Chelating Effect of Water Extract of *Mangifera foetida* L. Leaf inSerum of Thalassemia Patient by Ex Vivo Test

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ABSTRACT

Thalassemia is a genetic disorder, which is caused by the diminished synthesis of globin polypeptide chains. In Indonesia, 3-5% of cases are β-thalassemia and 2.6 to 11% are α-thalassemia. Regular blood transfusion is needed but iron overload is the consequence. That's why *Deferoxamine* is used as a chelating agent which function is bind iron and excrete them. Unfortunately, *Deferoxamine* needs high cost with high side effects. Therefore, an alternative natural medicine is required, that is mangiferin derived from aqueous extract of *Mangifera foetida* L. leaf. The aim of this research is to utilize the natural substance as a chelating agent of ferritin in thalassemia patient's serum. This was an experimental study, which used serumof patient with thalassemia. One Way Anova statistical test proved that the aqueous extract of *Mangifera foetida* L. leaf dose of 0.375 mg and 0.75 mg has a chelating effect compared with negative control (p=0.005). However, when subsequently tested with Post Hoc, 0.375 mg extract doesn't show a chelating effect compared with mangiferin (p=0.018).In the other hand, 0.75 mg extract has shown a chelating effect but not as good as mangiferin (p=0.259). It is considered that the low doses of extract and a possibility that the extract doesn't bind the iron directly are the factors which influence the result. That's why mangiferin still has better effectiveness in binding iron compared with aqueous extract of *Mangifera foetida* L. leaf dose of 0.375 mg and 0.75 mg.

Keywords: aqueous extract of *Mangifera foetida* L. Leaf, chelating, serum, thalassemia.

ABSTRAK

Talasemia penyakit herediter yang terjadi karena gangguan sintesis salah satu rantai polipeptida globin. Di Indonesia, 3-5% kasus merupakan talasemia β dan 2,6-11% merupakan talasemia α. Transfusi darahsecara teratur merupakan terapi yang dibutuhkan. Namun, tindakan ini memiliki konsekuensi berupa penumpukan besi di dalam tubuh. Untuk mengatasi hal tersebut, *Deferoxamine* digunakan sebagai agen kelator yang berfungsi mengikat besi dan kemudian mengeluarkannya dari tubuh. Penggunaan *Deferoxamine* membutuhkan biaya yang besar dan efek samping yang tinggi. Oleh karena itu, dibutuhkan pengobatan alternatif, yaitu mangiferin yang berasal dari ekstrak air daun *Mangifera foetida* L. Tujuan penelitian ini adalah untuk memanfaatkan bahan alami sebagai terapi kelasi besi pada penderita talasemia. Penelitian ini merupakan penelitian eksperimental dengan serum pasien talasemia sebagai subjek penelitian. Uji statistik *One Way* Anovamenunjukkan ekstrak air daun *Mangifera foetida* L. dosis 0,375 mg dan 0,75 mg memiliki efek kelasi bila dibandingkan dengan kontrol negatif (p=0,005). Namun, ketika selanjutnya diuji dengan *Post Hoc* didapatkan hasil ekstrak 0,375 mg tidak memiliki efek kelasi bila dibandingkan dengan mangiferin (p=0,018). Sementara itu, ekstrak 0,75 mg memiliki efek kelasi tetapi tidak sebaik mangiferin (p=0,259). Hal ini diduga berkaitan dengan beberapa faktor, yaitu: dosis ekstrak yang terlalu kecil dan adanya dugaan ekstrak tidak mengikat besi (Fe) secara langsung, melainkan mengikat apoprotein dalam serum. Oleh karena itu, dapat dikatakan mangiferin masih memiliki efektivitas yang lebih baik dalam mengikat besi bila dibandingkan dengan ekstrak air daun *Mangifera foetida* L. dosis 0,375 mg dan 0,75 mg.

Kata Kunci: ekstrak air daun *Mangifera foetida* L., kelasi, serum, talasemia.

INTRODUCTION

Thalassemia is a worldwide problem. There are 15 million people suffer from thalassemia which is separated in Syprus (16%), Thailand (1%), Bangladesh (3-8%), India, China, Malaysia, and Pakistan. In addition, there are 5-10% α-thalassemia in Mediterania, 20-30% in West Africa, 68% in South Pasific, and less than 1% found in Europe and Japan.[1,2,3]In Indonesia, the prevalence of thalassemia is quite high, 3-5% cases are β-thalassemia and 2.6-11% are α-thalassemia. Until 2008, there are 5000 person are detected as thalassemia patient.[4]

Thalassemia is a group of hereditary anemia that result from diminished synthesis of one of two globin polypeptide chains, which is caused by genetic disorder.[5,6] Clinically, there are 3 types of thalassemia: (1) major thalassemia, (2) intermediate thalassemia, and (3) minor thalassemia. Major thalassemia is the most severe type and needs blood transfusion every month.[6]But unfortunately, the consequnce of blood transfusion is iron overload which can stimulate the formation of reactive oxygen species (ROS) by Fenton reaction. These ROS can stimulate lipid peroxidation, protein disulfide bridges, and DNA cross-linking.[5,6] To prevent this iron overload, Deferoxamine is used as a chelating agent, which will bind the iron and inhibit the formation of reactive oxygen species (ROS) by Fenton reaction.[6] Beside that, Deferoxamine also excrete the overloading iron which is accumulated in cell (hemosiderin).Deferoxamine is given 5-7 times a week.[7,8]

Besides its increadible ability in preventing iron overload, Deferoxamine also has some harmful effects for thalasssemia patient. Oral administration of Deferoxamine is uncomfortable and decrease its effectiveness. Therefore, syringe driver is needed for subcutaneous injection of Deferoxamine (Portocath®). This therapy usually spend 3-5 million rupiahs each month with several adverse effects, such as: neurotoxicity, osteoporosis, defect on visual and auditorious function.[6,8]

Based on the fact above, we thought that Deferoxamine needs an alternative medicine which comes from nature. Natural alternative medicine is selected because it is expected to increase patient compliance with their cheaper cost and lower adverse effect. Based on *in vitro* study, it is known that mangiferin which comes from *Mangifera indica* L. stem bark has a chelating agent which is needed to inhibit formation of ROS. But, exploitation of *Mangifera indica* L. stem bark is environmentally harmful. That's why instead of using *Mangifera indica* L., we used aqueous extract of *Mangifera foetida* L. leaf which has higher level of mangiferin and doesn't environmentally harmful.[7,9]

MATERIALS AND METHODS

This was an experimental, placebo-controlled, parallel-unpaired-group study. This study used:

1. Serum of Thalassemia Patient
   Initially, We used 24 serum of thalassemia patientwhich regularly receive blood transfusion in Pediatric Health Department of Cipto Mangunkusumo Hospital in 2009-2010. But finally we used 7 serum as sample. Each 100 µl serum contain 100 µM iron. In this study we used 200 µl serum contain 200 µM iron
2. Aqueous Extract of *Mangifera foetida* L. Leaf
   Extraction process of *Mangifera foetida* L. leaf began with selection of leaffrom Depok which were identitified by Biology Research Center, LIPI Bogor. Then we washed, cut, and measured the amount of leaves until we got 400 gram of them and then made an infusion which then evaporated with Rotavapor Büche.Based on a research before, we have known that 400 gram of leaves

equivalent to 0,275 gram mangiferin. In addition, it is also known that 100 µg of stem bark) has an effect as a chelating agent.[7] To get the same chelating effect, we diluted*Mangifera foetida* L. leaf extract until we get 50 mg/ml concentration of aqueous extract of *Mangifera foetida* L. leaf. Therefore initially, we used 7,5 mg and 15 mg doses of extract of *Mangifera foetida* L. Leaf as the experimental substances. But, finally we decreased the doses into 0,375 mg and 0,75 mg.

3. Mangiferin Solution
   Mangiferin solution was made as a solution depot. It was made by adding 1 mg of mangiferin powder (product of Changyba Huir Biological-tech China)into 0,5 ml of aquades and 0,5 ml of ethanol. Then 100 µl of solution would contain 100 µg of mangiferin.
4. Desferal® Solution
   Desferal® solution was made by adding 2 mg of Desferal® into 1 ml of aquades. So, 50 µl of solution would contain 100 µg Desferal®.
5. Standard Medium
   Standard medium consist of sucrose 125 Mm, KCl 65 mM, potassium succinate 5 mM, phosphoric acid 2 mM, magnesium chloride 1 mM, and HEPES buffer (pH 7,2) 10 mM. In addition we also used 2 mM of citrate medium.

After preparing all of the experimental substances, we prepared the mangiferin (equivalent to 15 mg extract of *Mangifera indica* L. experimental subjects. The experimental subject was divided into 2 groups, they were: intervention groups and control groups. There were 2 intervention groups and 3 control groups, each group contain 1000 µl standard medium, 100 µl citrate medium, and 1000 µl aquades. Beside that, each group also contain different experimental substance, they were:

a. Group I : 200 µl serum only (as negative control).
b. Group II : 200 µl serum + 100 µg mangiferin (as positive control).
c. Group III : 200 µl serum + 100 µg Deferoxamine (initially as positive control)
d. Group IV : 200 µl serum + 0.375 mg Aqueous Extract of *Mangifera foetida* L. Leaf.
e. Group V : 200 µl serum + 0.75 mg Aqueous Extract of *Mangifera foetida* L. Leaf.

After preparing all of the experimental subjects, we then measured the absorbance of each groups by a spectrophotometer UV-VIS Optima 3000 at 28° C and λ = 190 nm-400 nm wavelength. According to a research before, we have known that the absorbance peak of mangiferin will appear in 275 nm and 380 nm.[7] The data then would be processed using a formula that we have prepared before:

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*abs=absorbance

After processing the data with this formula, the data was analyzed by One Way Anova statistial test using SPSS 13.0 for Windows®.

RESULTS

In the initial experiment, we used 24 serum of thealassemia patient. But, after weanalysed the experimental groups with spectrophotometer, we found that these 24 serum didn't show a similar pattern of absorbance, therefore we decided to reduce the number of sample into 9 serum. After doing the experiment with 9 serum, we

found that two serum didn't show a similar pattern, so finally we decided to use 7 serum as sample. The minimal sample we need is 5 sample (based on Federer formula). Initial volume of serumwe used was 100 µL which contain 100 µM iron. Then we increased the volume of serum into 200 µL, but it still contains 100 µM iron. We increased the volume of serum because the absorbance peak was not clearly visible on the graph. However, after the change of volume, the absorbance peak serum remained invincible. Therefore, we decided to increase the concentration of iron into 200 µM.

Initial experiment also used 7,5 mg and 15 mg doses of extract of *Mangifera foetida* L. Leaf as the experimental substances.But, after an initial analysis with spectrophotometer, we could not read the graphic clearly because the absorbance peak was out of the graphic (see Figure 1). That's why we decreased the extract doses into 0,375 mg and 0,75 mg. Beside that the concentration of aqueous extract of *Mangifera foetida* L. Leaf is also reduced into 15 mg/ml (see Figure 2).

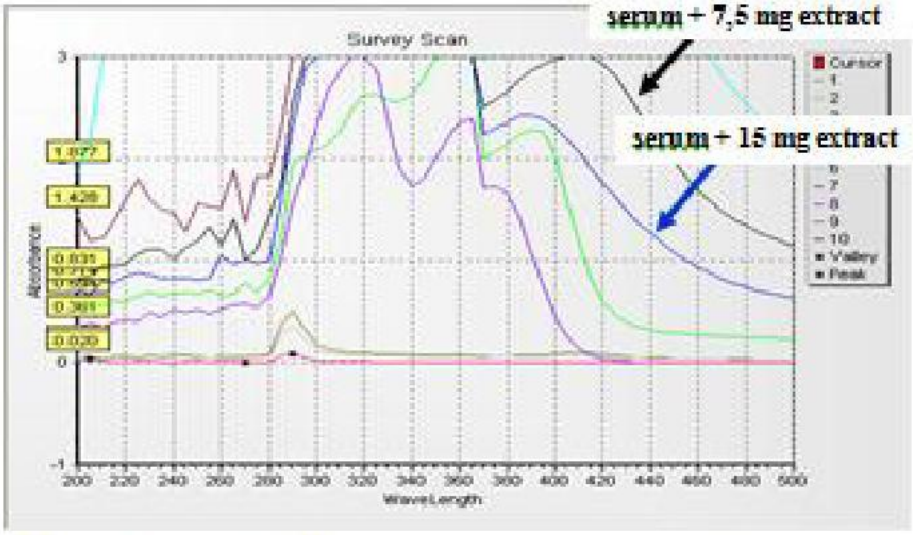

Figure 1. Spectrophotometer Output Graph of Aqueous Extract of *Mangifera foetida* L. Leaf Doses of
7,5 mg and 15 mg

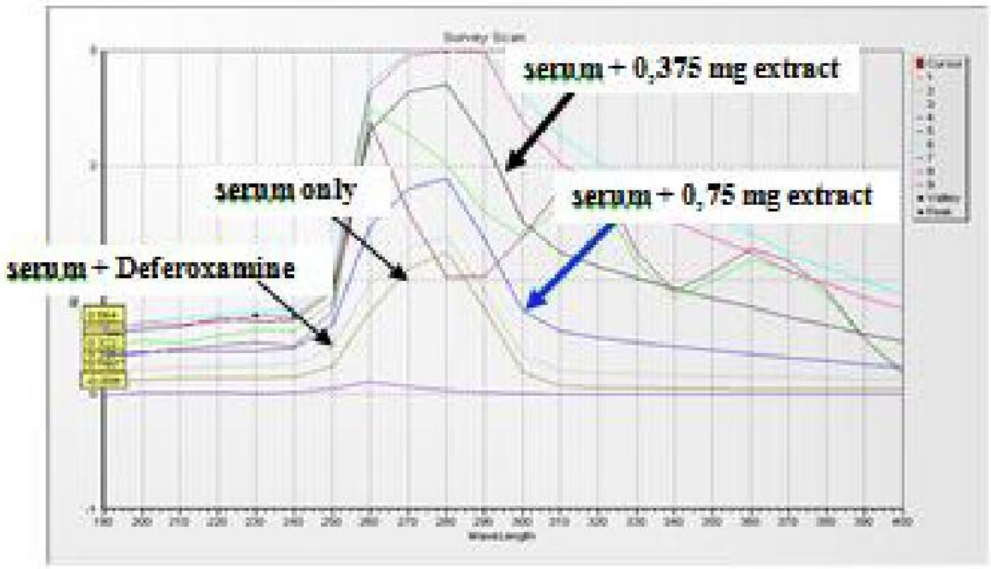

Figure 2. Spectrophotometer Output Graph after Lowering the Doses into 0,375 mg and 0,75 mg

As explained before, in the initial study, we used 3 groups of control, but lately we removed Deferoxamine as a positive control because its absorbance didn't show any significant differences with absorbance of serum (see Figure 2). It is thought that Deferoxamine (Desferal®) didn't bind iron directly, but apoprotein. That's why there was no formation of iron-Deferoxamine complex. The other possibility that caused insignificant differences between serum+Deferoxamine and serum only is inappropriate media used in this experiment. Based on the literature, we know that the appropriate media for Deferoxamine are ascorbic acid (28 mM) and ferric citrate (20 mM), but we didn't use those media in this experiment.[10]

After removing Deferoxamine as the positive control, we only used Mangiferin as the positive control. We compared the absorbance value of each group after it was processed by a formula that we have prepared before. By processing the data with the formula, we got the absorbance value of unbound mangiferin in each group. The comparison of unbound mangiferin absorbance value between intervention groups and control groups is showed below:

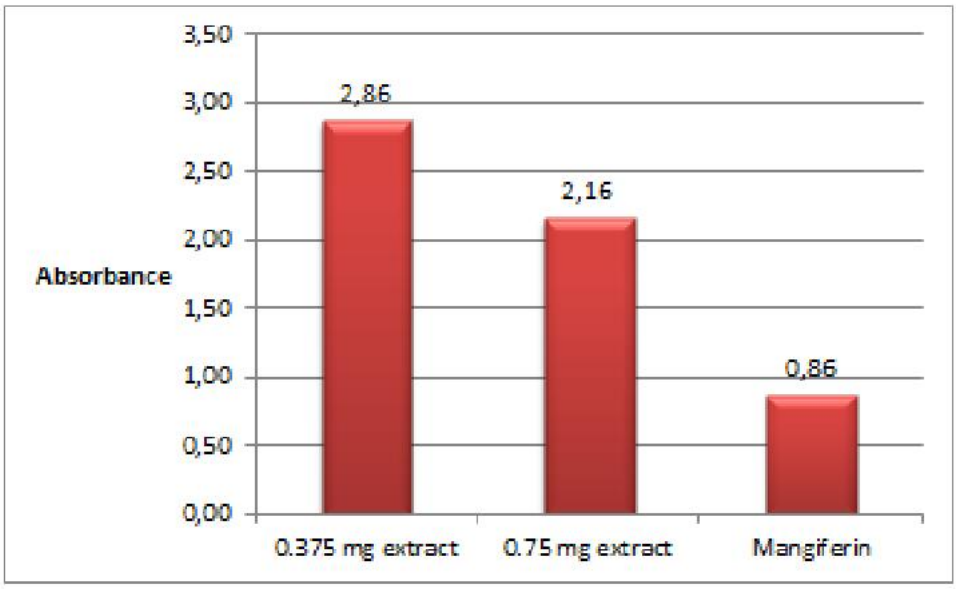

Figure 3. Mean Absorbent Value of 100 µg Mangiferin (Positive Control); and Water Extract of *Mangifera foetida* L. Leaf Dose of 0.375 mg and 0.75 mg (λ=280 nm)

DISCUSSION

This study used aqueous extract of *Mangifera foetida* L. leaf as the experimental substance. *Mangifera foetida* L. is one of *Mangifera indica* cultivar. Based on a study before, it was known that the concentration of mangiferin in this cultivar is highest than the other cultivar (2,56%).[9] Mangiferin will act as a chelating agent which will bind iron in the body, so it will prevent:

1. Lipid peroxidation which is induced by iron. Excessive iron in the body will stimulate oxygen consumption, and then the oxygen will be converted into superoxide and hydrogen peroxide by superoxide dismutase. Then hydrogen peroxide will be converted into reactive oxygen species (ROS) by Fenton reaction. This ROS is harmful because it can cause lipid peroxidation.[5,11]
2. Membrane mitochondria damage can cause the formation of non-selective pores, which will allow the moveable proton to through the membrane and finally mitochondria loss their ability to produce ATP.[5,11]

Beside contain the highest concentration of mangiferin, *Mangifera foetida* L. also contain high concentration of vitamin C (56 mg) which is needed to prevent the formation of human granulocyte macrophage-colony-stimulating factor (GMCSF). GMCSF will induce the formation of ROS. Based on a study before we have known that vitamin C can decrease 30% level of ROS in the body.[12]

Based on the One Way Anova statistical test, it was known that aqueous extract of *Mangifera foetida* L. leaf doses of 0,375 mg and 0,75 mg have a chelating effect in serum of patient with thalassemia. Aqueous extract of *Mangifera foetida* L. leaf doses of 0,375 mg and 0,75 mg had shown a significant differences with negative control (p=0,005), that's why the statistical test was followed by post Hoc. Post Hoc test proved that aqueous extract of *Mangifera foetida* L. Leaf dose of 0,375 mg had a significant differences (p=0,018) with positive control (mangiferin solution plus serum). This differences means extract 0,375 mg actually doesn't have a

chelating agent compare with positive control. In the other hand, aqueous extract of *Mangifera foetida* L. leaf dose of 0,75 mg didn't show a significant differences (p=0,259) with positive control, it means extract 0,75 mg has a chelating effect. But, we found that its chelating effect was not as good as chelating effect of positive control (mangiferin plus serum). Let's see Figure 3, in this figure the absorbance mean of extract 0,75 mg is higher than positive control. It means that the concentration of unbound mangiferin in extract 0,75 mg is higher than unbound mangiferin in positive control. The amount of unbound mangiferin could be a representationofthe chelating effect of experimental substances. From the fact above, we knew that the increasing amount of unbound mangiferin means the decreasing effect of chelating. Another point that we can get in the Figure 3 is the higher absorbance mean of extract 0,375 mg than extract 0,75 mg, it means aqueous extract of *Mangifera foetida* L. leaf dose of 0,75 mg has better chelating effect than dose of 0,375 mg. This fact was also been proved by statistical test. In the Post Hoc test, we found that there was a significant differences of chelating effect in extract 0,375 mg and 0,75 mg (p=1,000).

There were several points that we've thought to be the reason why extract didn't have better chelating effect when was compared with positive control. Some factors that was considered have a correlation with the result are low doses of extract and a possibility that the extract doesn't bind iron directly, but apoprotein.

After doing a conversion, we found that 40 grams of extract was equivalent to 0,275 grams of mangiferin, that's why 0,375 mg (375 µg) of extract was equivalent to 2,6 µg mangiferin and 0,75 mg (750 µg) of extract was equivalent to 5 µg of mangiferin. In the other hand, the control (mangiferin) dose we used is 100 µg, and it was based on a research before. Therefore the chelating effect of extract was not as good as the chelating effect of control (mangiferin).

The other factor that we have thought to be correlated with the result is the possibility that the extract didn't bind iron directly but apoprotein. That's why the chelating effect of extract didn't as good as mangiferin (positive control). Ferritin consists of iron in the inner and outer apoprotein (see Figure 4).[13] A chelating effect would be shown if there was a binding between iron and extract, but in this case we have thought that the extract didn't bind iron directly, it was proved by a fact that the absorbance peak didn't appear at 275 nm and 380 nm wavelength. Therefore, the chelating effect was not seen clearly in this experiment. To prove whether the extract bind iron directly or not, we need a nuclear magnetic resonance (NMR) spectroscopy. NMR would figure the actual bond formed, so we could know whether the extract bind iron directly or not.

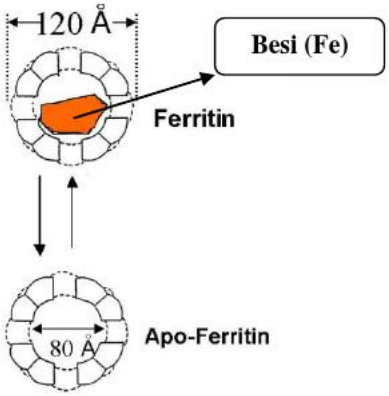

Figure 4.[13] Structure of Ferritin. Ferritin onsist of apoprotein (apo-ferritin) in the outer and inner iron.

CONCLUSION

Water extract of *Mangifera foetida* L. leaf dose of 0.375 mg and 0.75 mg has a chelating effect in serum of patient with thalassemia (p=0,005).

RECOMMENDATION

1. Futher experiment is needed to find an appropriate dose of Water extract of *Mangifera foetida* L. leaf so that the ability of this extract in binding iron will significantly observe.
2. It is recommended to use another dilution liquor to dilute the extract (such as: alcohol).
3. It is recommended to use Nuclear Magnetic Resonance (NMR) in futher experiment to observe the bond between extract, mangiferin, and Deferoxamine with serum.
4. It will be better if futher experiment try to use another part of *Mangifera foetida* L as the source of extract.

REFRENCES

1. Yaish H M. Thalassemia. WebMD Professional [materials on the internet] 2010 April [cited 2010 july 19]. Available from: http://emedicine.medscape.com/article/958850-overview
2. Anonymous. Thalassemia prevalence. Tha medical news [article on the internet] 2009 [cited 2010 November 25]. Available from: http://www.news-medical.net/health/Thalassemia-Prevalence.aspx
3. Anonymous. Prevalence and incidence of thalassemia. [article on the internet] 2010 [cited 2010 November 25]. Available from: http://www.wrongdiagnosis.com/t/thalassemia/prevalence.htm
4. Permanasari I. Talasemia: Transfusi Penyambung Hidup. [article on the internet] 2010 juny [cited 2010 July 19]. Available from: http://www.kesekolah.com/article-and-news/detail/9070-talasemia-transfusi- penyambung-hidup.html
5. Kumar V, Cotran R S, Robbins S L. Buku Ajar Patologi. 7th Ed. Jakarta: EGC; 2007. p. 452-5.
6. Bridges K R, Pearson H A. Anemias and Other Red Cell Disorders. 1st Ed. United State America: The McGraw-Hill; 2008. p. 277–300.
7. Andreu G P, Gelgado R, Velho J A, Curti C, Vercesi A E. Iron Complexing Activity of Mangiferin, a Naturally Occuring Glucosylxanthone, Inhibits Mitochondrial Lipid Peroxidation Induced by Fe^{2+}-Citrate. European Journal of Pharmacology. 2005;513:47-55.
8. Ganie R A. Thalassemia: Permasalahan dan Penanganannya. Universitas Sumatera Utara [materials on the internet] 2005 November [cited July 19]. Available from: http://www.usu.ac.id/id/files/pidato/ppgb/2005/ppgb_2005_ratna_akbari_ganie.pdf
9. Soetarno S, Soediro I, Padmawinata K. Isolasi dan Karakterisasi Mangiferin dari Daun Mangga Arumanis dan Perbandingan Kadarnya pada Daun Tujuh Kultivar *Mangifera Indica* L. Acta Pharmaceutica Indonesia. 2004;16:126-35.
10. Asbeck BS, Marcelis JH, Marx JM, Struyvenberg A. Inhibition of Bacterial Multiplication by The Iron Chelator Deferoxamine: Potentiating Effect of Ascorbic Acid. Eur. J. *Clin Microbiol*. 1983;2:426-31.
11. Chattopadhyay U, Chaudhuri L, Ghosal S. Immunostimulatory Activity of Mangiferin, A Naturally Occuring Xanthone-C_Glucoside. Pharmaceutical Research. 1986;3:307-8.
12. Carcamo JM, Oriana BO, Golde DW. Vitamin C Inhibit Granulocyte Macrophage-Colony Stimulating Factor-Induced Signaling Pathways. Blood Journal. 2003;99:3205-6.
13. Strongin D R. Structur of Ferritin. 2009 [cited 2010 November]. Available from: http://astro.ocis.temple.edu/~dstrongi/ferritin.jpg