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Anti Diabetes Mellitus Activity *in vivo* of Ethanolic Extract and Ethyl Acetate Fraction of *Euphorbia hirta* L. Herb

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Abstract: *Euphorbia hirta* L. is a plant used in traditional medicine and proven pre-clinically for antiinflanimation, asthma, wound healing and diarrhea. It also has antioxidant activity. However, the anti-diabetes mechanism of this plant has not been elucidated. A reliable technique to elucidate the anti-diabetes mechanism in the traditional medicine is required. Therefore, a new technique which was based on the glucose tolerance test was explored. Through various oral glucose tolerance test loading, the anti-diabetes properties of *Euphorbia hirta* L. could be elucidated. From the *in vitro* experiment, it was known that the ethanol extract and ethyl acetate fractions are the fractions with α-glucosidase inhibition activity, while *n*-hexane, chloroform, butanol and water fractions does not have α-glucosidase inhibitory effect. Therefore, only ethanol extract and ethyl acetate fractions were used for the *in vivo* tests. Based on the *in vitro* and *in vivo* test, *Euphorbia hirta* L. ethanolic extract and ethyl acetate anti-diabetes mechanism is related to its antioxidant capacity and also its α-glucosidase inhibitory properties.

Key words: *Euphorbia hirta* L., α-glucosidase inhibitor (*in vitro*), oral glucose tolerance test (*in vivo*)

INTRODUCTION

Due to the change in lifestyle, the number of the people in the world with diabetes has increased dramatically over recent years. It is estimated that by 2010, the diabetic population will increase to 221 million around the world (Aslan *et al*., 2007).

As diabetes mellitus is one of the most serious, chronic diseases that is developing with an increase in obesity and ageing in the general population, some drugs have been developed for diabetes. However, the best way to control postprandial plasma glucose level is with a medication in combination with dietary restriction and an exercise programme. One approach in the medication is to retard absorption of glucose by the inhibition of carbohydratehydrolysing enzymes, for example α-amylase and aglucosidase, in the digestive organs. Some established α-glucosidase inhibitors are acarbose and voglibose from microorganisms and nojirimycin and 1-deoxynojirimycin from plants and their effects are proven to reduce hyperglycemia post prandial (Kim *et al*., 2004).

On the other hand, the five types of oral antihyperglycemic drugs currently approved for the treatment of diabetes are biguanides, sulfonylureas, meglitinides, glitazones and alpha-glucosidase inhibitors have some limitations, especially in patients with Coronary Artery Disease (CAD). Regarding biguanides, gastrointestinal disturbances such as diarrhea are frequent and the intestinal absorption of group B vitamins and folate is impaired during chronic therapy. This deficiency may lead to increased plasma homocysteine levels which in turn, accelerate the progression of vascular disease due to adverse effects on platelets, clotting factors and endothelium. Current data indicate that combined glibenclamide/metformin therapy seems to present a special risk and should be avoided in the long-term management of type 2 diabetics with proven CAD. New possibilities for diabetic patients with heart disease are represented by incretin mimetic compounds, Dipeptidyl Peptidase (DPP)-4 inhibitors, inhaled insulin and eventually oral insulin (Fisman and Tenenbaum, 2008).

Acarbose delays digestion of complex carbohydrates and disaccharides to absorbable mono-saccharides by reversibly inhibiting α-glucosidases within the intestinal brush border, thereby attenuating postprandial blood glucose peaks. Although, acarbose seldom causes systemic adverse effects, it is associated with a high incidence of gastro-intestinal disturbances such as flatulence, abdominal distension, borborygmus and diarrhoea, caused by fermentation of unabsorbed

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carbohydrates. These symptoms tend to subside, with continued treatment and adherence to an appropriate diet. However, further long term efficacy and tolerability data are required, particularly in the latter indication (Balfour and McTavish, 1993).

Due to these limitations, antioxidants were also explored as one of the alternative medicine in diabetes. Marwah *et al.* (2007) stated that reactive oxygen species are involved in a number of degenerative diseases such as artherosclerosis, cancer, cirrhosis, diabetes an also in wound healing.

Euphorbia hirta is traditionally used as treatment of asthma and respiratory tract inflammation, cough, chronic bronchitis and other pulmonary disorders and proven pre-clinically for antiinflammation, asthma, wound healing and diarrhea (Ogbulie *et al*., 2007). There were experiments based on ethnobotanical information on some Euphorbiaceae in Mexico (*Euphorbia polystahia*, *Euphorbia preslii*), it was reported that there were hypoclygemic effects from these plants. Other plants earlier studied are *E. prostrata* (Ivorra *et al*., 1989) which having an effect in healthy rabbits, but not showing any activity in the diabetic model. These results suggest that some pancreatic function, or the presence of insulin, is required for the hypoglycemic activity of these anti-diabetes plants.

A general analysis of the results obtained at present with all the anti-diabetic Mexican plants whose hypoglycemic effect has been assessed in temporary hyperglycemic rabbits, including the plants studied in the present work, showed that 33 out of 62 plants studied have hypoglycemic activity. One of the plants with the highest hypoglycemic effect, denoting a significant decrease of over 15% of the area under the curve during the GTT is *E. prostrata* (15.9%), (Alarcon-Aguilara *et al*., 1998).

Considering that *E. prostata* was considered as excellent candidates for its hypoglycemic activity, we believe that *Euphorbia hirta* L., could be considered as candidates for future anti-diabetes medicine. On the other hand, in the tradional use, *Euphorbia hirta* L, hasn't been investigated for its activity as anti diabetes mellitus. Therefore, studies on determining the mechanisms of their hypoglycemic activity, as well as for the isolation and identification of active hypoglycemic substances should be carried out. In this research, the anti diabetes mellitus property was assayed *in vitro* using the alpha glucosidase inhibitor method and confirmed by *in vivo* oral glucose tolerance test with various loading method.

In addition to this study, further comprehensive pharmacological investigations, including experimental acute studies, will be carried out to assess the likely toxicological effects of the anti-diabetes plant.

MATERIALS AND METHODS

Materials: *Euphorbia hirta* L. ethanol extract and ethyl acetate fraction, α-Glucosidase, pnitrophenyl glucopyranoside (pNPG), phosphate buffer, Na_2CO_3, male Swiss webster mice, oral needle, *Manihot utilissima* amylum, sucrose (Merck, 1.07651.1000), maltose (Difco, 0168-17), glucose monohydrate (Merck, 5143356), aquadest.

Equipment: Plant grinding machine, digital scale (Mettler, Toledo AG204), maceration equipments, electric stove, volume pippettes, measuring pippettes, rotavapor (Buchi Rotavapor R-124 Buchi Waterbath B-480), water pump, distillation equipments, glasswares, spektrophotometer UV (Hewlett Packard 8452A)

Methods: The experiment was conducted from March until October 2009 in School of Pharmacy, Institut Teknologi Bandung and in LIPI Serpong.

Anti-diabetes activity *in vitro*: α-Glucosidase (0.075 unit) was premixed with the extract at various concentrations (0.01-200 μg mL^{-1}). The 3 mM p-nitrophenyl glucopyranoside (pNPG) as a substrate in phosphate buffer was added to the mixture to start the reaction. The reaction was incubated at 37°C for 30 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the p-nitrophenol release from pNPG at 400 nm. The IC_{50} value was defined as the concentration of α-glucosidase inhibitor to inhibit 50% of its activity under the assay conditions (Kim *et al*., 2004).

Anti-diabetes activity *in vivo*: The use of live animals in scientific research and other experimental activity is strictly controlled in accordance with the Cons 123 (2006) 3, Appendix A of the European convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123) Guidelines for accommodation and care of animals (Article 5 of the convention), which is approved by the Multilateral consultation.

Various loading of carbohydrates in oral glucose tolerance test: The loading was given successively in accordance to the classification of carbohydrate. Therefore, the result of 4 various loading was compared. The first loading was amylum 5 g kg^{-1} of body weight as amylum is polysaccharide. Then the second loading was maltose 3 g kg^{-1} of body weight. Another test was conducted using sucrose 3 g kg^{-1} of body weight to ensure the α-glucosidase inhibitor property. Then, the last loading was glucose 2 g kg^{-1} of body weight. The

calculation of the inhibition of the tested materials was compared by the regression value (R^2), which shows the rapidity of the tested materials to lower the blood glucose to normal state.

RESULTS AND DISCUSSION

The result of the α-glucosidase inhibitor activity *in vitro* is shown in Fig. 1. Quercetin and quercitrin was also tested its α-glucosidase inhibitor activity *in vitro* to confirm that quercetin and quercitrin are some of the compounds responsible for its anti-diabetes activity.

The quercetin α-glucosidase inhibitor activity *in vitro* was 14±0.25 μg mL^{-1}, which is almost similar to ethyl acetate (16 μg mL^{-1}). Therefore, the ethanolic extract IC_{50} value was 7.97±0.26 μg mL^{-1}, which is closer to the IC_{50} value of 1-deoxynojirimycin as standard reference (5.08±0.11 μg mL^{-1}). Therefore, it could be concluded that the ethanolic extract could contain the compounds that worked synergistically to exert its anti-diabetes activity.

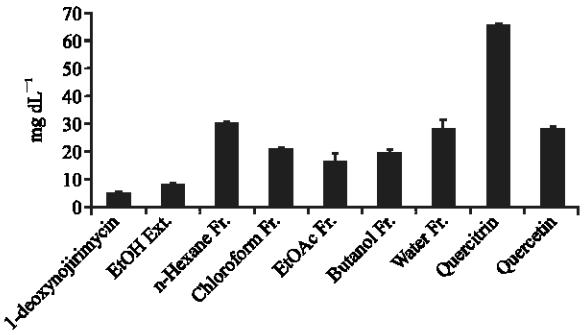

Fig. 1: Anti-diabetes activity *in vitro* and the *Euphorbia hirta* L. extract and fractions and quercetin with 1-deoxynojirimycin as a standard drug reference

The result of the antidiabetes activity *in vivo* using oral glucose tolerance test with various loading, starting from polysaccharide (amylum), disaccharide (maltose and sucrose) and monosaccharide (glucose) is shown in Fig. 2-9 and Table 1-4.

α-Glucosidase (EC 3.2.1.0) and α-amylase (EC 3.2.1.1) are the key enzymes involved in the metabolism of carbohydrates. The α-amylase degrades complex dietary carbohydrates to oligosaccharides and disaccharides, which are ultimately converted into monosaccharides by α-glucosidases. Liberated glucose is then absorbed by the gut and results in postprandial hyperglycemia. Inhibition of intestinal α-glucosidases limits postprandial glucose levels by delaying the process of carbohydrate hydrolysis and absorption, making such inhibitors useful in the management of type 2 diabetes. Plants and microorganisms have been a rich source of α-glucosidase inhibitors. For example, acarbose, 1-deoxynojirimycin and genistein have all been isolated from natural sources (Shinde *et al*., 2008). Therefore, *Euphorbia hirta* L herb as traditional plant used for various treatment, was tested for α-glucosidase inhibitor activity.

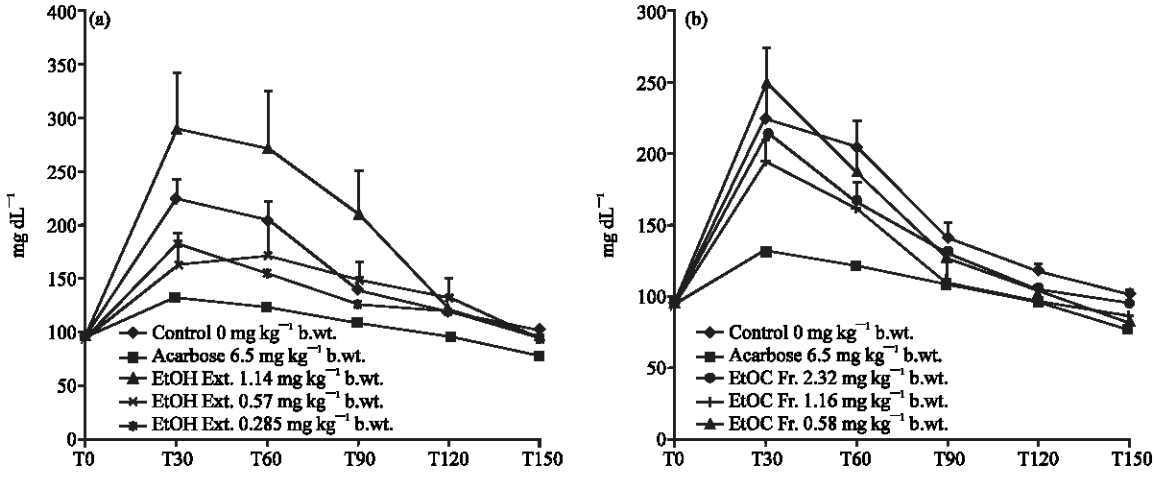

Fig. 2: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with amylum 5 g kg^{-1} b.wt. loading. T_0 is blood glucose level soon after the administration of the tested compounds, T_{30} is blood glucose level 30 min after carbohydrate loading and 60 min after the administration of the tested compounds, T_{60} is blood glucose level after 90 min after the administration of the tested compounds, T_{90} is blood glucose level after 120 after the administration of the tested compounds, T_{120} is blood glucose level after 150 after the administration of the tested compounds, $T15_0$ is blood glucose level after 180 after the administration of the tested compounds

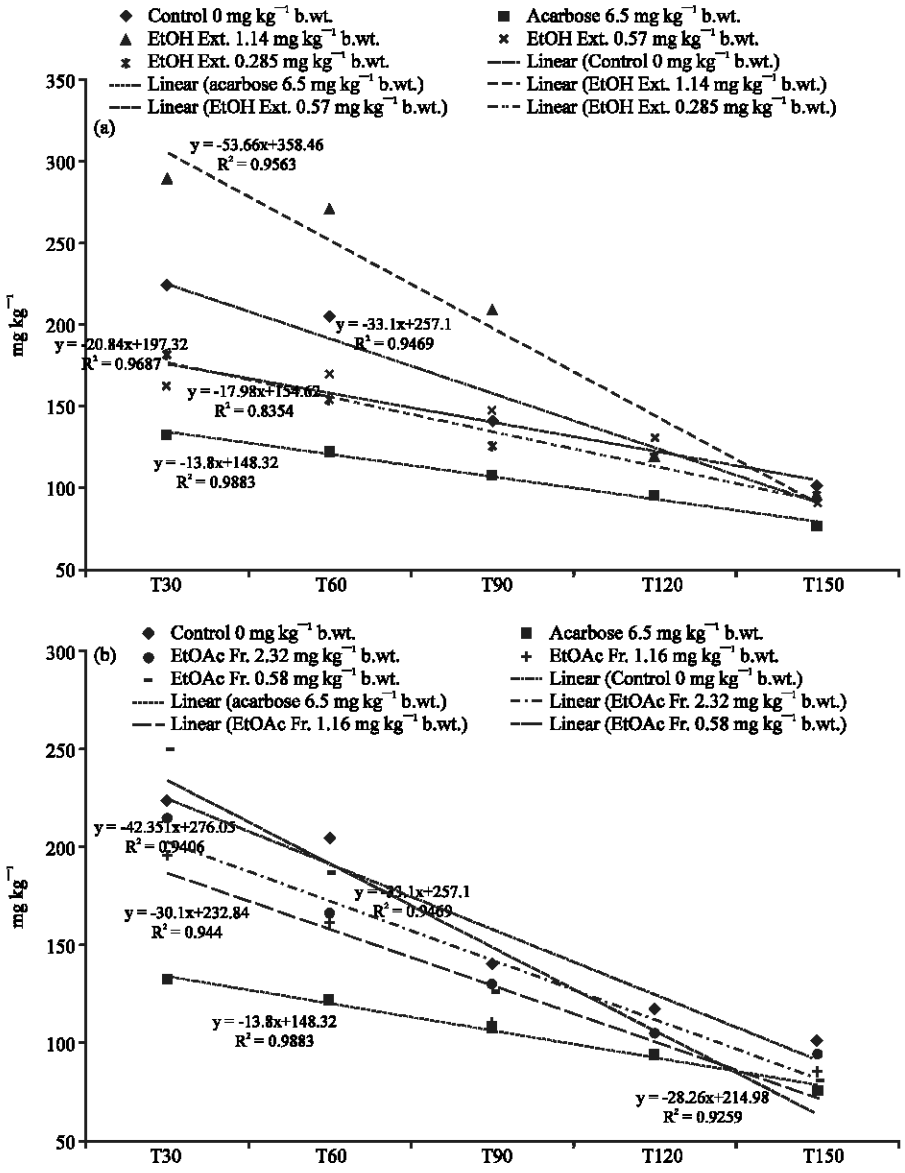

Fig. 3: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with amylum 5 g kg^{-1} b.wt. loading with its regression

Table 1: The average of the mice blood glucose level mg dL^{-1} in oral glucose tolerance test with amylum 5 g kg^{-1} b.wt. loading

Tested compounds (mg kg^{-1} b.wt.)	T_0	T_{30}	T_{60}	T_{90}	T_{120}	T_{150}
Control 0	94.00	223.80	204.60	141.00	118.00	101.60
Acarbose 6.5	95.40	132.20*	122.40*	107.80	95.60	76.60
EtOH Ext. 1.14.	95.60	289.60*	271.00*	209.40*	121.20	96.20
EtOH Ext. 0.57	99.40	162.20*	170.40	147.60	131.40	91.80
EtOH Ext. 0.285	96.20	181.40	153.80*	125.40	118.60	94.80
EtOAc Fr. 2.32	95.20	214.80	166.60	130.60	105.20	95.00
EtOAc Fr. 1.16	94.00	195.40	161.40	110.60	97.60	86.00
EtOAc Fr. 0.58	97.00	249.80	187.20	126.20	100.31	81.49

*Differs significantly towards control 0 mg kg^{-1} b.wt. with $p<0.050$

Anti-diabetes activity of a medicine usually tested by *in vitro* α-glucosidase inhibitor method. However, there could be found a difference between *in vitro* and *in vivo* test result. This is due to the difference between

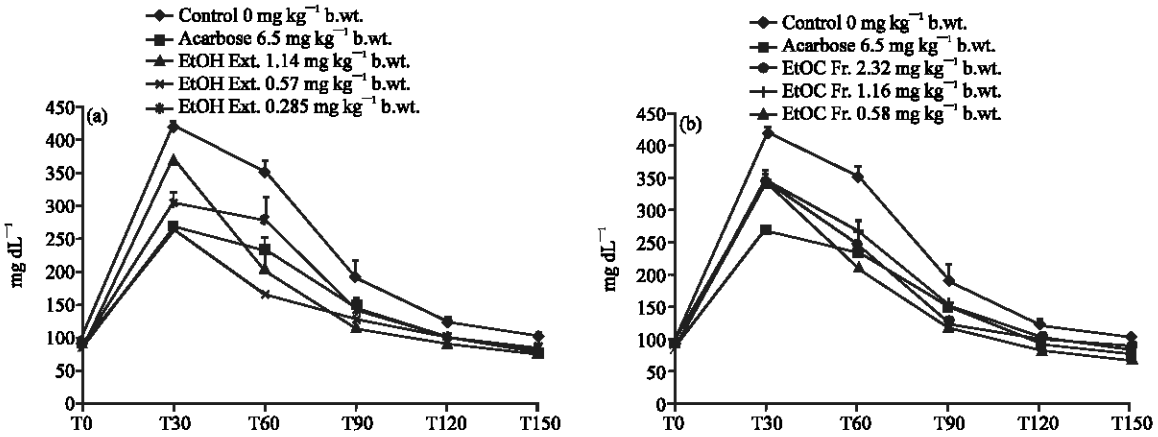

Fig. 4: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with maltose 3 g kg^{-1} b.wt. loading

Table 2: The average of the mice blood glucose level mg dL^{-1} in oral glucose tolerance test with maltose 3 g kg^{-1} b.wt. loading

Tested compounds (mg kg^{-1} b.wt.)	T_0	T_{30}	T_{60}	T_{90}	T_{120}	T_{150}
Control 0 mg	97.40	420.40	351.40	190.80	122.60	100.80
Acarbose 6.5	90.60	268.60*	233.00*	148.00	94.20	76.00
EtOH Ext. 1.14	90.40	370.60*	203.00*	116.00*	90.40	80.20
EtOH Ext. 0.57	90.20	264.40*	166.60*	127.00*	101.00	77.00
EtOH Ext. 0.285	85.20	306.00*	279.00*	143.00*	101.00	83.80
EtOAc Fr. 2.32	94.40	344.40*	246.80*	122.60*	101.00	87.80
EtOAc Fr. 1.16	89.40	347.00*	269.40*	153.60	103.60	84.40
EtOAc Fr. 0.58	91.20	340.80*	213.00*	116.75	81.37	66.69

Table 3: The average of the mice blood glucose level mg dL^{-1} in oral glucose tolerance test sucrose 3 g kg^{-1} b.wt. loading

Tested compounds (mg kg^{-1} b.wt.)	T_0	T_{30}	T_{60}	T_{90}	T_{120}	T_{150}
Control 0	93.20	221.00	166.60	137.40	126.60	101.60
Acarbose 6.5	90.20	124.80*	119.80*	110.60	95.60*	85.40
EtOH Ext. 1.14	93.80	167.40*	133.00*	104.40	112.40	88.60
EtOH Ext. 0.57	95.00	168.20*	143.00	119.80	109.20	98.40
EtOH Ext. 0.285	91.20	160.00*	125.80*	105.00*	93.40*	185.40
EtOAc Fr. 2.32	96.80	183.20*	151.00	120.00	110.80	98.20
EtOAc Fr. 1.16	94.00	185.00*	157.80	116.40	123.40	103.60
EtOAc Fr. 0.58	95.40	166.60*	135.20*	104.70	98.30	83.60

Table 4: The average of the mice blood glucose level mg dL^{-1} in oral glucose tolerance test with glucose 2 g kg^{-1} b.wt. loading

Tested compounds (mg kg^{-1} b.wt.)	T_0	T_{30}	T_{60}	T_{90}	T_{120}	T_{150}
Control 0	89.00	237.40	177.20	144.20	121.20	93.20
Metformin 130	95.60	182.60*	146.60*	104.60*	82.80*	68.60
EtOH Ext. 1.14	94.60	271.00*	182.60	148.00	107.40	84.00
EtOH Ext. 0.57	93.80	201.40*	141.00*	109.20*	94.20	79.20
EtOH Ext. 0.285	96.60	256.00	174.80	115.80*	93.00	78.20
EtOAc Fr. 2.32	87.60	235.80	156.20	126.80	106.40	87.00
EtOAc Fr. 1.16	92.80	248.60	168.20	123.40	95.00	79.40
EtOAc Fr. 0.58	91.60	229.00	144.20	124.20	104.80	83.80

α-glucosidase type. In *in vitro* test, the α-glucosidase involved is type 1 (produced in yeast *Saccharomyces cerevisiae*) while the α-glucosidase involve in *in vivo* test, is α-glucosidase type II (produced by mammalian source) (Kim *et al*., 2004). The α-glucosidase type 1 recognizes the glucosyl structure of the substrate, while the α-glucosidase type II recognizes the maltosyl structure (Kim *et al*., 2004). The example of the difference of the result between *in vitro* and *in vivo* method could be found in Sabu and Kuttan (2002) evaluation of Triphala, the combination of *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, for their antidiabetic activity and their relation with their antioxidant activity. *Terminalia belerica*, was found to be most active to reduce serum glucose level followed by *E. officinalis* and *T. chebula*. Triphala which is a combination of all the three produced a significant action in reducing the alloxan induced diabetic. The result is slightly different with the result by Anam *et al*. (2009) where, *T. chebula* has a higher α-glucosidase inhibitor activity compared to

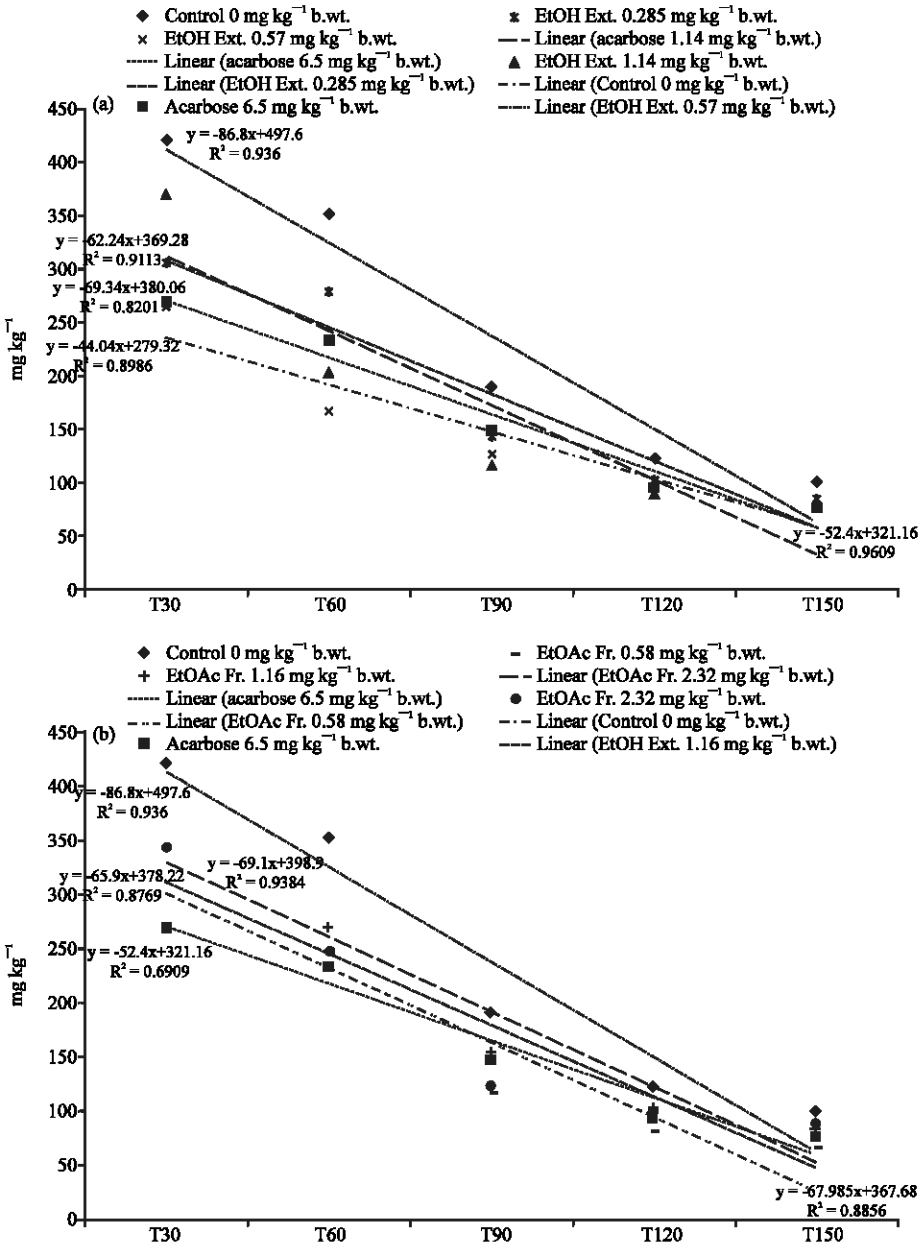

Fig. 5: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with maltose 3 g kg^{-1} b.wt. loading with its regression

T. belerica. This might be due to the location where the plants grew up and the experiment by Sabu and Kuttan was conducted *in vivo*, while in Anam *et al*. (2009), *in vitro* method was used.

Therefore, the *in vitro* test result must be confirmed by *in vivo* method. However, the *in vivo* method used, that is alloxan method, also gains critic due to the artificial way of diabetes induction. Therefore, a reliable method which is used clinically for years that could be applied as experimental model is needed, but some improvement should be made. Therefore, the oral glucose tolerance test was used, with improvement of various loading. Another improvement is the standard reference used in the oral glucose tolerance method. Previous experiments used acarbose for the oral glucose tolerance method with glucose loading, where it should be metformin as the mechanism of metformin is inhibiting the peripheral glucose uptake. Meanwhile, acarbose is used in the oral

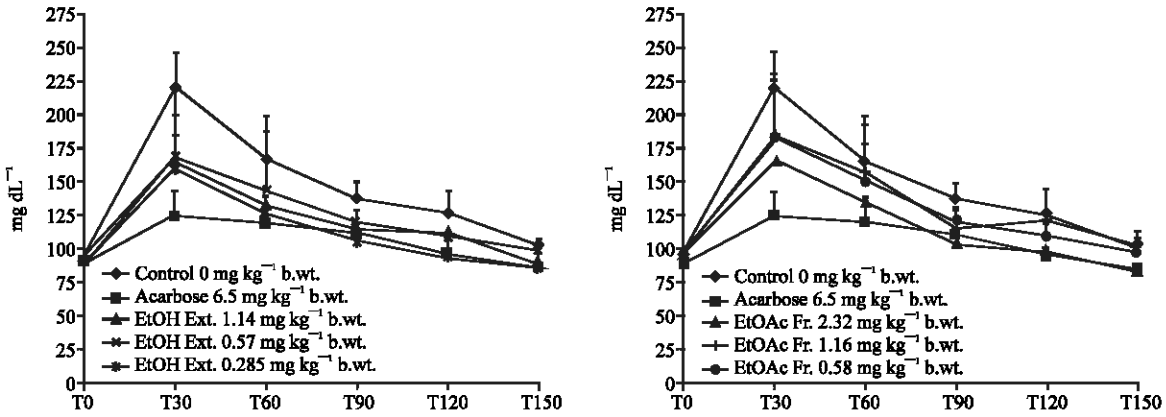

Fig. 6: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with sucrose 3 g kg^{-1} b.wt. loading

glucose tolerance method with amylum, maltose and sucrose loading as acarbose mechanism is to competitively inhibiting an enzyme (α-amylase) located at the brush border of the small intestine that is responsible for terminal carbohydrate digestion. This inhibition decrease glucose absorption, thereby reducing alimentary hyperglycemia and hyperinsulinemia (Hasegawa *et al*., 2008).

Based on the previous anti-diabetes *in vitro* experiment by alpha glucosidase inhibitor, ethanol extract and ethyl acetate fraction are the extract and fraction with alpha-glucosidase inhibition activity, while n-hexane, chloroform, butanol and water fractions do not have alpha glucosidase inhibitory effect. In *in vitro* test, acarbose was not used, but similar reference compound, miglitol, which contains 1-deoxynojirimycin was used. This is due to the polarity of the compound. Miglitol is the name of α-glucosidase inhibitor used to treat diabetes type 2. Miglitol contains 1-deoxynojirimycin. Therefore, 1-deoxynojirimycin is used as a reference compound as it is the most suitable reference compound in the *in vitro* experiment. This reference compound suitability is in accordance with the result of the reference compound used in Shinde's experiment (Shinde *et al*., 2008). For its *in vitro* α-glucosidase inhibitor activity using Baker's yeast and *B. stearothermophilus*, the reference compound 1-Deoxynojirimycin had the IC_{50} value of 83.4±2.1 and 0.175±0.003 μg mL^{-1} respectively, while acarbose showed no inhibition of α-glucosidase in Baker's yeast and *B. stearothermophilus*, but it has the IC_{50} of 210±1.2 and 233±4.0 μg mL^{-1}, respectively for sucrose and maltase in rat intestinal enzymes. It is concluded that acarbose is suitable as the reference compound for *in vivo* experiment and 1-deoxynojirimycin is suitable for *in vitro* experiment, due to the difference of the enzymes involved and the solvability of acarbose and 1-deoxynojirimycin in the solvent (Anam *et al*., 2009).

Based on this preliminary test, in order to confirm the anti-diabetes activity of the extract dan fraction, *in vivo* test using acarbose and metformin as reference was carried out. Acarbose was used in the oral glucose tolerance test with amylum, maltose and sucrose loading, as acarbose is effective for reducing blood glucose caused by polysaccharide or disaccharide administration. Meanwhile, metformin mechanism is the inhibition of the glucose peripheral uptake, therefore, metformin is used as a reference in the oral glucose tolerance test with glucose loading. The dose of carbohydrate given was in accordance with the molecular weight. Therefore, the loading of amylum is the highest between all of the carbohydrates (5 g kg^{-1} of body weight). The dissacharides (maltose and sucrose) dose was lower, 3 g kg^{-1} of body weight and the lowest is glucose, being 2 g kg^{-1} of body weight.

From the regression values, ethanolic extract and ethyl acetate fraction was not similar to the metformin mechanism. Therefore, it is known that ethanolic extract and ethyl acetate fraction mechanism is not by inhibiting the glucose uptake, but resembles acarbose. However, it is also known from the difference of the sucrose and maltose loading, that the extract and fraction was better to inhibit the raise of blood glucose caused by sucrose, rather than by maltose. The result of the sucrose and maltose loading confirms the previous experiment result, which described the α-glucosidase inhibitor property of *Euphorbia hirta* L. herb.

However, in all the loading, ethanolic extract showed a better result rather than the ethyl acetate fraction, which leads us to the conclusion of synergism of the

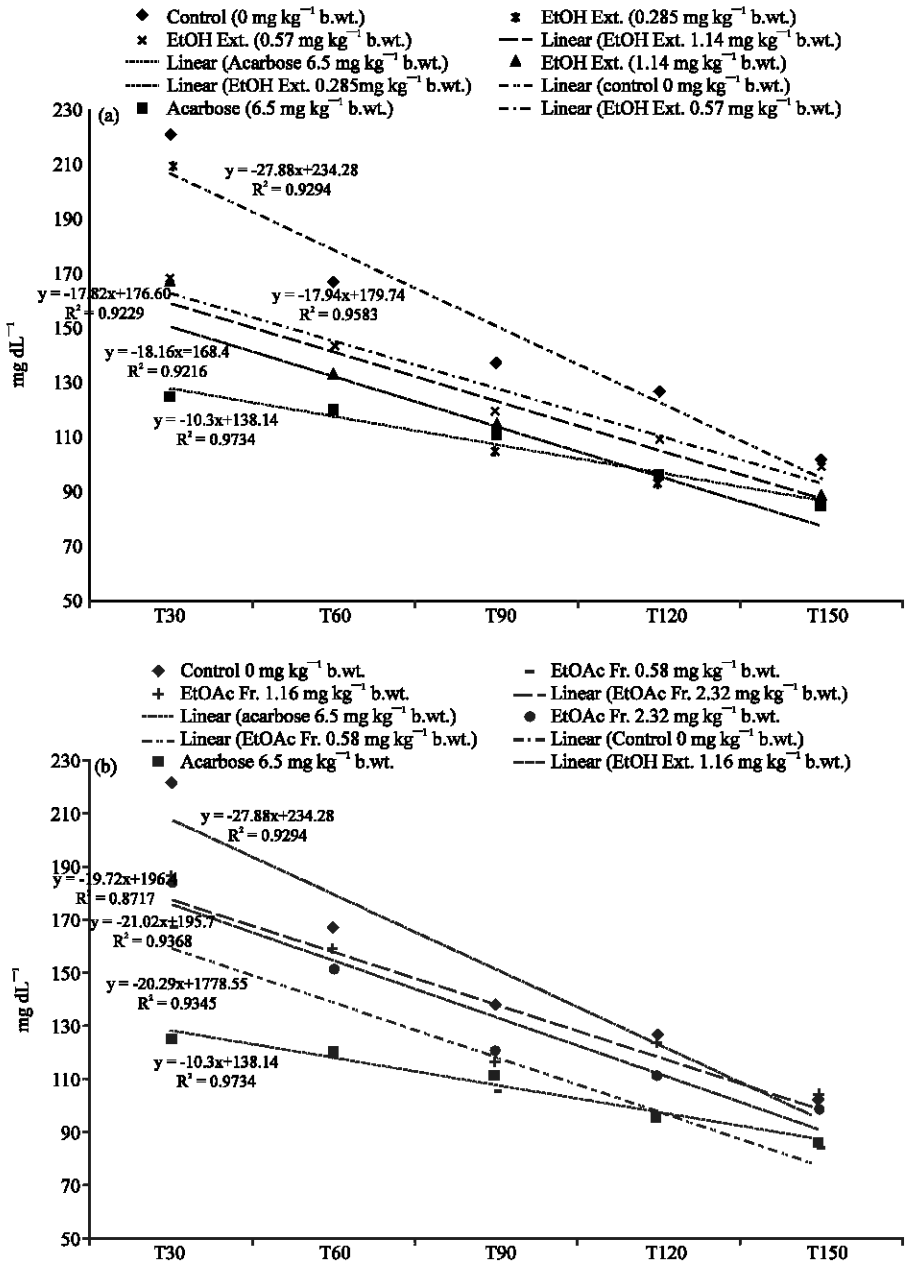

Fig. 7: (a-b)Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with sucrose 3 g kg^{-1} b.wt. loading with its regression

compounds contain in ethanolic extract is responsible for the anti-diabetes activity. The quercitrin contained in the ethyl acetate is known to be aldose reductase inhibitor, which prevents neuropathy and retinopathy as secondary complication of diabetes. Therefore, it is a valuable component of the fraction and it supports the use of the anti-diabetes medicine.

Another interesting finding is related to the characteristic of the herb. The dissolved in acid ash content was found to be high. This might be due to high content of metals. Choudhary and Bandyopadhyay (1999) stated that hypokalemia or potassium depletion could cause glucose intolerance. Calcium, potassium and traces of chromium play an important role in insulin release from the β cells of the Langerhans islet. With these compounds in the medicinal drugs, the plant could help the insulin release from the β cells of the Langerhans islet, which finally helps to lower the blood glucose level. However,

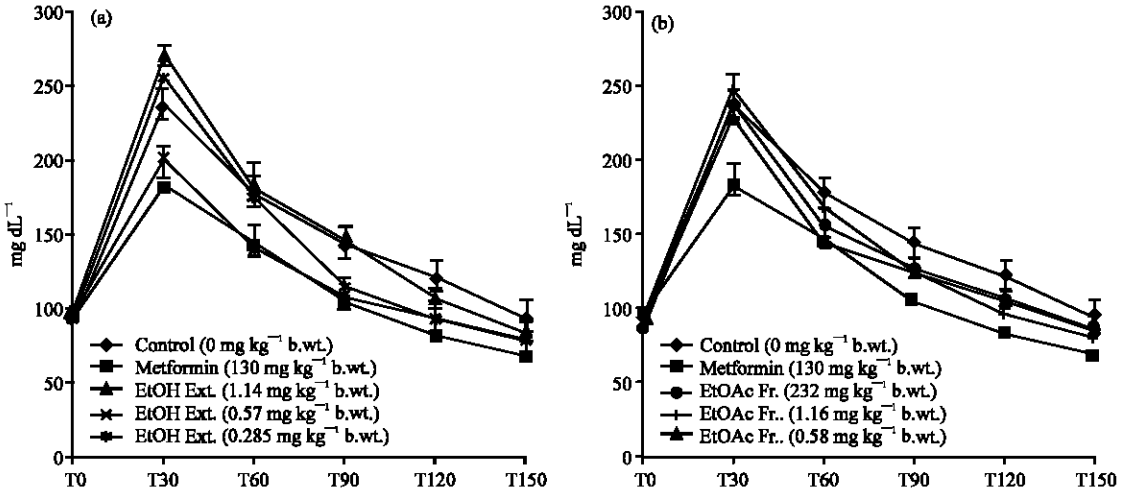

Fig. 8: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with glucose 2 g kg^{-1} b.wt. loading

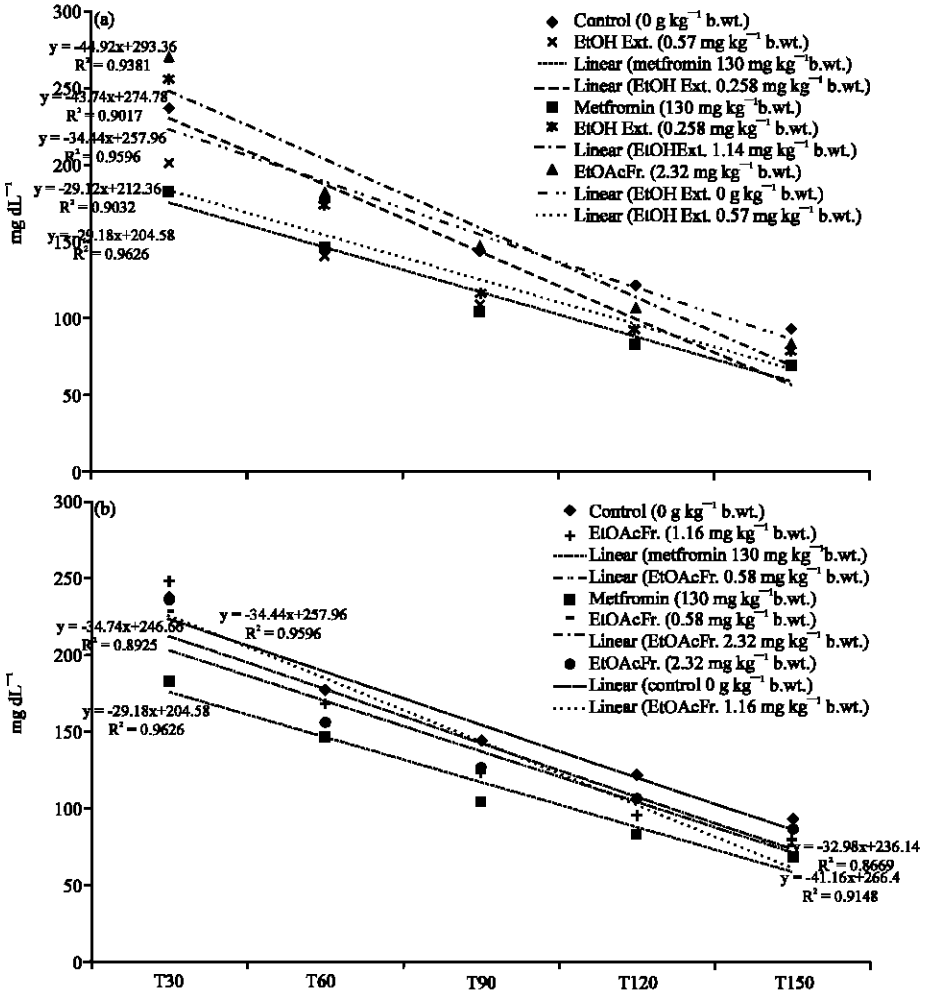

Fig. 9: (a-b) Correlation between the average of mice blood glucose level (mg dL^{-1}) with time in oral glucose tolerance test with glucose 2 g kg^{-1} b.wt. loading with its regression

the content of the metals in *Euphorbia hirta* L. herb was not measured and it could be confirmed in the future to confirm that these compounds also play a role in the anti-diabetes activity of the *Euphorbia hirta* L herb.

Quercitrin was also tested its α-glucosidase inhibitor activity *in vitro* to confirm that quercitrin are some of the compounds responsible for its anti-diabetes activity.

The quercitrin α-glucosidase inhibitor activity *in vitro* was 65.38±0.17 μg mL^{-1}, which is considered as not weak α-glucosidase inhibitor. Therefore, the ethanolic extract IC_{50} value was 7.97±0.26 μg mL^{-1}, which is closer to the IC_{50} value of 1-deoxynojirimycin as standard reference (5.08±0.11 μg mL^{-1}). Therefore, it could be concluded that the ethanolic extract could contain the compounds in ethyl acetate fraction that worked synergistically to exert its anti-diabetes activity.

CONCLUSION

The anti-diabetes activity of the ethanolic extract and ethyl acetate fraction of *Euphorbia hirta* L. is due to the synergism of the compounds contained and several mechanisms are involved, including the antioxidant activity, α-glucosidase inhibitory activity and increasing the activity of insulin release from β cells of the Langerhans islets.

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