Marrubiin: a potent \( \alpha \)-glucosidase inhibitor from \textit{Marrubium alysson}.

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Summary. \( \alpha \)-Glucosidase is an important target to discover new agents for treatment of diabetes-II and in slimming. The objective of this study is to investigate the effect of marrubiin, a major constituent of many medicinal plants including \textit{Marrubium alysson}, as \( \alpha \)-Glucosidase inhibitor. Bioassay-guided screening, isolation and purification of bioactive compounds of methanol extract of \textit{M. alysson} was carried out followed by identification using \(^1\)H and \(^13\)C NMR analysis, and comparing isolated compounds with the published data. Inhibition of \( \alpha \)-glucosidase activity bioassay at different concentrations of enzyme (0.3, 0.6, 1.5, 3 and 6 U/ml) and substrate (sucrose: 7.5, 15, 30, 60 and 120 mM), and at different pretreatment times. Alpha-acarbose used as positive control in comparison to isolated compounds. Molecular docking was done to find out the interaction between compounds and the \( \alpha \)-glucosidase receptor using MOE (molecular modeling environment). Bioassay-guided isolation led to the identification of three known labdane diterpenes; from which marrubiin \((1)\) showed strong inhibition with IC\(_{50}\) of 16.62 \(\mu\)M. Docking studies of compound \((1)\) against the \( \alpha \)-glucosidase enzyme gave comparable scores and hydrogen bond interaction (-12.474 kcal/mol) but different binding mode to the alpha-acarbose (-12.335 kcal/mol). These data suggest that marrubiin has an inhibitory effect on \( \alpha \)-glucosidase activity and these findings provide insight into the traditional uses of \textit{Marrubium} species for treatment of diabetes.

Industrial relevance. Natural products isolated from plants are rich source to new drugs for medicinal use. Docking studies of marrubiin diterpenes against the \( \alpha \)-glucosidase enzyme gave comparable scores and hydrogen bond interaction but different binding mode to the positive standard alpha-acarbose. These data suggest that marrubiin has an inhibitory effect on \( \alpha \)-glucosidase activity and these findings provide insight into the traditional uses of \textit{Marrubium} species for treatment of diabetes-II. Results of the present work provide a promising anti-diabetic compound, marrubiin, of great interest comprising many important benefits including lower level of risk on health than with synthetic drugs.

Keywords. \textit{Marrubium alysson}; Marrubiin; \( \alpha \)-Glucosidase; Diabetes; Docking.

INTRODUCTION

The genus \textit{Marrubium} comprises around 97 species widely spread over the temperate and warm regions indigenous to Europe, Mediterranean area and Asia. Many \textit{Marrubium} species are reported to be used in folk medicine for treatment of different pathologies. (Lewis, 1977; Watt and Breyer-Brandwizk, 1962) \textit{Marrubium alysson} (Labiatae) is a common plant in Egypt (Tackholm, 1956), and is native in the Mediterranean coastal strip from El- Sallum to Rafah and also in the Sinai desert. Its Arabic name is "Hashisha Rabiah" and is used in the form of a decoction as a remedy for asthma and as a diuretic. In North Africa, the tops are used as a flavouring agent. The genus \textit{Marrubium} is known to contain labdane type diterpenoids, such as marrubiin. (Al-Hazimi and Miana, 1994) The previous chemical investigation of \textit{M. alysson} resulted in the isolation of ursolic acid, \( \beta \)-sitosterol, choline, apigenin, apigenin-7-O-arabinoside and apigenin-7-O-glucoside. (Saleh et al., 1981). Medicinal plants play an important role in the management of diabetes mellitus including different \textit{Marrubium} species. Many studies have confirmed the benefits of medicinal plants possess hypoglycaemic effects in the management of diabetes mellitus. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. Moreover, during the past few years some of the new bioactive drugs isolated from plants showed antidiabetic activity with more efficacy than oral hypoglycaemic agents used in clinical therapy (Bnouham et al., 2006). One of the therapeutic approaches for decreasing of blood glucose rise after a meal is to retard the glucose absorption by inhibition of carbohydrate hydrolyzing enzymes, such as \( \alpha \)-amylase and \( \alpha \)-glucosidase. The latter is located in the brush border surface membrane of the intestinal cells and is the key enzyme of the carbohydrate digestion. The compounds that could inhibit the activity of \( \alpha \)-glucosidase can be considered as a potential agent for treatment of diabetes-II. (Kim et al., 2005)

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We have investigated the bioactive chemical constituents of the aerial parts of *M. alysson* and isolated marrubiin (1), marrubenol (2) in addition to premarrubiin (3). The inhibitory effect of the total methanolic extract of *M. alysson* as well as the isolated compounds on α-glucosidase enzyme was tested in the seek of a new drug from a plant origin.

**MATERIALS AND METHODS**

**Plant material.** Aerial parts of *Marrubium alysson* were collected from Mairut, El-Omayed, Egypt. A voucher specimen was identified by Prof. I. El-Garf as co-author of this article and deposited at the herbarium of Cairo University, El-Giza, Egypt.

**Extraction and isolation.** Air-dried aerial parts of the *M. alysson* (1.5 kg) were extracted with methanol at room temperature for 3 days (X 3). The combined extracts were concentrated in vacuo and the residue (55.0 g) was applied to silica gel column (15 X 40 cm). The column was eluted with a mixture of hexane and ethyl acetate in order of increasing polarity, similar fractions were pooled together according to the TLC profile to give 9 major fractions. The active fraction (No. 4, 7.5 g) was further purified using silica gel column and eluted with a mixture from hexane and ethyl acetate (7:3). Premarrubiin (3, 3.2 mg), marrubiin (1, 4.5 g, 0.3%) and marrubenol (2, 22 mg) were isolated and identified using ¹H and ¹³C NMR analysis, and comparing with the published data.

**Inhibition of α-glucosidase activity bioassay.** The enzyme inhibition studies were carried out spectrophotometrically in a 96-well microplate reader using a procedure reported by Li et al. (2005) with slight modifications. Briefly, A total 300 μL reaction mixture containing 50 μL of 100 mM phosphate buffer (pH 6.8), 50 μL of 37 mM sucrose in the buffer, and 200 μL of tested sample in DMSO up to 2% were added to each well, followed by 50 μL of 10 mM phosphate buffer (pH 6.8) containing 0.6 U/mL α-glucosidase to the mixture of treatment terminated wells. The plate was incubated at 37 °C and then heating at 90-100°C after 30 minutes to stop the reaction. The formation of glucose was determined. Inhibition rates were calculated as a percent of blank controls, and the IC₅₀ value was defined as the concentration required to inhibit 50% of the α-glucosidase activity under the assay conditions specified. The absorbance was recorded at 405 nm with a Tecan GENios multifunctional microplate reader (Männedorf, Switzerland). Controls contained the same reaction mixture except the same volume of phosphate buffer was added instead of tested sample solution. Acrarbose (Bayer) was dissolved in water and used as a positive control. The inhibition (%) was calculated as: (A戎 - A戎)/A戎 × 100%, where A戎 is the absorbance of the control, and A戎, the absorbance of the sample.

**Identification of isolated compounds.** NMR spectra were recorded using a Varian Mercury 200 MHz for ¹H-NMR and 50 MHz for ¹³C-NMR. Recorded spectral data of isolated compounds were compared with those published in literature. (El Bardai et al., 2003; Mughal et al., 2012)

**Molecular docking, docking study.** For the purpose of lead optimization and to find out the interaction between compound and the α-glucosidase receptor; molecular modeling calculations and local docking were done by using MOE (molecular modeling environment) to evaluate the binding free energies of this inhibitor into the target α-glucosidase receptor.

Molecular docking study was done to find out interactions between ligand and receptor and to compare affinities of the bioactive compound (marrubiin) to the target α-glucosidase receptor. For the docking calculations, the protein structure (PDB...
code: 3TOP) was first separated from the inhibitor molecule and refined using molecular minimization with added hydrogen. (Ren et al., 2011) Docking calculations were carried out using standard default variables for the MOE program. The binding affinity was evaluated by the binding free energies (S-score, kcal/mol), hydrogen bonds, and root-mean-square deviation (RMSD) values. The compound were docked into same groove of the binding site of the native co-crystallize ligand. The Dock scoring in MOE software was done using London dG scoring function and has been enhanced by using two different refinement methods, the Force-field and Grid-Min pose have been updated to ensure that refined poses satisfy the specified conformations. We allowed rotatable bonds; the best 10 poses were retained and analyzed for the binding poses best score. Energy minimization was done through Force-field MMFF94x Optimization using gradient of 0.0001 for determining low energy conformations with the most favorable (lowest energy) geometry. The experimental methods can be summarized in the following flowchart (Figure 2).

**Figure 2.** Experimental flowchart

**Statistical Analysis.** The results were subjected to statistical analysis. Values were reported as Mean ± SD. The data were analyzed using Student’s $t$-test to test for differences between treatment and control where a value of $P<0.05$ was accepted as significant.

**RESULTS**

**Isolation of the chemical constituents.** The phytochemical investigation of *M. alysson* led to the isolation of three known labdane diterpenes namely: marrubiin (1) (Appleton et al., 1967), marrubenol (2) (Savona et al., 1979) and premarrubiin (3) (Telek et al., 1997) (Figure 3).

**Figure 3: **Chemical structures of marrubiin (1), marrubenol (2) and premarrubiin (3)
Marrubiin, a bitter diterpenoid, was reported before from M. alysson while the other two related structures were reported for the first time. The presence of labdane-structured bitter diterpenoids in the genus Marrubium considered as a chemotaxonomical marker, which demonstrate both pre-furanic and furanic structures. The co-existence and instability of premarrubiin from Marrubium species gave some indications to the artifact nature of marrubiin, however, this fact (the instability of premarrubiin) could not rule out the natural existence of marrubiin in the plant tissues, as many report indicated the isolation of marrubiin from fresh plant materials.

**Biological activity and docking.** The assay showed a strong inhibiting effect of M. alysson total EtOAc extract on α-glucosidase (88.78% inhibition of the enzyme activity at 50 μg/mL with IC_{50}: 8.36±0.0019 μg/mL), significantly higher than that of the well known synthetic α-glucosidase inhibitor, acarbose (Positive drug-control, 54.98% inhibition at 250 μg/mL with IC_{50}: 64.14±0.0033 μM) (Table 1). The α-glucosidase inhibitory activity of EtOAc extract was further confirmed by examining enzyme inhibition activities of isolated compounds present in the extract. Interestingly marrubiin (1) which was isolated from the EtOAc fraction demonstrated potent inhibition with IC_{50}: 16.62±0.0024 μM. Marrubenol (2) was less active with IC_{50} of 262.2±0.0041 μM while premarrubiin (3) was the least active with IC_{50} of 283.54±0.0037 μM.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Inhibition^{ab} (%)</th>
<th>IC_{50} (μM)^{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total EtOAc extract</td>
<td>88.78^{a}</td>
<td>8.36 ± 0.0019 μg/mL</td>
</tr>
<tr>
<td>Acarbose</td>
<td>54.98^{d}</td>
<td>64.14 ± 0.0033 μM</td>
</tr>
<tr>
<td>Marrubiin</td>
<td>76.22</td>
<td>16.62 ± 0.0024 μM</td>
</tr>
<tr>
<td>Marrubenol</td>
<td>27.81</td>
<td>262.2 ± 0.0041 μM</td>
</tr>
<tr>
<td>Premarrubiin</td>
<td>25.27</td>
<td>283.54 ± 0.0037 μM</td>
</tr>
</tbody>
</table>

*{The result was an average of three determinations and expressed as Mean ± SD.}

*{Inhibition by 50 μM isolated compounds.}

*{Inhibition by 50 μg/mL total EtOAc extract.}

*{Inhibition by 250 μg/mL acarbose.}

*{Concentration required for 50% inhibition of the α-glucosidase under assay conditions.}

Ligand interaction and the binding mode of co-crystallized ligand alpha-acarbose with α-glucosidase receptor are shown in Figure 4, it exhibited two H-bond donor with ASP1157 with distances 1.69 and 1.81; a H-bond donor with ASP 1279 (distance 2.01); two hydrogen donor with ASP 1526 (distance 2.00 and 1.79) and two H-bond acceptor with ARG 1510 (distance 2.71 and 2.97) and one H-bond acceptor with HIS 1584 (distance 2.75). It gives score -12.335 kcal/mol.

**Figure 4.** Ligand interaction and the binding mode of co-crystallized ligand alpha-acarbose with α-glucosidase receptor
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DISCUSSION

Several pharmacological studies have demonstrated different activities of marrubiin like antinociceptive (De Jesus et al., 2000), cardioprotective (Mnonopi et al., 2011), vasorelaxant (Hussain et al., 2011), snoring reduction (Brand and Goedbloe 2007), gastroprotective (Brand and Goedbloe 2007; Paula de et al., 2011), antispasmodic (Hussain et al., 2011), immunomodulating

Figure 5. The superposition of marrubiin with green color and co-crystallized ligand alpha-acarbose with gray color.

Figure 6. Ligand interaction and the binding mode of marubiin (1) (green color) with α-glucosidase receptor.
characteristic (Karioti et al., 2007), antioedematogenic (Stulzer et al., 2006), analgesic (Meyre-Silva et al., 2005; De Souza et al., 1998) and antidiabetic (Boudjelal et al., 2012; Mnonopi et al., 2012). Only one toxicity report indicated the LD<sub>50</sub> of marrubiin as 370 mg/kg (oral, mice), but recently marrubiin was injected up to 100 mg/ml (i.p. in rats). (De Jesus et al., 2000).

Diabetes is considered as one of the world's greatest health problems that affects about 171 million people and most dominated by those suffering from type II diabetes (Gershell, 2005). Type II diabetes mellitus has become a serious medical concern worldwide which accounts for 9% of deaths that prompts efforts in exploring for new anti-diabetic agents. Despite the fact that drug treatment for type II diabetes mellitus has been improved to some extent during the last decade, drug resistance is still a big concern that needs to be dealt with effective approaches. One of the strategies to monitor blood glucose for type II diabetes mellitus is to either inhibit or reduce the production of glucose from the small intestine. α-Glucosidase inhibitors interfere with the digestion of carbohydrates. Thus, natural products of great structural diversity are still a good source for searching for such inhibitors.

The anti-diabetic activity of marrubiin was reported in vivo using an obese rat model (Mnonopi et al., 2011) which resulted in an increase in respiratory rate and mitochondrial membrane potential under hyperglycemic conditions. Hence, marrubiin increased insulin secretion. In vitro analysis carried out on marrubiin confirmed the significant stimulatory activity in hyperglycemic conditions. Insulin and glucose transporter-2 gene expressions were significantly increased by marrubiin (Stulzer et al., 2006).

The treatment goal for patients with type II diabetes mellitus is generally agreed to maintain near-normal levels of glycemic control and although diet and exercise are the first steps toward achieving treatment goals, 90% of patients with type II diabetes cannot maintain long-term glycemic control with diet and exercise alone. Thus, antihyperglycemic drugs are necessary for the treatment of type II diabetes (Ratner, 2001). A number of newer antidiabetes therapies—the meglitinides, α-glucosidase inhibitors, and insulin lispro and aspart (Mooradian and Thurman, 1999; Raskin et al., 2000) target blood glucose spikes, and these agents should be considered increasingly in the long-term management of patients with type II diabetes. Use of these agents may lead to achievement of overall target glycemic levels in a greater proportion of patients, thus helping to prevent the excessive morbidity and mortality associated with the disease.

α-Glucosidase is one of a number of glucosidases located in the brush-border surface membrane of intestinal cells, and is a key enzyme of carbohydrate digestion (Caspar, 1978). α-Glucosidase inhibitors block the actions of α-glucosidase enzymes in the small intestine, which is rate-limiting in the conversion of oligosaccharides and disaccharides to monosaccharides, necessary for gastrointestinal absorption. The main benefits attributable to α-glucosidase inhibitors are reductions in both glycemic levels and in the total range of glucose levels (Lebovitz, 1997). The clinical efficacy of α-glucosidase inhibitors is well established. However, it is well documented that synthetic α-glucosidase inhibitors have undesirable side effects, such as flatulence, diarrhea and abdominal cramping. In addition, some of them may increase the incidence of renal tumors and serious hepatic injury and acute hepatitis (Carrascosa et al., 1997; Kihara et al., 1997; Diaz-Gutierrez et al., 1998; Charpentier et al., 2000). Many herbal medicines have been used in the prevention and treatment of diabetes. They have in general not been associated with marked toxic or other side effects. As for most natural medicines, however, their action mechanisms need to be clarified.

The strong inhibiting effect of M. alysson total EtOAc extract on α-glucosidase (88.78% enzyme inhibition with IC<sub>50</sub>: 8.36±0.0019μg/mL) was significantly higher than that of acarbose (Positive drug-control, 54.98% inhibition at 250 μg/mL with IC<sub>50</sub>: 64.14±0.0033μM). Marrubiin isolated from the EtOAc fraction demonstrated superlative potent inhibition with IC<sub>50</sub>: 16.62±0.0024 μM than Marrubenol (IC<sub>50</sub> of 262.2±0.0041 μM) and premarrubiin (IC<sub>50</sub> of 283.54±0.0037 μM).

Eventually, the present study speculated that marrubiin had an inhibitory effect on α-glucosidase activity. Our findings provide insight into the traditional uses of Marrubium species for treatment of diabetes. This could be helpful to develop medicinal preparations or nutraceutical and functional foods for diabetes and related symptoms.

CONCLUSIONS

Marrubiin is a furanic diterpene represent a major constituent of many medicinal plants including M. alysson, it showed interesting activity against α-glucosidase, the key enzyme in treatment of both slimming and diabetes II. The binding power of marrubiin showed less interactive site than alpha-acarbose but it has comparable score. Marrubiin and plant extracts containing high quantity of marrubiin can be considered as a new potential source for reducing the risk of diabetes II and for treatment of slimming due to the safety and efficiency of them as indicted in literature including this article.

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CONFLICT OF INTEREST

No declarations of interests.