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*Research Paper*

# Potent α-glucosidase inhibitor and anti-glycemic agent from *Eclipta alba*

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**Abstract:** Decreasing postprandial hyperglycemia by inhibition of α-glucosidase is one of the effective therapeutic approaches in treatment of diabetes mellitus. Two coumestans; wedelolactone and nor-wedelolactone were purified from the aerial part of *Eclipta laba* during bio activity guided screening for α-glucosidase inhibitors. $IC_{50}$ values of wedelolactone and nor-wedelolactone were 46.6 and 432.5 µM against *Saccharomyces cerevisiae* α-glucosidase. Further, wedelolactone and nor-wedelolactone strongly inhibited rat-intestinal sucrase ($IC_{50}$ of 22.2 and 238.3 µM respectively) and rat-intestinal maltase ($IC_{50}$ of 20.2 and 360.6 µM respectively). The inhibition mechanism of these compounds was examined by measuring enzyme activity in different concentrations of substrate for Lineweaver–Burk plot analysis. Wedelolactone exhibited intensive inhibitory effect on α-glucosidase. Its inhibition was found to be noncompetitive. Oral administration of wedelolactone (10 to 100 mg/kg body wt.) significantly decreased blood glucose after maltose loading in normal and diabetic rats in a dose-dependent manner. These results suggest that wedelolactone might exert an anti-diabetic effect by suppressing carbohydrate absorption from the intestine and can reduce the postprandial glucose level.

## Introduction

Diabetes mellitus is a most serious and chronic disease caused by an absolute or relative lack of resistance to insulin and is characterized by hyperglycemia in the postprandial and/or fasting state. Incidence rates of diabetes mellitus are increasing with increasing level of obesity and also with aging of the general population over the world. Currently, an estimated 150 million people worldwide have diabetes and that this will increase to 220 million by 2010 and 300 million by 2025 [1]. Globally, type II diabetes (non insulin dependent diabetes mellitus) accounts for greater than 90% of the cases [2, 3]. Postprandial hyperglycemia plays an important role in development of type II diabetes and complications associated with the disease, such as micro- and macrovascular diseases [4]. The best way to control the postprandial plasma glucose level is to medicate in combination with dietary restriction and an exercise program [5]. Although several drugs for type II diabetes exist today, they have drawbacks such as liver toxicity and adverse gastrointestinal symptoms, thereby raising the symptoms and risk factors of heart disease [3].

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One therapeutic approach to decrease postprandial hyperglycemia is to retard absorption of glucose via inhibition of carbohydrate-hydrolysing enzyme, α-glucosidase, in the digestive organs [6]. Glycosidases are well known targets in the design and development of antidiabetic, antiviral, antibacterial, and anticancer agents. In type II diabetes, delaying glucose absorption after meals by inhibition of α-glucosidase is known to be beneficial in therapy [3]. α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is an exo-type carbohydrase that catalyzes the liberation of D-glucose from the non-reducing end of the substrate. This enzyme is widely distributed in microorganisms, plants, and animal tissues, although the substrate specificity of α-glucosidase differs greatly depending on the source [7]. Three types of α-glucosidase inhibitors exist: polyhydroxylated N-substituted heterocyclic compounds; polyhydroxylated cycloalkenes; and oligomers of pseudosugars. Most inhibit α-glucosidases by mimicking the pyranosyl moiety of the α-glucosidase. There are reports of α-glucosidase inhibitors such as acarbose [8, 9] and voglibose [10] from microorganisms and nojirimycin and 1-deoxynojirimycin from plants [11, 12, 13].

*Eclipta alba* is known to contain coumestan derivatives such as wedelolactone and demethylwedelolactone [14], thiophene derivatives like ecliptal [15]. Various dithienylacetylene esters (I, II, III) [16] are reported in root part. Saponin compounds like eclalbosaponins I-IV, Alkaloids [17], triterpene saponin eclalbatin [18], oleanane type triterpenoids, echinocysticacid, eclalbasaponin II, eclalbasaponin V, eclalbasaponin I and eclalbasaponin III [19] are also reported.

The objectives of this study were to; screen α-glucosidase inhibitors from *Eclipta aba*; to analyze the structure and biological activity of α-glucosidase inhibitors; and to compare the results with known α-glucosidase inhibitors.

## Materials and methods

*Reagents*

p-Nitrophenyl a-D-glucopyranoside (pNPG), *S. cerevisiae* α-glucosidase, rat intestinal acetone powder were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose assay kit was procured from Reckon Diagnostics, Vadodara, India. Sephadex LH-20 and Sephacryl HR-100 columns were purchased from Pharmacia Biotech Ltd. (Uppsala, Sweden). All other chemicals used in this study were of analytical grade.

*Plant material*

Fresh plant of *Eclipta alba* Hassk. was collected in the month of January 2011 from Munjaka village, Rajkot. The herbarium was prepared & preserved at Pharmacognosy Laboratory, Atmiya Institute of Pharmacy, Rajkot, Gujarat. The specimen was authenticaed by Dr. A. S. Reddy (Taxonomist), Bioscience department, Sardar Patel University, Vallabh vidhyanagar, Gujarat to meet their taxonomic identity (Specimen no. Ea-05). Aerial parts of the plant were separated and dried under shade at room temperature. Dried plant was ground in domestic grinder and sifted with a #10 sieve.

*Extraction and isolation of wedelolactone and nor-wedelolactone*

Plant powder (1.2 kg) was soaked in 2% acetic acid in methanol (2.5 l) and extracted under conditions where the solvent was heated until reflux began, this being maintained for 8 h. Extract was filtered and the marc was soaked in 2% acetic acid in methanol (1.0 l) and extracted as above. The extract was mixed with filtrate of previous step and filtered using Whatman No. 42 paper. Filtrate was then evaporated to 200 ml under reduced

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pressure at a temperature of 40 °C. The *E. alba* extract (200 ml) was added to the same amount of distilled $H_2O$. The whole (400 ml) was then partitioned using diethyl ether (400 ml) by standing for 30 min at room temperature after vigorous shaking, which was repeated five times until diethyl ether fraction was no longer fluorescent under long UV light (366 nm). Diethyl ether was evaporated to get concentrated solution (100 ml). On cooling at 0-4 $^0$C, a greenish yellow precipitates formed at the bottom of flask were separated. Further purification was performed by preparative TLC using silicagel G as stationary phase and tolene:acetone:formic acid (11 : 6 : 1 v/v/v) as mobile phase. Wedelolactone and nor-wedelolactone was identified at $R_f$ 0.56 and 0.45 respectively as bright blue fluorescent band under long UV light and eluted with methanol.

*Assay for α-glucosidase inhibitory activity*

The inhibitory activity of α-glucosidase was determined according to the modified method of Kurihara et al. [20]. A 3 mM p-nitrophenyl α-D-glucopyranoside (0.01 ml) and 20 U/ml α-glucosidase (0.01 ml) in 0.01 M phosphate buffer (pH 7) were added to the sample solution (2.2 ml) to start the reaction. Each reaction was carried out at 37 °C for 30 min and stopped by adding 0.1 M $Na_2CO_3$ (2 ml). Enzymatic activity was quantified by measuring absorbance at 405 nm. One unit of α-glucosidase activity was defined as amount of enzyme liberating p-nitrophenol (1.0 µM) per min. The $IC_{50}$ value was defined as the concentration of α-glucosidase inhibitor that inhibited 50% of α-glucosidase activity.

*Purification of rat intestinal α-glucosidase*

Rat intestinal α-glucosidase was purified according to the modified method of Kurihara et al. [20]. Commercial rat intestine acetone powder (10 g) was dissolved in buffer A (100 ml) (0.1 M potassium phosphate buffer containing 5 mM EDTA, pH 7.0), sonicated at 4 °C for 15 s, and then centrifuged at 27,000g at 4 °C for 60 min to obtain supernatant A. The precipitate was dissolved in buffer A (100 ml), sonicated, and then centrifuged (32,000g, 60 min, 4 °C) to obtain supernatant B. The combined supernatant was dialyzed against buffer B (0.05 M potassium phosphate buffer containing 0.4 mM EDTA, pH 7.0) for 48 h. This was concentrated to 20 ml by ultrafiltration (cut off membrane 10 kDa) and then loaded onto a Sephacryl HR-100 column (2.6 × 60.0 cm) equilibrated with buffer B in advance. Rat intestinal α-glucosidase was eluted with buffer B at a flow rate of 0.2 ml/min.

*Inhibitory assay for rat intestinal α-glucosidase (sucrase and maltase) activity*

Rat intestinal α-glucosidase inhibitory activity was determined according to the modified method of Kurihara et al [20]. Sucrase activity was determined in a mixture of 500 mM sucrose (0.1 ml), the isolated compound in DMSO (0.05 ml), and 0.1 M maleate buffer (pH 6.0, 0.75 ml). The mixture was pre-incubated at 37 °C for 5 min, and reaction was initiated by adding rat intestinal α-glucosidase (0.1 ml) to the reaction mixture. The mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 2.0 M maleate–Tris–NaOH buffer (pH 7.4, 1.0 ml). To measure maltase activity, maltose (500 mM) was used instead of sucrose. The glucose release in the solution was determined using a glucose assay kit based on the glucose oxidase/peroxidase method.

*Kinetics of α-glucosidase inhibitors*

The enzyme-inhibitory reaction was performed according to the mentioned above at varying concentrations of substrate (sucrose and maltose) in the absence and presence of test samples. The results were calculated according to Michaelis-Menten kinetics [21, 22]. The type of inhibition was determined using the Lineweaver–Burk plot [23].

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*Identification of α-glucosidase inhibitors*
The structures of isolated compounds were identified by comparing the spectroscopic data with that of reported in literature. Further, purity was assessed by thin layer chromatography of isolated compounds.

*Animals*
Male Wistar rats weighing 220–250g each were kept in individual cages, maintained under 12/12h light/dark cycles at (22–25 °C). They were fed commercial stock diet and water. Rats were deprived of food for 16h before experimentation, but allowed free access to tap water throughout the experiment. Maltose, voglibose and wedelolacrone were dissolved in distillated water and administered orally by intra-gastricroute (1ml) [24].

*Hypoglycemic activity of wedelolactone in normal rats*
Rats were classified into three groups of six animals each. Group 1 was treated with 2g/kg body wt. maltose as the control group. Group 2 was treated with 2g/kg body wt. maltose and 3 µg/kg body wt. voglibose. Group 3 was treated with 2g/kg body wt. maltose and one dose of 10 µg/kg body wt. of wedelolactone. Blood glucose level was determined just before and 30, 60, 120, 180 and 300 min after the administration of maltose, using an Accu-Cheks Glucometer (Roche Diagnostics India Pvt. Ltd., Mumbai, India)

*Induction of diabetes*
For induction of diabetes, 50 mg/kg body wt. streptozotocin (Sigma, USA) was dissolved in citrate buffer, pH 4.5, and was injected by a single intraperitoneal injection in rats previously fasted for 12h. Animals with fasting blood glucose over 250 mg/dl, 4 days after streptozotocin administration, were considered diabetic. Treatment of diabetic rats was entirely similar to normal rats.

*Hypoglycemic activity of wedelolactone in diabetic rats*
Rats were sorted into three, six-animal groups. They were treated entirely similar to the normal rats, as explained earlier.

*Dose dependency of hypoglycemic activity in diabetic rats*
Animals were divided into six groups of six animals each. Group 1 was treated 2g/kg body wt. maltose as the control group. Group 2 was treated with 2g/kg body wt. maltose and 3 µg/kg body wt. voglibose. Groups 3–6 were treated with 2g/kg body wt. maltose and doses of 10, 25, 50 and 100 mg/kg body wt. wedelolactone, respectively. Blood glucose levels were assayed at 0 (before administration), 30, 60, 120, 180 and 300 min after the administration of maltose, using an Accu-Cheks Glucometer.

*Statistical analysis*
All data represented as mean±SEM. Statistical analysis was performed by analysis of variance (ANOVA) and Post-Hoc Tukey test; p-values of less than 0.05 were considered to be significant.

## Results and Discussion

*Isolation and identification of α-glucosidase inhibitors*
Alcoholic extract of *E. alba* was preliminarily screened for α-glucosidase inhibitory activity. The α-glucosidase inhibition by methanol extract of *E. alba* at 1 mg/ml was 56.3%. Thin layer chromatography screening of diethyl ether fraction confirmed the presence of phenolic compounds when sprayed with ferric chloride solution (green coloured bands) [20]. Two kinds of positive spots were confirmed and classified as compound 1 (1.83 %w/w) and compound 2 (0.16 %w/w), respectively. Compound 1 and 2 were identified as wedelolactone and demethyl wedelolactone (Figure 1) by performing co-TLC and HPLC (Figure 2) with that of standard samples.

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*Assay for α-glucosidase inhibitory activity in vitro*

The α-glucosidase of *S. cerevisiae* was used to investigate the inhibitory activity of the isolated compounds. α-Glucosidase inhibitory activity isolated compounds against α-glucosidases was determined using pNPG as a substrate and this was compared with voglibose. The $IC_{50}$ values of wedelolactone and demethyl wedelolactone against *S. cerevisiae* α-glucosidase were 46.6 and 432.5 µM, respectively. The positive control, voglibose showed $IC_{50}$ value 11.6 µM (Table 1). The findings were contrary to the previous reports by Haslam [25], Stern et al. [26], and Cogoli and Semenza [27] which claimed that voglibose had high inhibitory effects on mammalian α-glucosidase, but no inhibitory activity against *S. cerevisiae* α-glucosidase. A study by Blanco and Iturbe [28] showed that (+)-catechin, an inhibitor of *S. cerevisiae* α-glucosidase, had no inhibitory effect on enzymes from mammalian species. In the present study, compounds from *E. alba* were shown to be effective inhibitors against *S. cerevisiae* α-glucosidase. Wedelolactone exhibited very powerful inhibitory activity as compare to demethoxy wedelolactone. The methoxy groups of wedelolactone may, therefore, have an important role in promoting inhibitory activity. Therefore, wedelolactone should bind to active or binding sites of the enzymes, resulting in inhibition of the enzyme activity.

*Inhibitory activity against rat-intestinal sucrase and maltase*

The inhibitory activities of *E. alba* compounds against rat intestinal sucrase and maltase were also compared with those of voglibose (Table 1). The $IC_{50}$ values of wedelolactone and demethyl wedelolactone were 22.2 and 238.3 µM against sucrase and 20.2 and 360.6 µM against maltase, respectively; thus, demethyl wedelolactone was a less effective inhibitor than wedelolactone. The $IC_{50}$ value of voglibose was 15.1 and 9.3 µM against sucrase and maltase, respectively. Potency of wedelolactone was equivalent to that of voglibose against mammalian α-glucosidase.

*Enzyme kinetic parameters*

Kinetic parameters like $K_m$ and $V_{max}$ have been estimated to understand the nature of inhibition of α-glucosidase by isolated compounds. Table 2 lists the kinetic parameters of α-glucosidase *in vitro*. Data analysis revealed that in case of sucrose as substrate, $V_{max}$ decreased ($P < 0.001$) by 0.42 and 0.3 mM/30 min in the presence of the wedelolactone and demethyl wedelolactone, respectively. Similarly, in case of maltose as substrate, $V_{max}$ decreased ($P < 0.001$) by 0.79 and 0.69 mM/30 min in the presence of the wedelolactone and demethyl wedelolactone, respectively. The apparent $K_m$ remained unaltered ($P > 0.05$) with wedelolactone and demethyl wedelolactone. It indicates that wedelolactone and demethyl wedelolactone inhibits α-glucosidase in a non-competitive manner.

*Acute hypoglycemic activity in normal rats*

Wedelolactone was first evaluated in an acute model, to check its hypoglycemic action in normoglycemic rats. Wedelolactone (10 µg/kg) induced a significant ($P < 0.01$) decrease of blood glucose concentration (approx. 15 mg/dl) at 60 min. Acute effect of oral administration of wedelolactone (10 µg/kg) and voglibose (3 µg/kg) on blood glucose is shown in Figure 3a.

*Dose dependent hypoglycemic activity in diabetic rats*

As shown in Figure 3b wedelolactone induced a significant decrease in the glycemic response, at a dose of 10 µg/kg compared to the control ($P < 0.01$). Dose dependent hypoglycemic effect was observed. The postprandial blood glucose was lower at 60 min and this activity was

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similar during 120, 180 and 300 min post-ingestion of carbohydrate (maltose). The blood glucose concentration lowering effect of wedelolactone was slightly lower than voglibose, a therapeutic drug used as positive control (3 µg/kg).

In previous studies, most mammalian α-glucosidase inhibitors did not effectively inhibit microbial α-glucosidases, whereas catechin, an inhibitor of *S. cerevisiae* α-glucosidase, did not inhibit mammalian α-glucosidases [28]. Therefore, the higher mammalian α-glucosidase inhibitory activity of voglibose compared to isolated compounds is likely due to the substrate specificities that depend on the source of α-glucosidases. This suggests that binding of phenolic compounds is less specific to the enzyme because they can bind to various proteins included in the crude enzyme solution. Bacterial, yeast, and insect enzymes (α-glucosidase I) show higher activity toward heterogeneous substrates such as sucrose and pNPG, and either no or less activity toward homogeneous substrates such as malto oligosaccharides; this implies that α-glucosidase I recognizes the ‘‘glucosyl structure” in the substrate.[25)] The mold, plant, and mammalian enzymes (α-glucosidase II) hydrolyze homogeneous substrates more rapidly than heterogeneous substrates, indicating that this class of α-glucosidases recognizes the ‘‘malto structure” [29]. The hydrolysis of p-nitrophenol 2-deoxya-D-arabino-hexopyranoside was catalyzed by α-glucosidase II [30], but no such reaction was observed with α-glucosidase I, suggesting that the 2-OH groups in the glucose moiety are essential for α-glucosidase I [31]. The α-glucosidase II catalyzed the hydration of D-glucal to produce 2-deoxy-α-D-arabino-hexose, but α-glucosidase I yielded no detectable hydration product [32].The strong enzymatic inhibitory activity against microbial α-glucosidases shown by wedelolactone and demethylwedelolactone, which is equivalent to that of commercial inhibitors such as voglibose at low concentration, will decrease the blood glucose level, adverse gastrointestinal effects, and abdominal discomfort caused by voglibose [3]. Therefore, wedelolactone can potentially be developed as novel natural nutraceuticals because of its high inhibitory activity against α-glucosidase. However, since the results reported herein were obtained *in vitro*, further studies need to be conducted *in vivo*. This is because if the structure of algal compounds is degraded in the human body by stomach acid or digestive enzymes, their inhibitory activity could be altered.

## Conclusion

In conclusions, reduction of post prandial hyperglycemia via inhibition of intestinal α-glucosidase is one of the therapeutic approaches for preventing diabetic complications. The search for α-glucosidase inhibitors from plant source is important because they are expected to suppress the postprandial hyperglycemia of diabetic patients. In this study, α-glucosidase inhibitors were isolated from aerial part of *Eclipta alba*. Two coumestans with α-glucosidase inhibitory activity wedelolactone and demethyl wedelolactone were isolated and identified. These compounds exhibited a non-competitive type of inhibition against *S. cerevisiae* α-glucosidase. Wedelolactone resulted in as higher inhibitory activity than demethyl wedelolactone. Similar was true against rat-intestinal sucrase and maltase. *In vivo* results supported the antiglycemic activity on normal and diabetic rats. Therefore, wedelocatone can potentially be developed as a novel natural nutraceutical to prevent diabetes mellitus because of their high α-glucosidase inhibitory activity.

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**Table 1: Sucrase and maltase inhibitory activity.**

| Sample | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | Against *S. cerevisiae* α-glucosidase | Against rat-intestinal sucrase | Against rat-intestinal maltase |
| Voglibose | 11.6 | 15.1 | 9.3 |
| Wedelolactone | 46.6 | 22.2 | 20.2 |
| Demethyl wedelolactone | 432.5 | 238.3 | 360.6 |

**Table 2. Effect of wedelolactone and demethylwedeloactone on enzyme kinetic parameters**

| Substrate | Kinetic Parameter | Control (no treatment) | Voglibose | Demethyl wedelolactone | Wedelolactone |
|---|---|---|---|---|---|
| Sucrose | $V_{max}$ | 1.280 ± 0.026 | 1.280 ± 0.010 | 0.980 ± 0.046* | 0.860 ± 0.055* |
| | $K_m$ (mM) | 0.350 ± 0.022 | 0.430 ± 0.050 | 0.350 ± 0.040 | 0.350 ± 0.071 |
| Maltose | $V_{max}$ | 1.530 ± 0.032 | 1.526 ± 0.010 | 0.840 ± 0.043* | 0.740 ± 0.033* |
| | $K_m$ (mM) | 0.370 ± 0.022 | 0.420 ± 0.070 | 0.364 ± 0.039 | 0.372 ± 0.068 |

Substrates were studied in the concentration range of 10 – 50 μg/mL.
$V_{max}$ is reported as mM glucose formed/30 min.
* indicate a statistically significant difference from the values in the absence of sample ($p<0.001$). Values are mean ± standard error mean (n=5).

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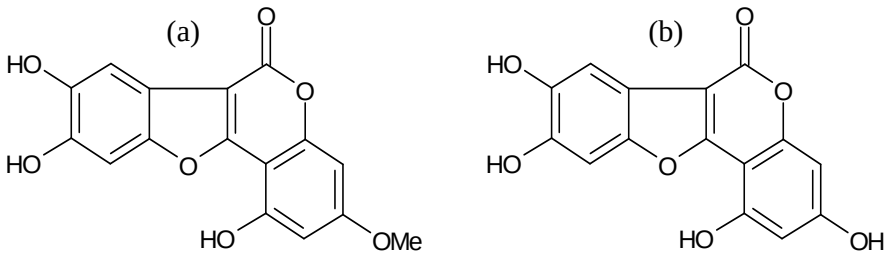


Figure 1: Chemical structure of (a) wedelolactone and (b) demethyl wedelolactone.

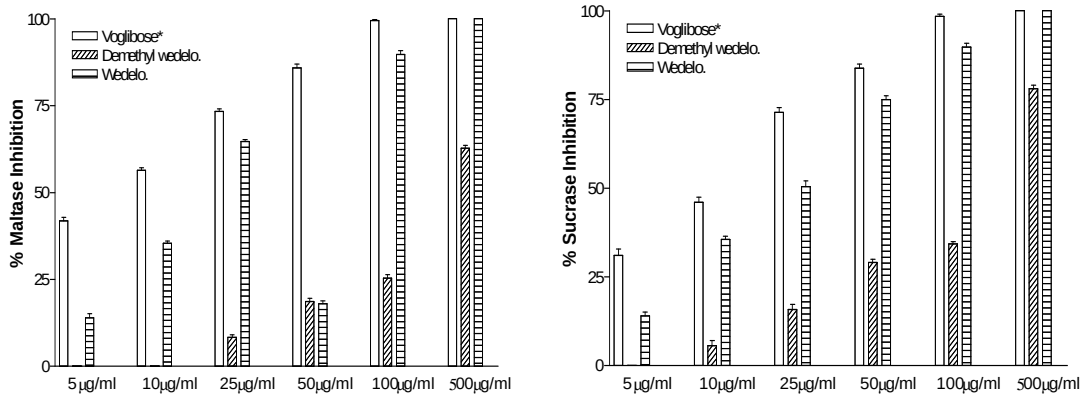


Figure 2: Maltase and sucrase inhibitory effect of wedelolactone and demethyl wedelolactone.

* Voglibose studied in the conc. range of 0.5 – 50 µg/mL.

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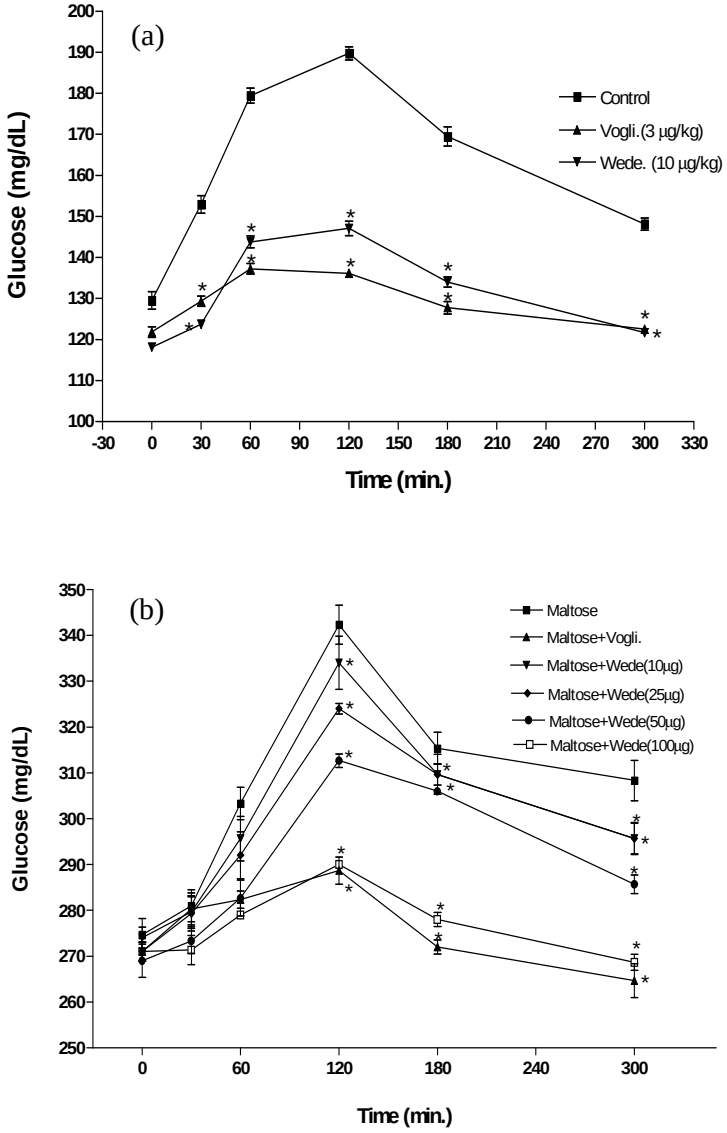


Figure 3: Hypoglycemic effect of wedelolactone in (a) normal rats administered with maltose (2 gm/kg) (b) diabetic rats administered with maltose (2 gm/kg); a dose dependent effect of wedelolactone (10-100 µg/kg) on blood glucose level.

* $p<0.05$ when compare with the control.

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