules of *Crotalaria juncea* infected with Rhizobium consist of amino acid such as asparagine, aspartic acid, glutamine, glutamic acid,alanine, lysine, serine and leucine were the main amino acids The N terminal amino acid sequence showed high similarities with several other leghemoglobins from other plants (Mendonça *et al*., 1999).

Vigna radiata root nodule extract that infected by rhizobium predicted beneficial as an orally available anemia therapeutic candidate. The leghemoglobin content in this extract could be considered for preventing iron and protein deficiency and would increasing erythropoiesis.

Erythropoiesis is the process by which red blood cells (erythrocytes) are produced (Sherwood, 2005)

Erythropoietin is a glycoprotein hormone synthesised principally in the kidney. Erythropoietin is synthesised and released in response to hypoxia and stimulates erythropoiesis.Erythropoietin (Epo) is a 34 - 39 kDa secreted glycoprotein that is a member of the type I cytokine superfamily. (Nagao, 1992)

The aim of these research is investigate the potential of root nodule extract of *V. radiata* that inoculated with Rhizobium as an orally anemia therapeutic candidate. Parameter that use in this research is physiological and biochemical approach, that measured from Hemoglobin concentration and protein profile of plasma blood.

The utilization of aquaeous root nodule extract could be another strategy to improve legume crop waste for food and medical role that economically, achievable and eco friendly.

Materials and Methods

Plant materials and inoculation of Rhizobium.

Seeds of *V. radiata* were mixed with Rhizobium inoculum that obtained from Microbiology Laboratory of Agriculture Faculty, Gadjah Mada University, Indonesia (dosage 150 g inoculums/ 15 kg seeds). (Rahayu, 2005). Then, it cultured in polybag media that consist of soil and compost (ratio 2:1) (Utami, 2007). The root nodules were yielded after 35 days culture age (or vegetative plant growth stage) (Rahayu, 2005)

Preparation of extract

One hundred grams of dried root nodules of *V.radiata* was powdered with mortar and mixed with 100 ml of distilled water and then filtered with filter paper These aqueous extract is added with distilled water until 100 ml, and it used as 100% of root nodule of *V.radiata (*rnVr) extract.

Experimental animals

Adult male mice *(Mus musculus) strain B Albino clone* (BALB/c) (n = 16) , average weight is 32 gram, 1.5 months old obtained from the Laboratory of Pre -Clinical Care for Experimental Animal at Gadjah Mada University. They were housed individually in large clean metallic cages and fed with pellet C 524-2 Charoen pokphand (nutrient composition : 17-18 % protein (0.9 gram/ day) and amount Fe is 16.7 gram (85 mg/day). They administered with water *ad libitum.*The animals were kept in the laboratory condition for one week before the commencement of the experiment for acclimatization.

Experimental induction of anemia in mice

Anemia was induced by diet of *"aking rice pellet"* that representing the low nutrient food . Previous study suggested if the mice fed aking rice only obtain 0,17 gram protein /day and 0,01 mg Fe /day. (Rahmawati, 2008).Meanwhile, the optimum diet for the normal growth mice is 1,25 gram protein /day and 25-30 mg Fe /day (Kusumawati, 2004). After 21 days of aking rice administration, blood haemoglobin

(Hb) was measured.The average Hb concentration were showed between 9.68 – 9.85 mg/dL. These mice level concentration are categorized suffered anemia and used for treated mice (n=12). Meanwhile, the positive control mice (n=4) that fed with standart pellet (representing the high nutrient food) showed the normal Hb (average 13.5gr/dL). Sundari (1998) reported the normal Hb for mice is12.8-13.4 gr/dL

Animal grouping

Mice were divided into three groups designated**:** Positive and Negative Control and Treated . Mice in treated groups still fed with aking rice and received the root nodule extract of *V. radiata* (rnVr) in difference concentration. While animals in negative control only fed aking rice and positive control group were fed with pellet C 524-2 Charoen pokphand that representing the high nutrient food. The water and extract were administered to each mice using a blunt needle syringe tube 1 ml at 08.00–09.00 am for 14 days. Below is the grouping of treated and control mice :

Group I (Positive control): administered with high nutrient pellet + 1 ml of distilled water

Group II (Negative control): administered with aking rice pellet + 1 ml distilled water (0% rnVr extract rnVr extract)

Group III: administered with aking rice pellet + 1 ml of rnVr extract (33%)

Group IV: administered with aking rice pellet + 1 ml of rnVr extract (67%)

Group V: administered with aking rice pellet + 1 ml of rnVr extract (100%)

Blood collection and assays

Blood was collected from the orbital sinus of eye using sterile micro haematocrite tubes from each of the mice before treatment and 14 days after treatment. Plasma was separated by centrifugation at 3000 g for 10 min. Plasma protein profile were analyzed with *electrophoresis (SDS-PAGE).*

Haemoglobin measurement

Haemoglobin concentration were measured using cyanmethaemoglobin methods. The 0.02 ml blood were mixed with 5 ml Drabkins solution and after 2–4 hours the blood was analysed using a spectrophotometer. The *optical density* were readings at 540 nm.

Analyzing of blood plasma protein profile

Samples were collected 12-20 µl and then it were analyzed using elektroforesis with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according Laemmli (1970), using polyacrylamide vertical slab gel Bio-Rad Mini Protean II Dual Slab Cell contain 7.5 % separating gel 0.375 M Tris pH 8.8 dan 4% stacking gel 0.125 M Tris pH 6.8 . Electrophoresis were operate with constanly voltage (200 volt) for 45 minute. Then it was stained for 30 minute with 0.1 % Coomassie brilliant blue R250 in fixtaive solution (40 % MeOH, 10 % HOAc). And it was destained to eliminate the *background* color for about 1-3 hours (until the blue color eliminated). It stopped by putting the gel in solution of 10% glacial acetat acid. The existing of protein that contain in blood plasma was known by comparing the mobility and molecule weight of each protein band with the bovine marker. The molecule weight was determined by count the value of Rf (*Retardation factor*) from band (Rachmawati, 2006)

Statistical analysis

Statistical analysis for the increasing of Hb concentration was by Analysis of Variace (ANOVA) and followed with Multiple Comparison Dunnet test (2 sides), to comparison between treated mice with positive control, $\alpha = 0.05$.

Result and Discussion

Haemoglobin (Hb) concentration

According the statistical analysis showed if the p=0.000, that's mean there are relationship between rnVR treatment with increasing of hemoglobin concentration animal test. After 14 days, if compare with the positive control, all anemia mice groups that treated with extract of rnVr tend to showed significance increasing of Hb concentration. It showed in table 1. The increasing of extract of rnVr concentration (33%; 67 % and 100%, respectively) were followed by Hb concentration (0.92; 1.88 and 3.04 gr/dl respectively). The anemia mice group that treated with 100 % extract of rnVr had showed recovery and reach normal categorized (from Hb: 9.85 to 12.68 g/dL).

Table.1 : Hemoglobin concentration of anemia mice after and before treated with extract of rnVr.

Group	*Hb concentration before treatment (g/dL)*	*Category*	*Hb concentration after treatment (g/dL)*	*Category*	*Hb increasing (g/dL)*
I	13.56	N	13.25	N	_
II	9..02	A	nd	_	_
III	9.68	A	10.6	A	0.92 *
IV	9.77	A	11.65	A	1.88 *
V	9.85	A	12.88	N	3.04 *

Note : The group I and II is a positive and negative control respectively , the anemia groups that treated with extract of rnVr are grouped in III, IV and V. **A** is abbreviated of anemia mice and **N** is normal mice. After treated, the anemia mice showed significant increasing of Hb than the control. *: showed significant differences with the group I (control). The group V reach Normal Hb (recovery). The blood content from negative control mice (group II) was very low so it was not available to analyze.(nd= *not detectable*).

Meanwhile the group II or negative control mice (the anemia mice that fed with aking rice without treated with extract of rnVr (0%)) suffered acute malnutrition and the most weight body lost than the other anemia mice. The change of mice weight after experiment were showed in table 2.

Table 2. The change of body weight of mice after experiments

Body weight	Animal Group (Gram)				
	I	II	III	IV	V
Weight lost	16.7	11.8	10,5	9.8	
Weight gain	4.4				

Note: I = the positive control (healthy mice that fed with high nutrient pellet ; II= the negative control, (the anemia mice that only fed with aking rice without treated with rnVr extract) and group III; IV and V are treated anemia mice fed with aking rice without with rnVr extract.

Profile protein of Blood Plasma from treated and control mice

Total protein that showed in plasma blood from positive control mice is 23 protein bands. Meanwhile, the treated mice of 33%, 67% and 100 % showed 25; 24; and 23 protein band, respectively.

Some protein that observed from the blood plasma of the control and also in treated mice are the proteins that have molecule weight 35,7 and 36.5 kDa. It was indicated there was an Erythropoietin (Epo) in blood plasma. Epo is glycoprotein hormone that have range of weight molecule 34 - 39 kDa and have a role in erythropoesis (erythrocyte production).

The treated mice that used in these research were suffered anemia. This condition should be undergoing hypoxia that could triggered erythropoietin (Epo) production. It is stimulated by decreased O_2 delivery to the kidneys, which then secrete the hormone erythropoietin This activates increased erythropoiesis (Sherwood, 2005)

The Epo production was determined

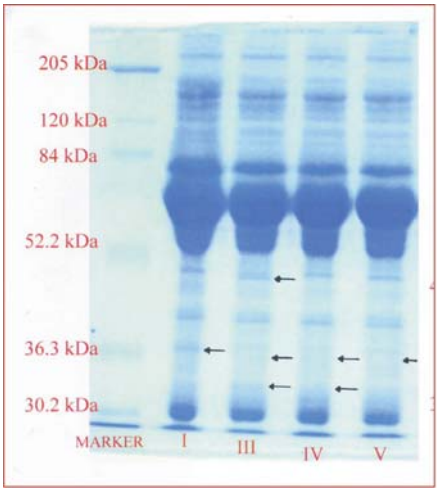

Figure1: The protein profile of blood plasma from positive control mice (I); and from the treated mice (III; IV and V). There was showed the protein 35.7 and 36.5 kDa that include the range of erythropoietin weight molecule (34-39 kDa

according the existence of protein profile that have weight molecule 35,7 and 36.5 kDa (Figure 1) in blood plasma of mice after 14 days treated with rnVr. These protein were include the range of erythropoietin weight molecule (34-39 kDa) (Nagao, 1992)

For supporting erythropoesis , the row material such as amino acid and heme are very important. So that, the anemia mice that treated with rnVr (group III, IV and V) more successful in erythropoesis than anemia mice that not treated with rnVr. It was proved by the increasing of hemoglobin concentration in all treated mice.

All anemia mice were fed with the aking rice (representing low nutrient) which then caused decrease body weight (Table 2). In treated mice, although body weigth were lost, showed increasing hemoglobin (Table 1) and active behaviour. Meanwhile the mice that only fed aking rice without administered with rnVr showed extremely body weight lost and showed an inactive behaviour

The variance of amino acid and heme structure in rnVr extract have an important role to increasing hemoglobin of anemia mice. Heme from the leghemoglobin that absorbed by duodenum was predicted in heme form. Fe in the heme formation would absorbed effectively. Heme would be degradated by heme oxygenase in mucosa epithel to be free Fe and tetrapirole (Rodak 2005). It would be as an effective Fe source in hemoglobin formation

The increasing of haemoglobin concentration and erythropoietin production suggested if extract of rnVr could increasing red blood production and potential as an orally available anemia therapeutic candidate.

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