Journal of Oleo Science
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doi : 10.5650/jos.ess15026
J. Oleo Sci. **64**, *(8) 835-843 (2015)*

The α-Glucosidase and α-Amylase Enzyme Inhibitory of Hydroxytyrosol and Oleuropein

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Abstract: To date, numerous studies have reported on the antidiabetic properties of various plant extracts through inhibition of carbohydrate-hydrolysing enzymes. The objective of this research was to evaluate the inhibitory effect of the hydroxytyrosol and the oleuropein against α-amylase and α-glucosidase. The hydroxytyrosol was purified from olive leaves. The result shows that the hydroxytyrosol had the strongest α-glucosidase inhibitory effect with IC_{50} values of 150 μM with mild inhibition against α-amylase. The enzyme kinetic studies, using Lineweaver–Burk indicated that, in the presence of the hydroxytyrosol, the Michaelis–Menton constant (K_m) remained constant but the maximal velocity (V_{max}) decreased, revealing a non-competitive type of inhibition with inhibition constants; K_i for the formation of the inhibitor-enzyme complex and K_{is} for the formation of the inhibitor-enzyme-substrate complex of 104.3 and 150.1 μM, respectively. On the other hand, oleuropein showedan uncompetitive inhibition. The concentrations used in this work were below cytotoxic levels observed at 400 μM. However, at 600 μM, the hydroxytyrosol significantly decreased viability of the Caco-2 cells ($p < 0.05$) and in the case of the oleuropein, there's an increase in cell number compared to control ($p < 0.05$). These results suggest that the hydroxytyrosol and oleuropein are two potential effective α-glucosidase inhibitors for management of postprandial hyperglycemia.

Key words: α-amylase, α-glucosidase, diabetes, hydroxytyrosol, oleuropein

1 INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder caused by deficiency in insulin secretion. The disease is characterized by hyperglycemia, impaired glucose tolerance, insulin resistance and hyperlipidemia. One of the therapeutic approaches for decreasing the postprandial hyperglycemia is to retard the digestion and assimilation of starch at the early steps. Complex polysaccharides are hydrolyzed by amylases to dextrins or oligosaccharides that are further hydrolyzed to glucose by intestinal α-glucosidase before being absorbed into the intestinal epithelium. Consequently, the inhibition of these hydrolytic enzymes may help to reduce postprandial hyperglycemia and hence may delay the absorption of glucose[1, 2].

Currently, there are some antidiabetics drugs, namely, acarbose and miglitol which act by inhibiting α-amylase and α-glucosidase activity[3]. While efficient in attenuating the rise in blood glucose levels in many patients, the continuous use of these drugs is often associated with undesirable side effects, such as liver toxicity and adverse gastrointestinal symptoms[4, 5]. To this end, there is a need for natural α-amylase and α-glucosidase inhibitors which have low or unwanted secondary effects.

Olea europea L. leaves are known to contain various bioactive substances with diverse health benefits. Oleuropein appears to be the principal phenolic compound. Its concentration varies with cultivar and climate[6]. Following hydrolysis, oleuropein can produce other bioactive substances, especially hydroxytyrosol. Several *in vitro* and *in vivo* studies have shown that oleuropein and its derivate hydroxytyrosol possess a wide range of biochemical and pharmacological properties[7]. It was also reported that olive leaves extract possess an inhibitory activity against pancreatic amylase[8] and could improve the hypercoagula-

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Accepted May 1, 2015 (received for review February 1, 2015)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

ble state in diabetes[9]. In fact, the inhibitory activity of oleuropein´s aglycon and luteolin glucosides (luteolin-7-O-β-glucoside and luteolin-4´-O-β-glucoside) against pancreatic amylase were determined[8]. However, the anti-diabetic effects of hydroxytyrosol component have not yet been investigated against α-glucosidase and α-amylase. Therefore, the aim of this study is to evaluate the inhibitory effect of hydroxytyrosol against α-glucosidase and pancreatic α-amylase and to compare those observed effects to oleuropein. Enzyme kinetic studies using Michaelis–Menten and the derived Lineweaver–Burk (LB) plots were performed to understand the possible mode of inhibition of these phenolic compounds.

2 EXPERIMENTAL

2.1 Chemicals

Saccharomyces cerevisiae α-glucosidase (≥ 10 U/mg), pancreatic α-amylase (≥ 250 U/mg), and acarbose (BJ05882) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for the analyses were obtained from sigma-Aldrich.

2.2 Olive leaves extract preparation

Olive leaves (200 g) were dried in a microwave oven three times for 2 min at maximum power (1250 W). Dried leaves were powdered and stored under dryness in the dark until extraction.

Olive leaves powder (60 g) was extracted with a mixture of methanol and water (300 ml, 4:1 vol/vol) overnight under agitation in the dark. Then, the solution was filtered using GF/F filter paper. The filtrate was hydrolyzed at 100℃ for 1 h using a 2 M HCl solution (4:1 vol/vol). The sample was then cooled and monomeric phenolics were extracted by a separatory funnel three times with ethyl acetate which was subsequently removed by evaporation and the residue was stored for further analyses.

2.3 HPLC analysis

Chromatographic analyses were achieved on an Agilent series 1260 HPLC-DAD instrument (Agilent, Waldbronn. Germany). The instrument includes a quaternary pump, an online degasser, an auto sampler and a thermostatically controlled column compartment. Chromatographic separation was carried out on a ZORBAX Eclispe XDB-C18 column serial number USNH027266 (4.6 mm I.D. × 250 mm × 3.5 μm particle size). The mobile phase was made of solvent A (0.1% acetic acid) and solvent B (Acetonitrile).

The running gradient was as follows: 0-22 min, 10% -50% B, 22-32 min, 50%-100% B; 32-40 min, 40-44 min, 100-10% B. Re-equilibration duration lasted 6 min. The flow rate was adjusted at 0.5 mL/min, the injection volume of 10 μL and operating temperature was set at 40℃. The DAD detector scanned from 190 to 400 nm and the samples were detected at 254, 280 and 330 nm.

2.4 Purification of hydroxytyrosol using semipreparative HPLC

The hydroxytyrosol was purified using semipreparative HPLC (Knauer, Germany). The experiment was conducted on a model liquid chromatograph fitted with a eurospher C18 column (Knauer, Germany) with dimensions of 250 mm × 9.4 mm I.D. Here, 0.1% phosphoric acid aqueous solution (A) and 100% acetonitrile (B) were used as the mobile phases at a flow rate of 2 mL/min. The gradient started at 10% B and was kept for 8 min, then increased to 100% B for 32 min. The isocratic gradient was kept for 8 min, and finally acetonitrile was decreased to 10% B at 56 min. An eluted peak of hydroxytyrosol was collected using a model sampler. The collected hydroxytyrosol solution was pooled, concentrated using a rotary evaporator and further. The purity level of oleuropein was determined using LC–MS/MS (Agilent 1100 LC, Germany) equipment.

2.5 Maintenance of cell line

Human colonic carcinoma Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% non-essential amino acids. The cells were grown at 37℃ at 5% CO_2 in a humidified incubator. Exponentially growing cells were used throughout.

2.6 Assessment of cell viability

Cytotoxicity of the pure compounds was measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. This assay requires cells that are metabolically active and able to reduce MTT to an insoluble formazan and which later dissolved in 10% sodium dodecyl sulfate (SDS) and detected at 570 nm. Briefly, cells were treated with oleuropein or hydroxytyrosol (100-600 μM) in a 96-well plate for 24 h and 48 h. Cell viability was reported as a percentage of viable cells compared to control cells (cells treated with medium only).

2.7 α-Amylase assay

Appropriate compounds at different concentrations (100 μM - 600 μM) and 500 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/ml) were incubated at 25℃ for 10 min. Then, 500 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixture was further incubated at 25℃ for 10 min and stopped with 5.0 ml of 3,5-dinitrosalicylic acid color reagent and the mixture was incubated in a boiling water for 10 min at 100℃. The reaction mixture was then diluted by adding 10 ml of distilled water,

and the absorbance was measured at 540 nm. The α-amylase inhibitory activity was expressed as percentage inhibition[10].

2.8 α-Glucosidase inhibition assay

The α-glucosidase assay was performed, using the method of Liu *et al*. on 96-well plates with some modifications[11]. 50 μl of appropriate dilution of extracts and 100 μl of α-glucosidase solution (1.0 U/ml) in 0.1 M phosphate buffer (pH 6.9) was incubated at 25℃ for 10 min. Then, 50 μl of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) solution in 0.1 M phosphate was added. The mixtures were further incubated at 25℃ for 5 min, before reading the absorbance at 405 nm in the spectrophotometer. The α-glucosidase inhibitory activity was expressed as percentage inhibition[12].

2.9 IC_{50} determination

Active compounds were assessed for their potency through the determination of their IC_{50} values. This is defined as the concentration of an inhibitor required for reducing 50% of the enzyme activity obtained from an activity versus concentration plot. The data points were fitted into a nonlinear sigmoid plot to take into account non-linear concentration dependent of enzyme -inhibitor interaction at low and high concentrations[13].

2.10 Determination of the inhibitory mode of action

The kinetic mode of inhibition of the active compounds against α-glucosidase was determined by preparing a series of sample solutions in which the concentration of the substrate was varied (1 - 5 mM) in the absence or presence of different concentrations of the inhibitor. The inhibition mode of the test compounds was evaluated on the basis of the inhibitory effects on K_m (dissociation constant) and V_{max} (maximum reaction velocity) of the enzyme[13]. This can be determined using the Lineweaver-Burk plot, which is the double reciprocal plot of enzyme reaction velocity (V) versus substrate (pNPG) concentration (1/V versus 1/[pNPG]).

2.11 Statistics analysis

The results of four experiments were pooled and expressed as mean ± standard deviation (SD). Data were subjected to a one-way analysis of variance (ANOVA) using SPSS 19 statistical package (SPSS Ltd., Woking, UK) followed by Tukey post hoc tests. Differences were considered significant when $p < 0.05$.

3 RESULTS

3.1 Preparation of olive phenolic compounds

HPLC chromatograms of the olive leaves extract and purified hydroxytyrosol are depicted in Fig. 1 (A and B), respectively. The acid hydrolysis of the methanolic extract of olive leaves resulted in the cleavage of oleuropein, the principle polyphenolic compound of olive leaves, into the ortho-diphenolic compound hydroxytyrosol (Hyd). The UV-HPLC spectra of the olive leaves extract before and after purification have shown respective pics of oleuropein (OLE) and hydroxytyrosol. The extraction yield of oleuropein was 8 % of the dry leaves weight and purified hydroxytyrosol was 10% molar yield of extracted oleuropein.

The chromatogram of Fig. 1B depicts a sharp monomodal peak of hydroxytyrosol at a retention time of 10 min. The hydroxytyrosol purity, determined by LC-MS-MS was higher than 99%.

3.2 Inhibitory effect of active compounds on yeast α-glucosidase

The α-glucosidase inhibitory activity was measured in the concentration range of (100 - 600 μM), for both oleuropein and hydroxytyrosol. Results are illustrated in Fig. 2. α-Glucosidase inhibitory activity was increased significantly ($p < 0.05$) in a dose dependent manner relatively to the concentration of active compounds. The hydroxytyrosol reached the highest α-glucosidase inhibitory (75%) activity at 600 μM and at this concentration, the inhibitory activity of hydroxytyrosol, acarbose and oleuropein were about 75, 65 and 55%, respectively. Based on IC_{50} values, it can be seen that the hydroxytyrosol was the potent inhibitor of the α-glucosidase, with an IC_{50} of 150 μM which was higher than of acarbose ($IC_{50} = 200$ μM) and much higher than that of oleuropein (400 μM).

3.3 Inhibitory effects on pancreatic α-amylase

The inhibitory activities of olive compounds against pancreatic amylase were also studied. At the highest concentration, the hydroxytyrosol and the oleuropein were not effective as less than 25% and 10%, activity inhibition were observed, respectively (data not shown).

3.4 Mode of inhibition

In the present study, the initial velocity 'v' of the hydrolysis reactions catalyzed by α-glucosidase was measured at various substrate concentrations [S] in the presence or absence of test compounds [I], as illustrated in Fig. 3. The slope 's' and vertical axis intercept 'i' increase with increasing hydroxytyrosol concentration. This result indicates that hydroxytyrosol affected the velocity of the reaction catalyzed by α-glucosidase, without affecting the Michaelis constant K_m. Therefore, we conclude that a non-competitive inhibition occurs in the presence of hydroxytyrosol. Kinetic constants for the inhibition of α-glucosidase are shown in determined Fig. 3A. The α-glucosidase has a Michaelis–Menton constant (K_m) of 4×10^{-3} M for p-nitrophenyl-α-D-glucopyranoside and V_{max} value of 125 μmoles

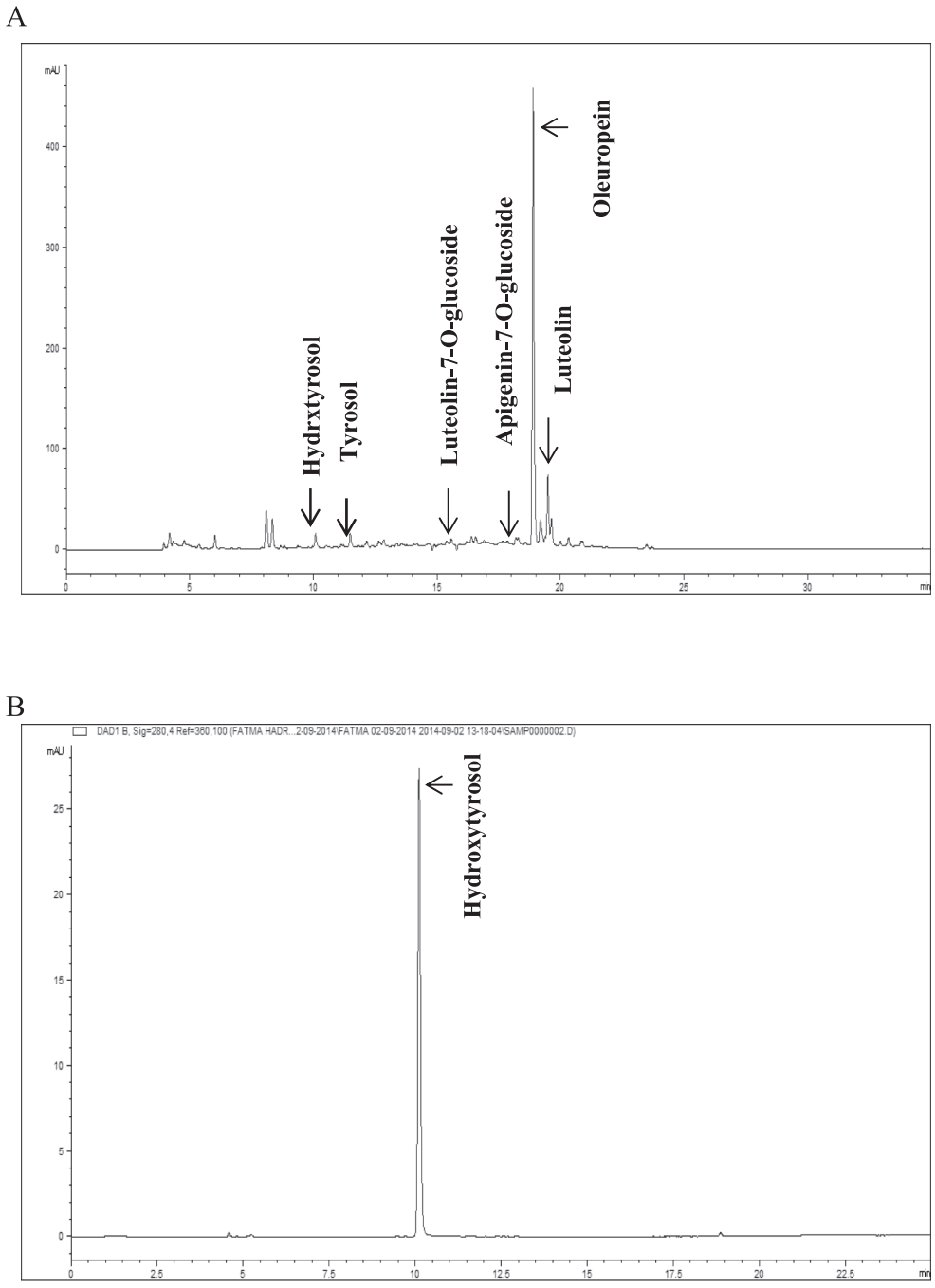

Fig. 1 HPLC chromatograms at 280 nm of olive leaves extract (A), and highly purified hydroxytyrosol (B).

min^{-1}. Apparent V_{max} values in the presence of 100 and 200 μM of hydroxytyrosol were found to be 71.10 and 50.5 μmoles min^{-1}, respectively. The inhibition constants for inhibitor binding with the free enzyme (to form EI) or with the enzyme–substrate complex (to form ESI) were determined using the secondary plot (Fig. 3B) and the secondary replot (Fig. 3C), respectively. Using the secondary plot representing slopes (K_m/V_{max}) of the double reciprocal plots against the inhibitor concentration an EI dissociation constant (K_i) of 104.1 μM was determined, whereas using the secondary replot representing intercept of the double reciprocal relation against the inhibitor concentration an ESI dissociation constant (K_{is}) of 150.1 μM was obtained.

In contrast, the presence of oleuropein in the reaction mixture resulted in no intersection of the straight lines (lines are parallel) in the LB plot (Fig. 4). In this case, we conclude that an uncompetitive took place, where the inhibitor only binds to the enzyme substrate complex resulting in a change of both K_m and V_{max}. The uncompetitive inhibition exerted, indicated that binding of starch at the

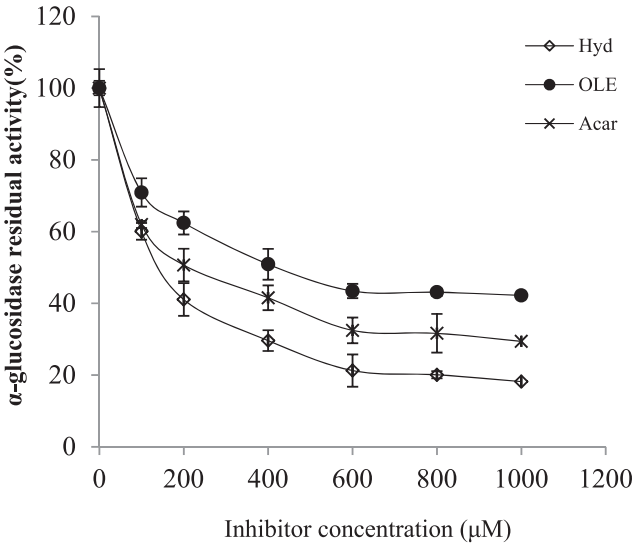

Fig. 2 Inhibitory effect of hydroxytyrosol (-◇-), oleuropein (-●-) and acarbose (-×-) on the activity of yeast α-glucosidase. The residual activity vs the concentration of the tested compounds were plotted. Error bar shows SD of mean (n =3). $p < 0.05$ vs 0 μM.

catalytic site may have modified the confirmation of α-glucosidase, making the putative inhibitor binding site available.

4 Cell viability

While the antidiabetic effects of oleuropein and hydroxytyrosol have been established, this study also aims to validate the efficacy of the extracts as prospective functional food ingredients. The determination of the toxicological potential is important to predict the consequences of exposure at different dose levels[14]. Following 24 h exposure and 48 h exposure to both compounds, no cytotoxic effects were observed at least for IC_{50} values being 150 and 400 μM, previously determined for hydroxytyrosol and oleuropein, respectively ($p > 0.05$). Whereas, at the highest concentration (600 μM), the hydroxytyrosol significantly decreased viability of the Caco-2 cells ($p < 0.05$) (Fig. 5A and B). Also, it is worth noting that after 48 h exposure, the hydroxytyrosol had a substantial inhibitory effect on Caco-2 viability (~40%), however, in the case of the oleuropein, there' s an increase in cell number compared to control ($p < 0.05$).

5 DISCUSSION

The control of postprandial hyperglycemia is critical in the early intervention and prevention of diabetic complications for type 2 diabetes management[15]. α-Glucosidase and α-amylase are two key enzymes related to carbohydrate digestion and elevation of blood glucose levels within fasting subjects. It is now believed that the inhibition of these enzymes might be recognized as a therapeutic strategy for reducing hyperglycemia[16]. Plant phenolic compounds, as potential inhibitors have been shown to inhibit these digestive enzymes. In fact, α-glucosidase and pancreatic amylase activities were effectively reduced by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/(−)-epicatechin, diadzein and epigallocatechin gallate[17].

In this present study, we investigate for the first time the effect of hydroxytyrosol and oleuropein on digestive enzymes. Our results revealed that hydroxytyrosol exert a higher inhibition against α-glucosidase compared to the oleuropein. Indeed, the inhibition rate for α-glucosidase was close to that of acarbose, and the inhibition rate for α-amylase was obviously lower than that of acarbose. This indicated that hydroxytyrosol was a strong inhibitor for α-glucosidase with a mild inhibition against α-amylase. This findings support earlier reports by Loizzo *et al*. who showed that olive oil extracts were weaker inhibitors of α-amylase compared to α-glucosidase[18]. Pharmaceutically, strong inhibitors of α-glucosidase with mild inhibitory activity against α-amylase have been sought due to their great importance in order to prevent the abnormal bacterial fermentation of undigested carbohydrates in the colon, which results in flatulence and diarrhea[4]. Given that oleuropein and hydroxytyrosol have a lower inhibitory effect against α-amylase and stronger inhibitory activity against α-glucosidase they may form the basis of a particularly effective therapy for postprandial hyperglycemia with minimal side effects.

Several reports have investigated the inhibitory effect of luteolin and luteolin-7-O-glucoside against α-glucosidase and α-amylase. In fact, luteolin inhibited α- glucosidase by 36% and was stronger than acarbose, whereas its inhibitory effect against α-amylase was less potent than acarbose[19]. On the other hand, gallic acid, vanillic acid, quercetin and p-coumaric acid isolated from finger millet were shown to be potent inhibitors against α-amylase with respective inhibition of 67.7%, 71.9%, 73.5% and 62.5% and showed uncompetitive inhibition[20].

In order to determine the inhibition constant and the enzymatic model of the inhibition, competitive, noncompetitive or uncompetitive type and whether it occurs in the steady state, we have outlined the Lineweaver–Burk representation. Experimental data showed that hydroxytyrosol has the capacity to inhibit the activity of the α-glucosidase with K_i value of 104.1 μM. In addition, hydroxytyrosol was revealed to exert a noncompetitive inhibition whereas oleuropein exhibited an uncompetitive inhibition. The difference in the inhibitory potencies of hydroxytyrosol and

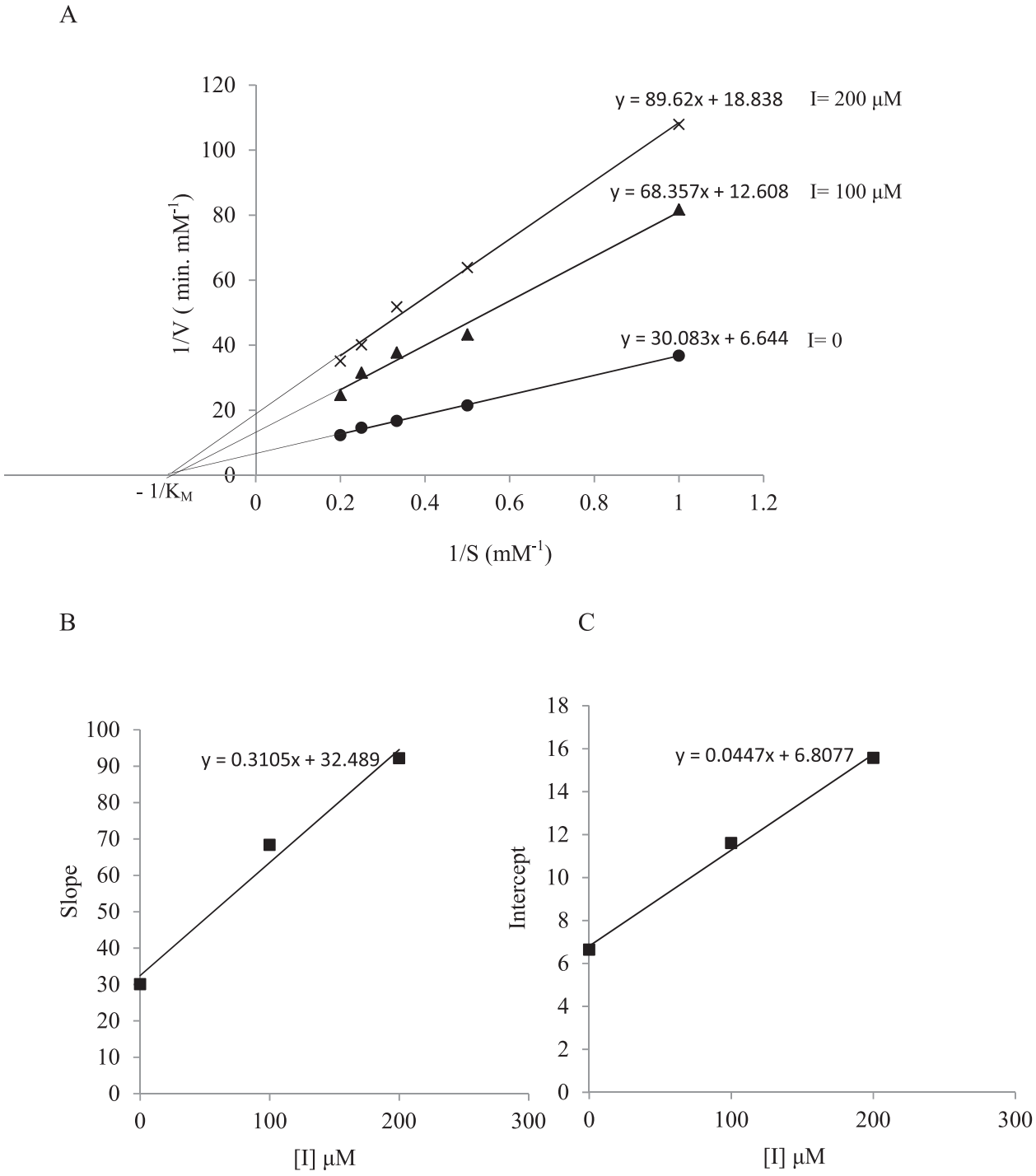

Fig. 3 Double reciprocal diagram of hydroxytyrosol (A) as inhibitor of α-glucosidase using 4-nitrophenyl-α-D-glucopyranoside (pNPG) as substrate. (B) Secondary plot representing slopes of the double reciprocal plot versus the concentrations of hydroxytyrosol for the determination of Ki and (C) secondary plot of intercepts of the double reciprocal plot versus the concentrations of the hydroxytyrosol for the determination of KIS.

oleuropein can be associated with variation in the molecular structures. In fact, hydroxytyrosol had a polar structure giving it a high capability to interact directly with the enzymes (α-glucosidase) which the enzyme and phenolic glycosidic conjugates is expected to be higher than the non-glycosidic- molecules.

The effect of hydroxytyrosol and oleuropein on Caco-2 was established and these findings demonstrate that the

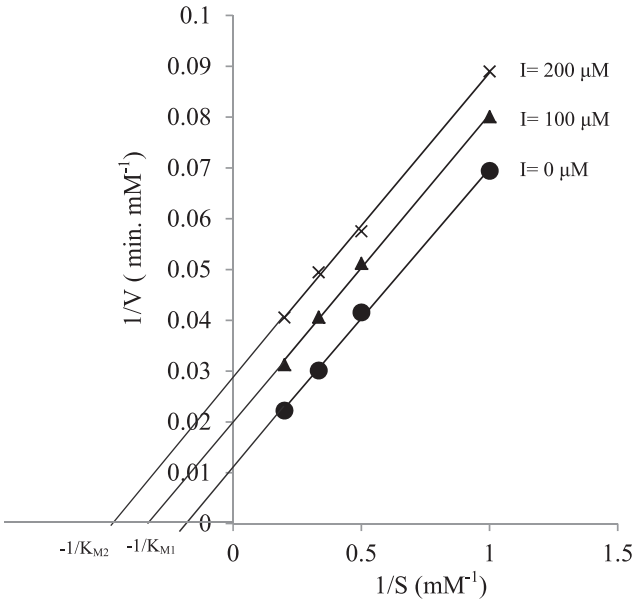

Fig. 4 Lineweaver-Burk plot of the α-glucosidase hydrolysis reaction with variable substrate concentrations (1–5 mM) in presence of oleuropein. Data are means of three independent experiments.

IC_{50} of hydroxytyrosol and oleuropein for α-glucosidase inhibition were greatly below cytotoxic levels. These results are somewhat in contrast with those reported by Mateos *et al*. who showed that the hydroxytyrosol affect the viability of the Caco-2 from 100 μM. However, the authors only document the cytotoxic effects at 24 h[22].

On the other hand, the inhibition of Caco-2 cells proliferation by olive compounds has been found to be related to the antioxidant properties of such compounds[23]. Therefore, antioxidants may prevent the progressive impairment of pancreatic beta-cell function and thus reduce the occurrence of type 2 diabetes[24]. Researchers pay much more attention to antioxidant activities when antidiabetic activities of phytochemicals, especially phenolics, were evaluated, and it was reported that several phenolic compounds with inhibitory activities against α-glucosidase and α-amylase had moderate antioxidant activity[25–27].

Although this study focuses on the antidiabetic potential of olive leaves compounds (hydroxytyrosol and oleuropein), and the Caco-2 cell line which was used as a model for cultured colonocytes, the observed findings could also be looked at from a chemotherapeutic perspective. Owing to their cytotoxic effects, 400 μM of the hydroxytyrosol could potentially be employed in the inhibition of cancer development.

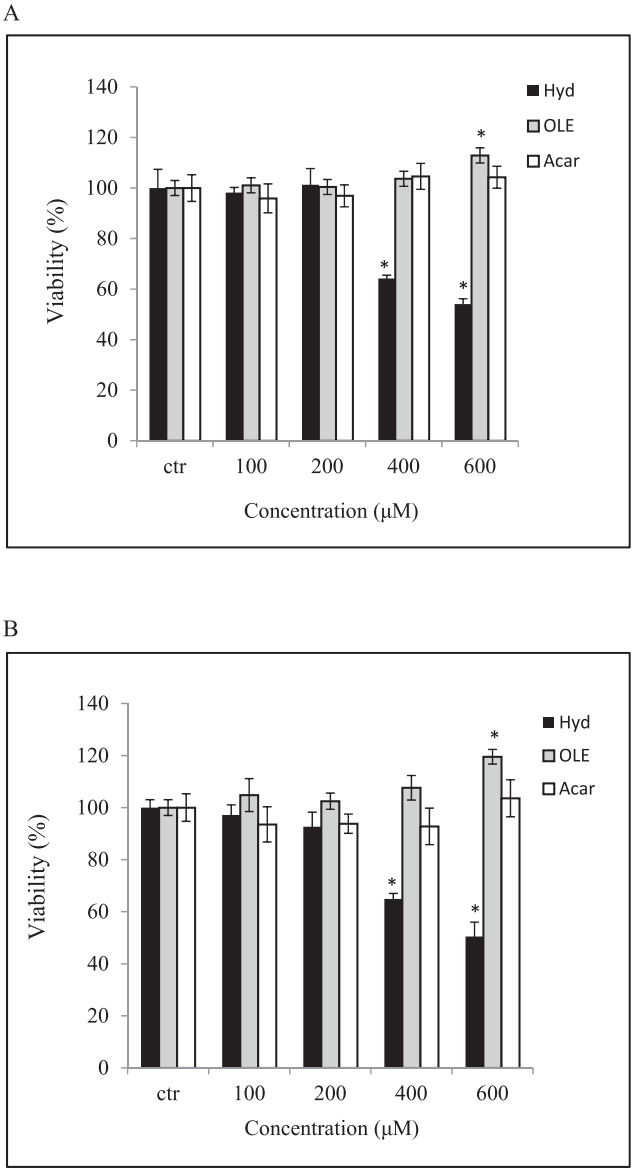

Fig. 5 The effects of hydroxytyrosol (■), oleuropein (▒) and acarbose (□) treatment on Caco-2 cells viability measured by MTT assay following exposure to 24 h (A) and 48 h (B). The asterisk indicates $p < 0.05$ vs ctr in each treatment.

6 CONCLUSION

With the increasing prevalence of type II diabetes, efforts are being made to identify natural therapies that can control hyperglycaemia. In the present study, we demonstrate the efficacy of hydroxytyrosol to inhibit enzymes involved in intestinal carbohydrate digestion and assimilation, namely α-glucosidase. Due to it availability and strong inhibitory properties, hydroxytyrosol has potential for use in functional food applications. Additionally, the strong α-glucosidase inhibition at non-cytotoxic levels and mild inhibition of α-amylase makes the extracts a good source

for the management of type-2 diabetes with minimum side effects currently observed with some of the drugs being used for the management of non-insulin dependent diabetes mellitus.

Acknowledgments

Ministry of Higher Education & Scientific Research – Tunisia supported the study design in relation with the sampling of olive leaves and the preparation and chemical characterization of olive leaves extract, collection, analysis, interpretation of data and manuscript writing. Ministry of Education, Culture, Sports, Sciences and Technology Japan supported the study design, collection, analysis and interpretation of cellular and molecular data. The Mitsui & Co., Ltd., supported the study design, collection, analysis and interpretation of cellular and molecular data.

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