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The aldose reductase inhibitory capacity of *Sorbus domestica* fruit extracts depends on their phenolic content and may be useful for the control of diabetic complications

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Received March 23, 2008, accepted April 25, 2008

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Pharmazie 63: 693–696 (2008) *doi: 10.1691/ph.2008.8567*

Aldose reductase (ALR2) is a rate-limiting enzyme in the polyol pathway associated with the conversion of glucose to sorbitol and whose activity is implicated in the development of the long-term diabetic complications. Upon previous years, several scientific efforts were focused towards the development of ALR2 inhibitors as effective secondary anti-diabetic drugs. To this regard and during our extensive phytochemical analysis of *Sorbus domestica* (fam. *Rosaceae*), twenty nine different extracts, fractions and residues of five different maturity stages of *Sorbus domestica* fruits were evaluated for their *in vitro* ALR2 inhibitory capacity. The data obtained thus far have indicated that the diethyl ether and ethyl acetate fractions possess high aldose reductase inhibitory activity. Furthermore, detailed phytochemical LC-DAD-MS (ESI+) analysis of such extracts has shown that this aldose reductase inhibitory activity could be attributed to the high content of flavonoids and hydroxycinnamoyl esters. These results suggest that *Sorbus domestica* fruit consumption may be a promising way for lowering the incidence of long-term complications of diabetes mellitus, especially at early stages, a possibility being discussed in this paper.

1. Introduction

Diabetes mellitus is considered as one of the worldwide epidemics in the 21st century and is becoming a major threat to human health. The diabetic individual is prone to late onset complications that are largely responsible for the morbidity and mortality observed in these patients (Miyamoto, 2002). It has been demonstrated that the more severe and sustained the degree of hyperglycemia, the more likely it is that the chronic complications of diabetes will develop. To this respect, it is very difficult to maintain normoglycemia (normal glycose concentration in the plasma), nowadays, by any available therapeutic manner in patients with diabetes mellitus (Ziegler, 2004). Therefore, pharmaceutical intervention of hyperglycemia-induced diabetic complications is actively pursued (Nicolaou et al. 2004), (Demopoulos et al. 2007).

Aldose reductase enzyme (AR, ALR2, E.C. 1.1.1.21, aldi-tol/$NADP^+$ oxidoreductase) is the first enzyme of the polyol metabolic pathway that is found to be implicated in the etiology of the long-term diabetic complications, such as peripheral neuropathy, nephropathy, retinopathy, and cataract (Matthew et al. 2002). As a matter of fact, upon previous years several scientific efforts have been focused on the development of effective ALR2 inhibitors as new anti-diabetic drugs. To this respect, carboxylic acid and cyclic imide derivatives in general are among the most active inhibitors of ALR2 (Constantino et al. 1999a). The presence of an acidic functionality in these compounds has been suggested to be an important feature for the inhibition of ALR2. The negatively charged carboxylates or cyclic imides form hydrogen bonds to three key enzyme residues, Tyr48, His, 110 and Trp111, and exert strong electrostatic interactions with the positively charged of the oxidized form of the nicotinamide ring of $NADP^+$ (Rastelli et al. 2000).

Many naturally occurring compounds are known to possess strong aldose reductase inhibitory (ARI) activity *in vitro*. In general, flavonoids are known as potent inhibitors of this enzyme. A comparative study among different flavonoid structures indicated that pentahydroxy flavones and their derivatives are the most effective ARI agents (Varma et al. 1976). Similarly, among the aglycons, quercetin was found to be the most effective. On the contrary, the 2,3-dihydroquercetin (taxifolin) was less potent than quercetin, which indicates that the presence of a 2–3 unsaturation in the quercetin ring enhances activity. Furthermore, specific scientific efforts have shown that the unsubstituted 7-OH group is significant for the effectiveness of such structures (Constantino et al. 1999a). Reports on the existing structure-activity relationship indicate that the phenolic 7-hydroxyl group is a main structural feature for the inhibition of ALR2, since it forms a hydrogen bond with the amino-acids Tyr48 and His110 that are important for the

activity of ALR2 (Constantino et al. 1999b). The 2-benzyl substitution, due to its aromatic and lipophilic nature, as well as its specific spatial conformation, was found to fit to a hydrophobic pocket of the enzyme lined by the amino-acids Trp111 and Leu300 (Urzumtzev et al. 1997). Finally, the 4′-hydroxyl group seems to play an important role for exhibiting these compounds ARI activity, as it could form a bond to amino-acid Thr113 (Rastelli et al. 2000)

p-Hydroxybenzoic acid has been found as an inactive agent in assays evaluating its ARI activity even at the high indicative concentration of 100 μM (Demopoulos et al., unpublished data). However, this compound is present, as a core structure, in a number of naturally occurring products, such as vanillic acid and protocatechuic acid. To this respect, in similar ARI activity assays, it has been indicated that simple hydroxycinnamic acids are weak inhibitors of ALR2 *in vitro*. For example, although ferulic acid had weak ARI activity (Yawadio et al. 2006), caffeic acid was found inactive (Koukoulitsa et al. 2006). On the contrary, however, hydroxycinamoyl esters have strong ARI activity. Thus, salvianolic acid A, salvianolic acid K, lithospermic acid B and rosmarinic acid are all reported as good ALR2 inhibitors (Koukoulitsa et al. 2006; Du et al. 1995; Kasimu et al. 1998). Furthermore, chlorogenic acid (5-*O*-caffeoylquinic acid) as well as 3,5-dicaffeoylquinic acid, showed effective inhibitory activity (Varma et al. 1976; Ravn et al. 1990; Terashima et al. 1990). Finally, 4,5-dicaffeoylquinic acid was the most powerful inhibitor of ALR2 among all the constituents of *Artemisia dracunculus* (Logendra et al. 2006), even more potent than the reference compound quercitrin.

Sorbus domestica fruits (fam. Rosaceae) are widely used in northern Europe as antioxidant agents in beverages (Olschlager et al. 2004). Previous research work from our laboratory has confirmed the good antioxidant potential of these fruits (Termentzi et al. 2006). The tree is shelf-grown in the mountainous regions of Europe, northern Africa and Minor Asia. In addition to its use as antioxidant agents, *Sorbus domestica* fruits are consumed by many local populations in Europe as a traditional herbal supplement for patients suffering from diabetes mellitus. Such a use is supported by the belief that systematic consumption of the fruit delays and ameliorates long-term diabetic complications (peripheral neuropathy, nephropathy, retinopathy, and cataract). Even some local drugstores sell fruit pulp in sterilized bottles for this usage. To this respect, and upon our extensive phytochemical analysis of *Sorbus domestica* fruits published elsewhere (Termentzi et al. 2006, 2008), we reasoned in this study firstly to evaluate the *in vitro* inhibitory ALR2 activity of various *Sorbus domestica* fruit extracts isolated from different fruit maturity stages, and secondly to correlate this activity with the extracts' phenolic content.

2. Investigations, results and discussion

For comparison reasons five different fruit categories were tested, each one partitioned with increasing polarity solvents (see experimental). As it is shown in the Fig. and the statistical analysis data presented in the Table, the diethyl ether and ethyl acetate fractions isolated from *Sorbus domestica* fruits exhibited high ALR2 inhibitory activity at a final concentration of 50 μg/mL ranging from 72 to 93%. Among the diethyl ether extracts of the five different categories of the fruits, the raw yellow fruits were the strongest inhibitors (93% at 50 μg/mL). Interestingly,

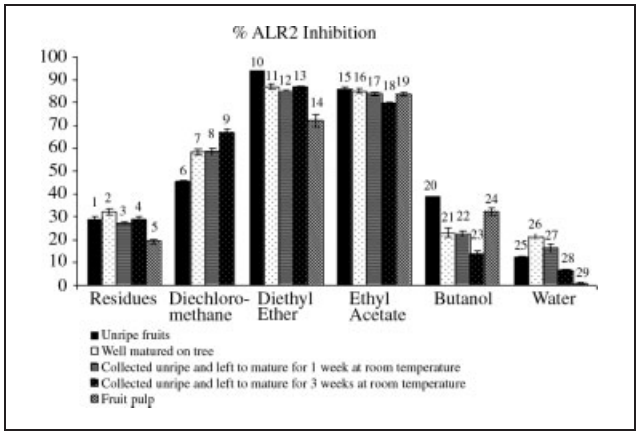

Fig.: Inhibitory effect of *Sorbus domestica* fruits' extracts on aldose reductase enzyme (in final concentration that of 50 μl)

this fruit category has shown no significant difference in ALR2 inhibitory activity from those fruits left to mature for either 1 or 3 weeks as well as those that are well matured on the trees (86%, 85% and 86%, respectively, at 50 μg/mL). On the contrary, the fruit pulp exhibited the lowest, but still significant inhibitory activity (72% at 50 μg/mL). Regarding the ethyl acetate fraction, the different maturity stages of the fruits do not seem to affect their ALR2 inhibitory activity (ranging 80–86% at 50 μg/mL). Furthermore, the dichloromethane fractions possessed inhibitory activity above 50%. In comparison, those of the well-matured fruits on the trees and those collected unripe and left to mature for either 1 or 3 weeks at room temperature, the ALR2 inhibitory activity appears weak below 40% for the butanol and water extracts as well as for the residues (see Fig.).

Table: Statistical analysis of the experimental ALR2 inhibitory activity data by correlating pairs of samples according to Welch's approximation through the use of program Stata 8.

Pairs of Samples	*P > |t|
2–24	0.860
1–3	0.083
3–4	0.083
1–4	1.000
5–26	0.096
5–27	0.057
7–8	0.959
11–12	0.070
11–13	0.744
11–16	0.095
11–15	0.344
12–16	0.939
12–17	0.093
12–15	0.063
12–19	0.083
13–16	0.112
13–15	0.437
16–17	0.209
16–15	0.238
16–19	0.149
17–19	0.715
21–22	0.652
21–26	0.210
22–26	0.267
23–27	0.059
23–25	0.172

* When $p > 0.05$, samples are not statistically different

The detailed phytochemical analysis of all extracts isolated from *Sorbus domestica* fruits indicated that ethyl acetate and diethyl ether fractions, that possessed the best ALR2 inhibitory activities, are rich in flavonoids and hydroxycinnamoyl quinic acid esters (Termentzi et al. 2006, 2008). To this end, the qualitative analysis showed that most of the flavonoids were quercetin aglycon, glycosides and esters. All quercetin glycosides and esters had only 3-hydroxyl group bonded, in contrary to 7- and 4′-OH groups that were free in all cases. The latter, may be related to the observed high ALR2 inhibitory activity of these extracts, since, as it has been already mentioned above, the presence of 7- and 4′ hydroxyl groups on quercetin structures are important features for the exhibition of such biological activity of the flavonoids. Furthermore, all extracts were rich in hydroxycinnamoyl quinic acid esters, especially chlorogenic acid derivatives, substances with known ALR2 inhibitory activity, as it has been already discussed. Furthermore, the quantitative analysis of extracts isolated from *Sorbus domestica* fruits revealed great differences among the samples of different fruit categories (Termentzi et al. 2006, 2008). Fruit categories A, C (unripe fruits) and E (fruit pulp), were rich in flavonoids, while categories B and D (well matured fruits) had lower flavonoid content. All categories, however, had similar hydroxycinnamoyl quinic acid ester content. To this respect, the significant ALR2 inhibitory activity seen in both diethyl ether and ethyl acetate fractions of all fruit types can be attributed to their flavonoid and hydroxycinnamoyl content (Termentzi et al. 2008). Interestingly, at the dilution concentrations of these extracts tested for ALR2 inhibitory activity, their phenolic content of all fruit types is high enough to explain the high inhibitory capacity of ALR2 activity.

On the contrary, the assessment of dichloromethane and butanol fractions of *Sorbus domestica* fruits has revealed their poor content in flavonoids and their moderate ALR2 inhibitory activity. The latter biological activity, however, could be attributed to their high hydroxyl cinnamoyl quinic acid esters (Termentzi et al. 2008).

To our knowledge, this is the first report on the inhibitory capacity of *Sorbus domestica* fruits extracts on ALR2 activity *in vitro*. The data obtained add new information and highlight the plant's significance for its use in the diet of patients suffering from diabetes mellitus as a potential protective agent against the long-term diabetic complications, especially at early stages. As a result, the use of *Sorbus domestica* fruit extract may be considered as a natural ALR2 inhibitor in food and medicinal products with potent pharmacological activity and therapeutic role against the long-term diabetic complications, justifying the traditional use of the fruit as food beneficial to health. Such a possibility, however, needs further exploitation and experiments designed to justify this potential use of *Sorbus domestica* fruits are underway in our laboratory.

3. Experimental

3.1. Reagents and solvents

All reagents and solvents mentioned in this work are commercially available and were obtained from Merck, Aldrich, Fluka and Pfizer.

3.2. Plant material and preparation of crude extracts

All fruit samples were collected in September 2003 from the mountain regions of Rodopi (Greece). Five fruit categories were tested for comparison reasons: A, unripe fruits; B, well matured on tree; C, collected unripe and matured for one week in dark, at room temperature; D, as in C but prolonged maturation at three weeks (form consumed by the local population) and E, sterilized pulp from well matured fruits (disposed at local drugstores). Fruits of category A, C and D were harvested on the 10th of September, while that of category B ten days later.

All plant material was directly extracted following the procedure suggested by Mellidis et al. (1993). The fruits were segmented and their seeds were removed carefully. They were then directly put into a Soxhlet apparatus 1 L and extracted exhaustively with methanol. Concerning the pulp extraction, the content of the sterilized bottle was directly put into methanol after its opening and filtrated until the discoloration of the solvent. The extracts obtained were evaporated under vacuum to dryness. The dry residual of the methanolic extracts were dissolved in 1.5 L of boiling water and then directly filtered. The water solutions were partitioned with dichloromethane, diethyl ether, ethyl acetate and butanol three times X 500 ml each. The organic layers were dried with anhydrous sodium sulphate, and evaporated under vacuum to dryness. All extracts, fractions and initial residues were kept at 0 °C under nitrogen atmosphere.

3.3. Assessment of the inhibition of ALR2 activity in vitro

The studied crude extracts were dissolved in 10% aqueous solution of DMSO. Lenses were quickly removed from Fischer-344 rats of both sexes following euthanasia and the enzyme preparation and assay were performed as previously described (Nicolaou et al. 2004). Briefly, the enzyme activity was measured by monitoring the change in absorbance at 340 nm and 30 °C, which accompanies the oxidation of NADPH catalysed by ALR2. The assay mixture contained 2.4 mL phosphate buffer 0.067 M (pH = 6.2), 100 μL NADPH 0.104 mM, 300 μL enzyme preparation, 100 μL tested crude extract of final concentration 50 μg/mL and 100 μL DL-glyceraldehyde 10 mM to start the reaction, in final volume 1.1 mL. The reaction was monitored for 5 min. Each crude extract was assayed in triplicate. A reference blank containing all the above reagents except DL-glyceraldehyde was used to correct the non enzymatic oxidation of NADPH.

All data are presented as % inhibition in the Figure and are mean values from 3 measurements with SD < 10%. According to T test the significant level P = 0.0001 in comparison to control. Sorbinil was used as a positive control and showed inhibition 45% at a concentration of 0.25 μM (indicative reported IC_{50} values range from 0.07 to 0.9 μM (Zaher et al. 2002)).

3.4. Statistical analysis

Two-sample T test with unequal variances where observations are not paired (Welch's approximation) was realized for the statistical process of all results. The statistical program used for this analysis was "Stata 8". Table 1 presents all pairs of samples that are not statistically different.

3.5. Phytochemical analysis

The qualitative and quantitative phytochemical analysis of the extracts of *Sorbus domestica* fruits was performed from our laboratory by applying a LC-DAD-MS (ESI+) system, as it has been published elsewhere (Termentzi et al. 2008).

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