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Preliminary Studies of Indonesian *Eugenia polyantha* Leaf Extracts as Inhibitors of Key Enzymes for Type 2 Diabetes

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Eugenia polyantha leaves are widely used in traditional diabetic treatment and as food additives in Indonesia. Extracts of the leaves showed potential inhibitory effect on alpha glucosidase activity *in vitro*. This study is an effort to evaluate the antidiabetic properties of *E. Polyantha* leaves extracts. The methanol-water extracts showed the highest activity (IC_{50} 71 μg mL^{-1}), more than the methanol extracts (92 μg mL^{-1}) and water extracts (73 μg mL^{-1}). The extracts at 100 mg kg^{-1} BW dose also demonstrated a hypoglycemia effect in the *in vivo* assay test against alloxan induced diabetic rats. Repeated bioassay-guided fractionation of the methanol-water extracts afforded two active compounds, 4-hydroxy-3-methoxy benzoic acid and 4-hydroxy-3, 5-dimethoxy benzoic acid, with 27 and 35% inhibition of alpha glucosidase activity, respectively.

Key words: *Eugenia polyantha*, antioxidative, isolation, bioassay guided

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INTRODUCTION

Diabetes mellitus is a major chronic disease caused by imbalance of glucose homeostasis (Rossetti *et al*., 1990). Type 2 diabetes mellitus non-insulin-dependent diabetes mellitus which is one of the main adult diseases is a heterogeneous disease resulting from a dynamic interaction between defects in insulin secretion and insulin action. Diabetes is followed by other clinical disorders which can result in chronic disease. The long-term manifestations of this disease can result in the development of vascular disorders such as retinopathy, nephropathy, neuropathy and angiopathy. In the cardiovascular system, diabetes alters capillary vesicles in the kidney, eye and foot. Complications in the kidney glomelurus capillary system can result in nephropathy diabetes. Changes in the eye's capillary system can cause retinopathy and possibly blindness. Alterations to the brain's capillary system can cause cerebrovascular disease and stroke. Even though the death rate due to diabetes mellitus is lower than that due to heart failure, the consequences remain alarming. People with diabetes need to watch their daily dietary intake as well as insulin intake (ADA, 1994).

Diabetes mellitus has a tendency to be inherited and is mainly caused by an unhealthy life style, especially daily food intake. People with a history of diabetes are more likely to have offspring who are also affected. Diabetes is also triggered by being overweight, minimum physical exercise and alcohol consumption. Based on epidemiological studies, the prevalence of diabetes in Indonesia ranges from 1.4-6% of the total population of 250 million and diabetes patients in Indonesia were estimated to number 8 million in 2010. The diabetes prevalence in the world is also alarming. In 1994, the prevalence was 110.4 million while in 2010, it reached 239.3 million (Tjokroprawiro, 2006). The aim of antidiabetic therapy for IDDM (insulin dependent diabetes mellitus) and NIDDM patients is to achieve normal glycemia and to reduce insulin resistance in NIDDM therapy, thereby improving metabolic control with the intention of preventing late complications (Ye *et al*., 2002). The most beneficial therapy for NIDDM is said to be one that achieves optimal blood glucose control after meals. Among therapeutic drugs to prevent a high blood glucose level are the alpha glucosidase (EC 3.2.1.20) inhibitors. Alpha glucosidase is a membrane-bound enzyme at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharide (De Melo *et al*., 2006). The impending digestion and adsorption of glucose attenuates postprandial plasma glucose level (Alarcon-Aguilara *et al*., 1998).

Indonesia is a tropical country with a huge biodiversity of plant resources that have been used traditionally as medicines. These natural resources play a prominent role in pharmacy, the cosmetics industry and also Indonesian traditional remedies known as jamu. The use of herbs and medicinal plants dates back to the ancient Chinese, Indian and Egyptian. Research has been conducted to understand the pharmacological effects and mechanisms of traditional medicine. *Eugenia polyantha* is widely used in Indonesia as a culinary additive. Its leaves are directly added to cooking and food as a way to enhance flavor. The tincture of the leaves is known to lower blood glucose levels. The bark and stem are known to have anti diarrheal activity.

This study was conducted to evaluate aqueous extracts of *Eugenia polyantha* for activity to inhibit alpha glucosidase *in vitro* as a parameter of antidiabetic. The active compounds present in the leaf extracts are predicted to inhibit alpha glucosidase activity, resulting in a lower postprandial glucose level.

MATERIALS AND METHODS

Plant materials: Leaves of the ten plant species were collected from Ehime Prefecture Garden, Shigenobu town, Toon City, Ehime Prefecture: *Hypericum chinense*; *Thuja orientalis* L., (Cupressaceae), *Datura innoxia*, (Solanaceae), *Ligustrum japonicum* T., (Oleaceae), *Juniperus chinensis* var. *procumbens*, (Cupressaceae), *Gardenia jasminoides* E., (Rubiaceae), *Mallotus japonicus* M., (Euphorbiaceae), *Taxus cuspidate* var. *nana*, *Cinnamonium camphora*, *Eucommia ulmoide*. Pictures of the plants selected are shown in Fig. 1.

The leaves were dried and cut into small pieces. The *Eugenia polyantha* leaves were collected in Sukabumi and Bogor, Indonesia in mid July 2009, with the same type of soil and air-dried prior to extraction. A voucher specimen was deposited in the Laboratory of Plant Chemistry, Faculty of Agriculture, Ehime University, Japan.

Chemicals: Dimethysulfoxide, p-nitrophenyl-D-glucopyranoside, alpha glucosidase enzyme (EC 3.2.1.20) and bovine serum albumin were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). The silica gel used for column chromatography was Wakogel C-200 (Wako Pure Chemical Industries Ltd., Osaka, Japan). TLC (Thin layer chromatography) aluminum sheet (0.25 mm) (Silica gel 60 F254, 20×20 cm) and PLC (preparative thin layer chromatography) glass plates (2 mm) (Silica gel 60 F254, 20×20 cm) were obtained from Merck (Darmastadt, Germany). All solvents were of the highest purity or high performance chromatography (HPLC) grade.

Fig. 1(a-k): Plant leaves for screening of inhibitory effects on alpha glucosidase activity (a) *Thujaorientalis* L., (b) *Daturainnoxia*, (c) *Ligustrum japonicum* T., (d) *Juniperus chinensis* var. *procumbens*, (e) *Gardenia jasminoides* E. *Mallotus japonica*, (f) *Mallotusj aponicus* M., (g) *Taxus cuspidate* var. *nana*, (h) *Cinnamonium camphora*, (i) *Eucommia ulmoides*, (j) *Eugenia polyantha* and (k) *Hypericum chinensea*

Apparatus: Melting points were measured on a Yanaco micro melting point apparatus (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. Ultraviolet (UV) spectra were measured with a Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). HPLC was conducted with a Waters system consisting of a 600 Delta pump controller equipped with a Charatec Sample Loader SL964, Waters module column oven, Waters Photodiode Array Detector 2998, UV-visible Detector 2489 and Fraction collector III. Peak analysis and assignment were conducted using Class-LC10/M10. Mass spectra were recorded at electron energy of 70eV with a direct inlet and on a Shimadzu GC-MS QP 5050A (Shimadzu Corp., Kyoto, Japan).

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay: The free radical-scavenging activity of each extract was determined. Plant extracts were added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was measured under constant mixing at room temperature after 30 min and percent inhibitory activity was calculated from:

$$\frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard.

Beta-carotene bleaching assay: β-carotene (10 mg) was dissolved in 10 mL of chloroform. The carotene-chloroform solution (0.2 mL) was pipetted into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40. The chloroform was removed using a rotary evaporator under reduced pressure at 40°C for 5 min and 50 mL of distilled water was added slowly to the residue with vigorous agitation in order to form an emulsion. A 4.8 mL aliquot of the emulsion was added to a tube containing 0.2 mL of the sample solution and the absorbance at 470 nm was immediately measured against a blank that was an emulsion without β-carotene. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored spectrophotometrically by measuring the absorbance at 470 nm for 60 min. Control samples contained 0.2 mL of ethanol instead of the sample extracts. Ascorbic acid was used as the reference. The antioxidative activity was expressed as percent inhibition with reference to the control after 60 min of incubation using the following formula:

$$AA = 100 \frac{DR_C - DR_S}{DR_C}$$

where, AA is the antioxidative activity; DR_C is the degradation rate of the control:

$$DR_C = \frac{\ln(a/b)}{60}$$

DR_S is the degradation rate in the presence of the sample:

$$DR_S = \frac{\ln(a/b)}{60}$$

where, a is the absorbance at time 0 and b is the absorbance after 60 min.

Inhibition assay for alpha glucosidase activity: The reaction mixture consisting of 500 μL of 20 mM p-nitrophenyl-D-glucopiranoside and 1 mL of phosphate buffer (pH 7.0) with BSA was added to a flask containing 250 μL of sample dissolved in DMSO or water at various concentrations. The mixture was pre-incubated for 5 min at 37°C, the reaction was started by adding 250 μL of alpha glucosidase (EC 3.2.1.20) and incubation was continued for 15 min. The reaction was stopped by adding 2.5 mL of 200 mM Na_2CO_3. The activity of alpha glucosidase was determined by measuring the release of p-nitrophenol at UV max 400 nm. Quercetin was used as a positive control for the alpha glucosidase inhibitor.

Hyperglycemia *in vivo* assay: Wistar male adult rats, weighing from 300-400 g and having normal activity were used as test animal. The test animals were obtained from Veterinary Research Center, Bogor. The test animals were conditioned prior to the test subject to adapt to their new surroundings. Thirty adult rats were divided into 6 groups of treatment. Body weight and glucose blood level were recorded prior to treatment and continued at day 3rd, 7th, 14th and 21st. The hyperglycemia effect of the group was obtained by interperitonial injection of 120 mg kg^{-1} BW of alloxan. The additions of *E. ployantha* extract were conducted on the 3rd day after the rats were diabetically induced. Group A were treated as normal group with induction of NaCl 0.9% w/v and given aquadest as daily liquid intake. Group B were treated as negative control group, whereas given alloxan and aquadest. Group C were treated as positive control group with the alloxan treatment and given Daonil (0.16 mg kg^{-1} BW). The last three groups (D, E and F) were acting as the test groups, with the addition of 100, 200, 300 mg kg^{-1} BW of *E. polyantha* extracts, respectively. Glucose blood level and body weight level were recorded during the treatment.

Screening, extraction and isolation of antdiabetes compounds: Eleven plants, *Hypericum chinense*,

Thujaorientalis L., (Cupressaceae), *Daturainnoxia*, (Solanaceae), *Ligustrum japonicum* T., (Oleaceae), *Juniperus chinensis var. procumbens*, (Cupressaceae), *Gardenia jasminoides* E., (Rubiaceae), *Mallotus japonicus* M., (Euphorbiaceae), *Taxus cuspidate* var. *nana*, *Cinnamonium camphora*, *Eucommia ulmoides* and *Eugenia polyantha* Wight, were screened for antidiabetes activity *in vitro*. *Eugenia polyantha* was most potential and selected for further analysis. *E. polyantha* leaves were air dried, cut into small pieces and extracted with methanol, methanol-water (50:50) and water at 60°C for 8 h. The extracts were concentrated with a rotary evaporator under reduced pressure. Activity-guided isolation was conducted by an alpha glucosidase assay to screen for antidiabetes activity in the aqueous extracts. Fractions showing potential activity were further purified by column chromatography. For the isolation of antidiabetes compounds, the methanol-water extract of *E. polyantha* leaves (100 g) was suspended in water and then partitioned with n-hexane, chloroform, ethyl acetate, n-buthanol and methanol to give the respective solubles. The n-buthanol soluble was found to be the most active fraction. The fractions collected were first checked by TLC and spots were detected under a UV lamp (254 nm). Preparative HPLC and repeated bioassay-guided fractionation using silica gel column chromatography, Sephadex LH-20 and preparative TLC, of the active fractions were conducted to isolate the active compounds.

Statistical analysis: All data are given the Mean±SD of three measurements. Statistical analyses were performed using SPSS 15 Software for Windows. Data were subjected to an Analysis of Variance (ANOVA) followed by the Duncan test.

RESULTS

Screening of plants for inhibitory effects on alpha glucosidase activity *in vitro*: From eleven plants selected, *E. polyantha* Wight demonstrated potential antidiabetic activity through inhibition of alpha glucosidase (95%) and DPPH free radical-scavenging assay (87%). *Hypericum chinensis* and *Mallotus japonicus* were also have high anti alpha glucosidase inhibition assay. The results are shown in Fig. 2.

E. polyantha was subjected to evaluation based on activity, availability and ethenopharmacological use. Extraction using aqueous solvents, i.e., methanol, methanol-water (1:1) and water, was conducted prior to the partitioning using organic solvents. Comparisons of the aqueous extracts are shown in Fig. 3. The methanol-water extract exhibited more activity (IC_{50} 70.90 µg mL^{-1}) than the methanol extract (IC_{50} 91.52 µg mL^{-1}) or water extract (IC_{50} 72.72 µg mL^{-1}).

Hyperglycemia *in vivo* assay: The *E. polyantha* aqueous extracts, demonstrated a potent activity in lowering the glucose blood level in the test animal at a dose of 100, 200 and 300 mg kg^{-1} BW, respectively. In group A, acting as the normal group, the glucose blood level remain stable during the treatment. In group B which is the negative control, the death of test animals occurs in the 3rd day of treatment while in positive control group (C), the glucose blood level decrease 51, 2% on the 21st day of treatment. The treatment group D with 100 mg kg^{-1} BW of *E. polyantha* extract gives the highest impact in lowering the glucose blood level at 53, 45% on the 21st day of treatment, followed by group F treatment of 300 mg kg^{-1} BW *E. polyantha* extract with 42, 4% decrease of glucose blood level and group E treatment of

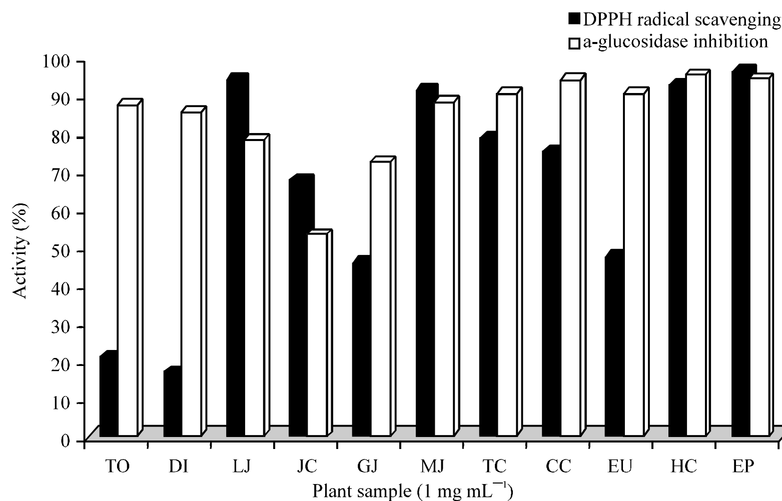

Fig. 2: Screening of selected plants for potential antidiabetic activity

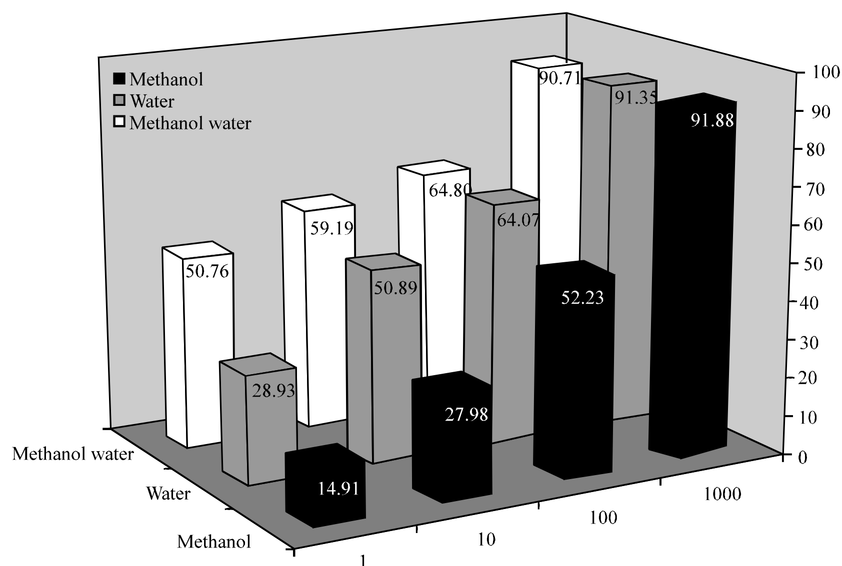

Fig. 3: Inhibition of alpha glucosidase by *E. polyantha* aqueous extracts *in vitro*

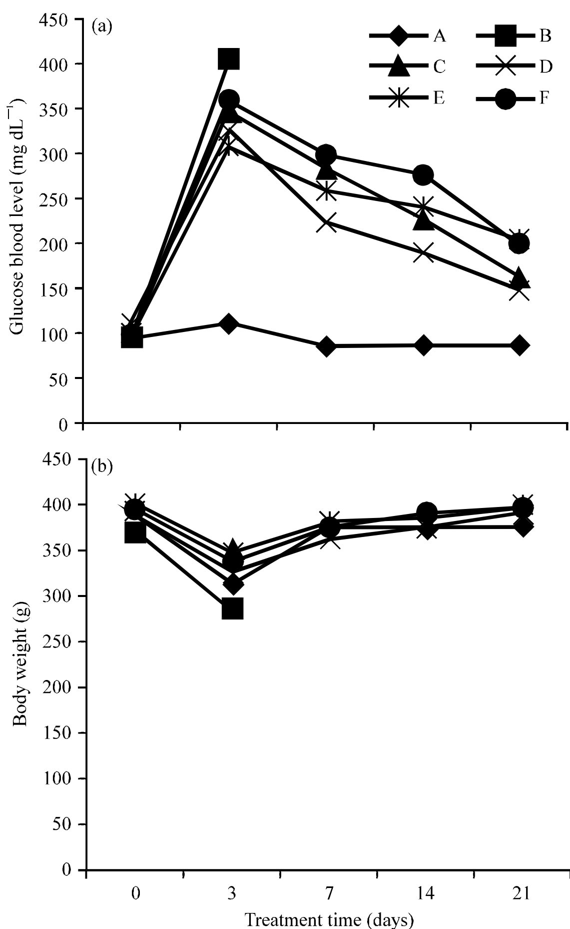

Fig. 4(a-b): (a) The mean of test animal body weight and (b) Glucose blood level during treatment

200 mg kg^{-1} BW of extract with 34, 5% of lowering glucose blood level in the test animal. The change of glucose level and body weight during the 21 day of treatment of the six test groups are presented in Fig. 4a, b.

Isolation of antidiabetes compounds: The methanol-water extract of *E. polyantha* leaves was suspended in water and then partitioned with n-hexane, chloroform, ethyl acetate, n-buthanol and methanol, to give the respective solubles. Each of the solubles was subjected to an *in vitro* alpha glucosidase assay. One fraction; n-buthanol demonstrated potential activity towards alpha glucosidase assay.

HPLC of n-buthanol solubles was conducted to separate the active fractions. Three hydroxyl benzoic acid derivatives were fractionated using a HPLC preparative system from the *E. polyantha* methanol-water extract, i.e., 3, 4, 5-trihydroxy benzoic acid, 4-hydroxy 3-methoxy benzoic acid and 4-hydroxy 3, 5-dimethoxy benzoic acid. The HPLC profile is shown in Fig. 5.

The active compound isolated from methanol-water extract had a similar basic structure that is, phenolic with a benzoic acid moiety. To evaluate the structure-activity relationship of the compounds, similar compounds were tested for antioxidative properties using DPPH free radical scavenging and beta carotene bleaching protection (BC) and antidiabetic activity using inhibition of alpha glucosidase activity (AG), the results are presented in Table 1.

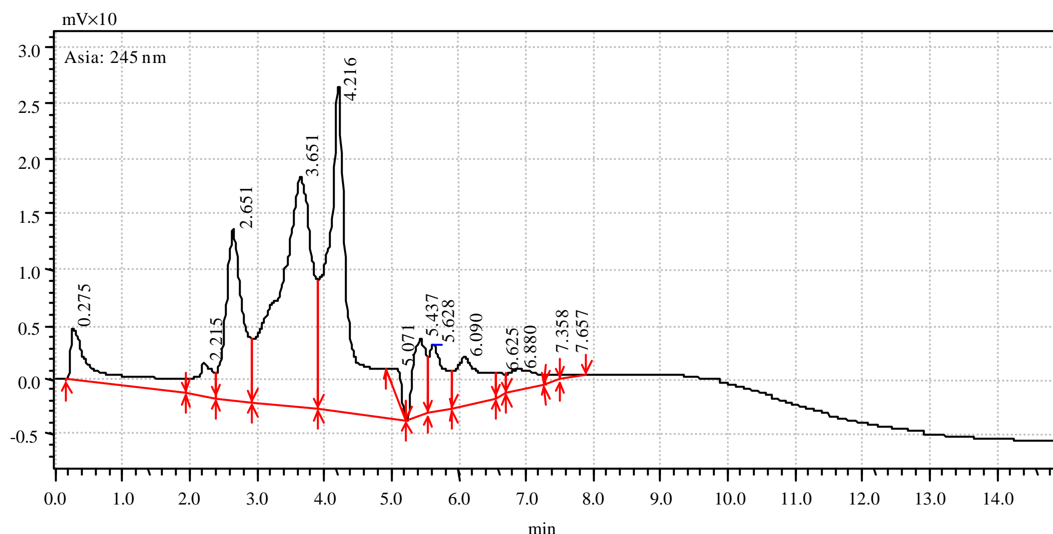

Fig. 5: HPLC profile of *E. polyantha* n buthanol soluble

Table 1: Antioxidative and antidiabetic activity of isolated compounds

Sample	Σ OH	DPPH (%)	BC (%)	AG (%)
3,4,5-trihydroxy benzoic acid	4	95	-21	20
3,5-dihydroxy benzoic acid	3	11	14	25
Ellagic acid	4	95	86	-61
Pyrogallol	3	94	3	-19
4-hydroxy-3-methoxy benzoic acid	2	6	-7	27
4-hydroxy-3, 5-dimethoxy benzoic acid	2	89	44	35
Buthylated hydroxyl toluene	1	70	90	-35
Eugenia polyantha MeOH-water	*na*	95	70	62

DISCUSSION

Eugenia polyantha leaves are widely used in traditional medicine to treat diabetic and as food additives in Indonesia. Although, reported that *E. polyantha* has high antioxidative properties, evidence of the antidiabetic properties and lowering glucose blood level effect is still lacking. The aim of this study is to evaluate aqueous extracts of *Eugenia polyantha* for activity to inhibit alpha glucosidase *in vitro* as a parameter of antidiabetic properties. Three plants i.e., *Hypericum chinensis*, *Mallotus japonicus* and *Eugenia polyantha* from eleven screened plants showed the most potent antidiabetic and antioxidative properties (Fig. 2). *E. polyantha* was selected to evaluation based on activity, availability and ethenopharmacological use. *E. polyantha* extracts demonstrated potential antidiabetic activity in dose-dependent manner. The methanol-water extracts showed the highest activity (IC_{50} 71 μg mL^{-1}), more than the methanol extracts (92 μg mL^{-1}) and water extracts (73 μg mL^{-1}). The high phenolic content in the *E. polyantha* extract (Lelono *et al*., 2009) is correlated to the antioxidative properties and possible also responsible to the inhibition of alpha glucosidase activity. In this study we found that free ellagic acid and pyrogallol did not demonstrate any alpha glucosidase inhibition activity while ellagic acid derivatives; 3,4'-di-O-methylellagic acid 3'-O-beta-D-xylopyranoside (Tabopda *et al*, 2008) showed significant activity towards alpha glucosidase. The presence of gallic acid and its benzoic acid derivatives (Li *et al*., 2008) and polyphenols (Hanamura *et al*., 2006) have significance role in alpha glucosidase inhibition activity, whereas from the n-buthanol solubles of *E. polyantha* we afforded 3, 4, 5-trihydroxy benzoic acid, 4-hydroxy 3-methoxy benzoic acid and 4-hydroxy 3, 5-dimethoxy benzoic acid. 4-Hydroxy-3, 5-dimethoxy benzoic acid (35%) showed the highest level of inhibition followed by 4-hydroxy-3-methoxy benzoic (25%) acid and 3, 4, 5-trihydroxy benzoic acid (20%). As a mixture, the three compounds inhibited 42.38% of alpha glucosidase activity. It is suggested that gallic acid, 4-hydroxy-3-methoxy benzoic acid and 4-hydroxy-3, 5-dimethoxy benzoic acid act synergistically on alpha glucosidase activity. The presence of other active compounds in the fraction should be evaluated further in an attempt to clarify the potential effects. *E. polyantha* posses the antidiabetic effect through lowering glucose blood level and also posess strong free radical scavenging

activity and beta carotene bleaching protection. Alpha-glucosidases are an enzymes located on the intestinal brush-border which are important carbohydrates in food, are hydrolyzed to monosaccharide, such as glucose and fructose, by the enzyme and then absorbed into the blood, increasing the glucose blood level (Shen *et al*., 1998). Every group of test animals suffered from weight loss on the third days due to environmental stress, decoction and injection of 120 mg kg^{-1} BW alloxan (Szkudelski, 2001). According to Duncan test, the body weight of normal control has no significance difference to the the control treated with 100 mg kg^{-1} BW of *E. polyantha* extracts, but differs from treatment in group B, C, E and F ($p<0.005$). The highest lowering impact of glucose blood level during the 21 day of treatment also demonstrated by the decoction of 100 mg kg^{-1} BW of *E. polyantha* extract. This showed that decoction of 100 mg kg^{-1} BW of *E. polyantha* extracts could act as the optimum concentration of antidiabetic treatment. These finding of alpha glucosidase inhibition activity is a correlation of the lowering effect of the glucose blood level are in accordance of findings by Heinz *et al*. (1989).

CONCLUSION

The extracts of *Eugenia polyantha* leaves exhibited potential antidiabetic effects through the inhibition of alpha glucosidase activity and lowering glucose blood level. Bioassay-guided separation of the active fraction indicated phenolic acid with a benzoic acid moiety; 3, 4, 5-trihydroxy benzoic acid, 4-hydroxy 3-methoxy benzoic acid and 4-hydroxy 3,5-dimethoxy benzoic acid to be the most antidiabetic active compounds. The number and the position of the hydroxyl groups affect the antioxidative and antidiabetic activity.

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