Original Article

Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase *in vitro*

Patrick P McCue MS[1] and Kalidas Shetty PhD[2]

[1]*Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003, USA*
[2]*Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA*

Porcine pancreatic alpha-amylase (PPA) was allowed to react with herbal extracts containing rosmarinic acid (RA) and purified RA. The derivatized enzyme-phytochemical mixtures obtained were characterized for residual amylase activity. These *in vitro* experiments showed that the amylase activity was inhibited in the presence of these phytochemicals. The extent of amylase inhibition correlated with increased concentration of RA. RA-containing oregano extracts yielded higher than expected amylase inhibition than similar amount of purified RA, suggesting that other phenolic compounds or phenolic synergies may contribute to additional amylase inhibitory activity. The significance of food-grade, plant-based amylase inhibitors for modulation of diabetes mellitus and other oxidation-linked diseases is hypothesized and discussed.
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Key Words: amylase inhibitors, oregano, lemon balm, herbal extracts, rosmarinic acid, diabetes mellitus

Introduction

Rosmarinic acid (á-*o*-caffeoyl-3,4-dihydroxyphenyllactic acid; RA) is a diphenolic compound common to many species of herbs and spices, particularly in the families of *Boraginaceae* and *Lamiaceae*.[1-3] RA is regarded as a potential pharmaceutical plant product and is noted for its potent antioxidant properties.[4,5] Herbs, many of which that contain RA as the dominant phenolic constituent, have long been used in traditional medicines in Southern Europe, Japan, and India for treatment of numerous maladies, from stomachache, headache, and diabetes mellitus to insect bites and acne.[4-7] Various RA-containing extracts from the leaves of herbs and spices have been reported to possess antioxidant, antimutagenic, anti-tumorigenic, anti-HIV, anti-proliferative, and anti-cyclooxygenase properties.[8-14]

Type 2 diabetes mellitus is a major metabolic disorder that is increasing worldwide. Normally, the entry of glucose into pancreatic â-cells stimulates the release of insulin into the blood, where it stimulates peripheral cells to take up glucose. But in Type 2 diabetes, insulin is secreted in inadequate or inappropriate amounts and/ or peripheral cells become resistant to the action of insulin, resulting in hyperglycemia.[15] Long-term hyperglycemia can lead to damage in cells that cannot block sugar from entering. In these cells (especially those lining blood vessels), as mitochondria utilize entering sugars, harmful byproducts accumulate.

Much research has focused on glycosidase inhibitors to control hyperglycemia, but many forms of starch are also digested as rapidly as glucose absorption.[15-20] Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes.[15,17] Inhibitory activity against amylase by flavonoids and anthocyanins has been reported.[21,22] Exogenous RA has been reported to have inhibitory activity against plant á-amylase during seed germination and seedling growth.[23] Herbs used in traditional Indian medicine for diabetes mellitus treatments are known to contain RA, as well as the flavonoid quercetin. We hypothesize that the health benefits of these herbs against diabetes mellitus may be due to the amylase-inhibiting activity of the phenolic compounds, such as RA, that are found within them. It is unknown whether or not the putative anti-amylase activity of RA is dose-dependent.

In the current study, we investigated the effect of RA extracts from lemon balm and oregano on porcine pancreatic amylase compared to pure (97%) RA. Our results suggest that amylase inhibition by the RA extracts correlates with the concentration of RA present in the extracts. Further, a possible role for phenolic synergies in amylase inhibition by herb extracts is discussed. The significance of plant-based amylase (PPA) inhibitors for oxidation-linked disease modulation were hypothesized and discussed.

Methods

Materials

á-Amylase from porcine pancrease in solution (Duramyl 300™, 300U (1.08g)/mL) was purchased from Sigma Chemical Co. (St Louis, MO). Pure RA (97%) was purchased from Sigma. Lemon balm (*Melissa officinalis* L.)

Correspondence address: Prof Kalidas Shetty, Dept of Food Science, Chenoweth Lab., University of Massachusetts, Amherst, MA 01003, USA.
Tel: +1-413-545-1022; Fax: + +1-413-545-1262
kalidas@foodsci.umass.edu
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50% RA extract was from CMS International (Oxford, UK). Oregano (*Origanum vulgare* L.) 7% RA extract (Origanox™) was generously provided by Barrington Nutraceuticals (Harrison, NY).

Treatment of á-amylase with RA and RA-containing extracts

Treatment of amylase was performed according to the method of Rohn *et al.*,[24] with some modifications. A 300 mg aliquot of enzyme was added to 25mL of distilled water (dH_2O) and diluted to 27mL (with dH_2O). The solution was adjusted to pH 6.9 with 0.1 N NaOH. For the non-treated enzyme controls, 3mL of 50% EtOH was added and the solution readjusted to pH 6.9, if necessary. For the treatments, to the 27mL of enzyme solution (pH 6.9) was added 100mg of RA extract in 3mL of 50% EtOH. The pH of the solution was readjusted to 6.9 after mixing. The enzyme-RA extract solutions were stirred ~24 h at 4 C.

Characterization of á-amylase activity

á-Amylase activity was determined by the method of Worthington[25] using starch as a substrate in a colorimetric reaction using 3, 5-dinitrosalicylic acid. A standard curve was generated for the splitting products (reducing groups) using D-(+)-maltose monohydrate. Activity is reported as units/ mg protein, where 1 unit is defined as the amount of enzyme required to liberate 1 micromole of maltose under assay conditions. Protein content was assayed by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Each experiment was repeated three times and the data averaged for reporting. Data is given as the mean ± standard deviation (SD). Statistical analysis of data was determined by analysis of variance (ANOVA) single factor F test and *P*-value analysis using Microsoft Excel XP.

Results

Effect of RA and RA-containing herbal extracts on amylase activity

The aim of this study was to show the different reactivity of porcine pancreatic amylase with herbal RA extracts of different concentrations. The experimental treatments were done under conditions of pH 6.9 and a total concentration of the herbal extracts of 100mg. The influence of different concentrations (97%, 50%, and 7%) of RA in the herbal extracts on the reaction was investigated.

The RA-containing herbal extracts (Table 1) react with á-amylase causing a decrease in the starch-degrading activity as illustrated in Figure 1A. The extent of the inhibition of amylase activity appears to correlate with the RA content of the herbal extract applied. This amylase-inhibiting effect is most dramatic when varying concentrations of pure (97%) RA are treated (Fig. 1B). The inhibition of amylase by pure RA appears to be dose-dependent, and was found to have a correlation of r^2=0.982.

Table 1. Rosmarinic acid extracts used in this study

Extract	Concentration of rosmarinic acid
Pure RA	97%
Lemon balm	50%
Oregano*	7%

*Origanox, a commercial oregano powder produced by Barrington Nutritionals (NY).

Pure (97%) RA inhibited amylase activity by 85% and lemon balm-based extract with 50% RA inhibited amylase activity by 50%, while oregano-based extract with 7% RA inhibited amylase activity by 42% (Fig. 2).

The effect of the reaction on á-amylase with oregano-based extract with 7% RA was higher than expected (Fig. 1,2). In their phenolic profiles, oregano and lemon balm both contain similar levels of protocatechuic acid, caffeic acid, coumaric acid, and quercetin, but lemon balm contains 4-fold more rosmarinic acid (S. Chun and K. Shetty, unpublished observations). Considering that the pure (97%) RA extract had close to twice the RA content as the lemon balm-based extract with 50% RA, we expected that the oregano-based extract with 7% RA would therefore have demonstrated much weaker amylase-inhibiting activity, but it did not. We hypothesize that phenolic synergies may have played a role in the high amylase-inhibiting activity observed with the oregano extract with 7% RA, wherein the combined phenolics have more activity than the sum of the activities of the components alone.[26] Alternatively, other phenolic compounds present in the oregano extract may also possess amylase inhibitory activity. This possibility is being researched further.

Discussion

Our results show that herbal extracts with RA react with porcine pancreatic amylase, inhibiting its enzymatic activity against starch as a substrate in *in vitro* experiments. As illustrated, the activity of á-amylase decreased depending on the RA content of the extract. Ninety-seven percent (97%) RA showed the strongest reactivity, followed by lemon balm-based extract with 50% RA, and then by oregano-based extract with 7% RA. Surprisingly, the oregano-based extract with 7% RA showed amylase-inhibiting activity that was only slightly less than the extract with 50% RA, suggesting that other phenolic components of the oregano extract may support the anti-amylase activity, perhaps through synergistic mechanism(s). Our results agree with other reports, demonstrating the ability of phenolic substances to interact with and/or inhibit proteins/enzymes.[24,27-29]

Rohn *et al.*,[24] demonstrated that phenolic substances that are able to form quinones (ie caffeic acid, chlorogenic acid, gallic acid, etc) are more reactive than those phenolics that cannot form quinones, and suggested that semi-quinones formed may react with amino acid side chains and free thiol groups on the enzyme. While there is no published report in the literature to date that has tested this type of mechanism of action for rosmarinic acid specifically, we do note that the similarity in structure

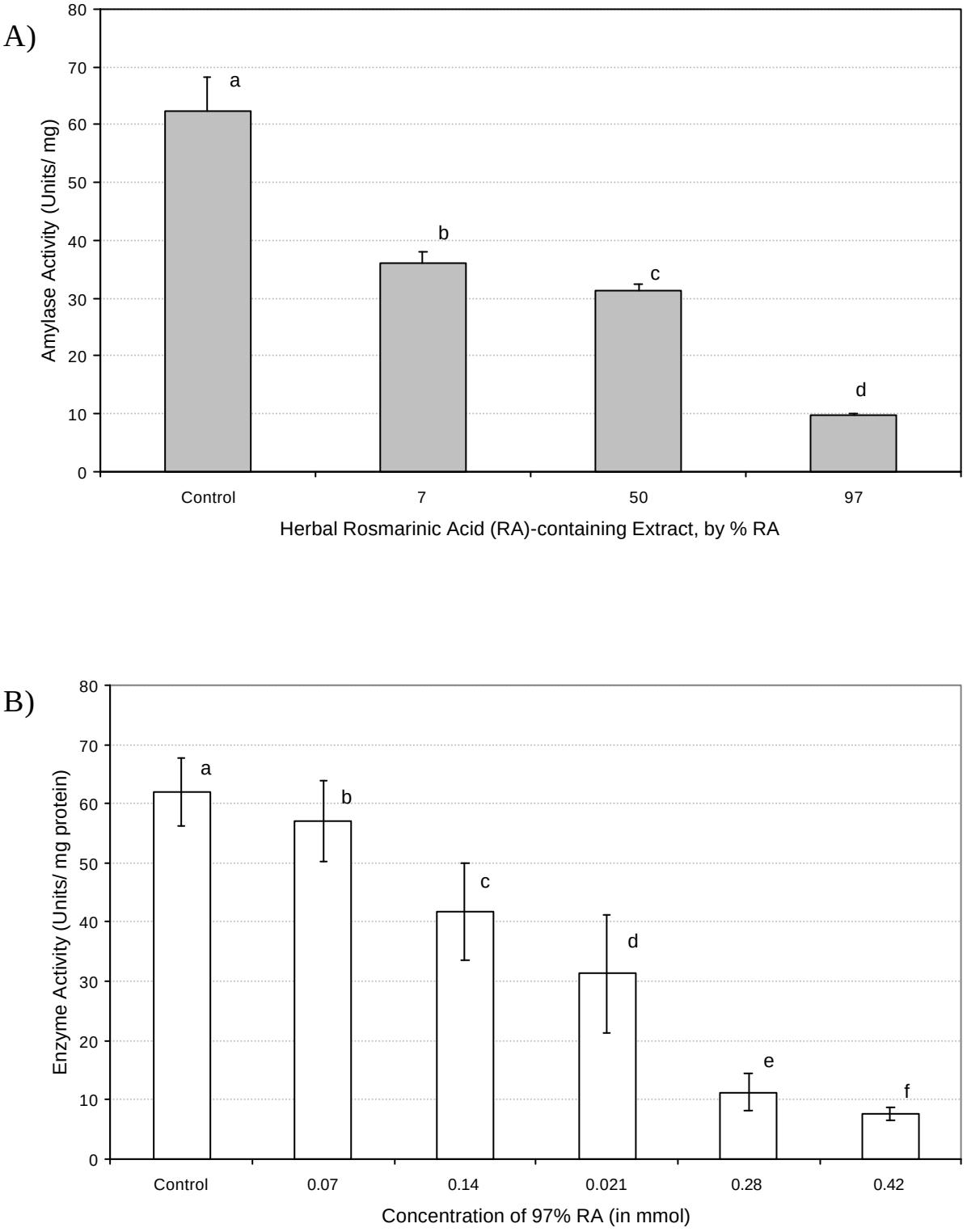

Figure 1. Residual activity of amylase-phytochemical mixtures against starch at pH 6.9. A) Mixtures with herbal extracts containing RA; B) Mixtures with pure RA. Data sets with different letters are significantly different by ANOVA at $P<0.05$.

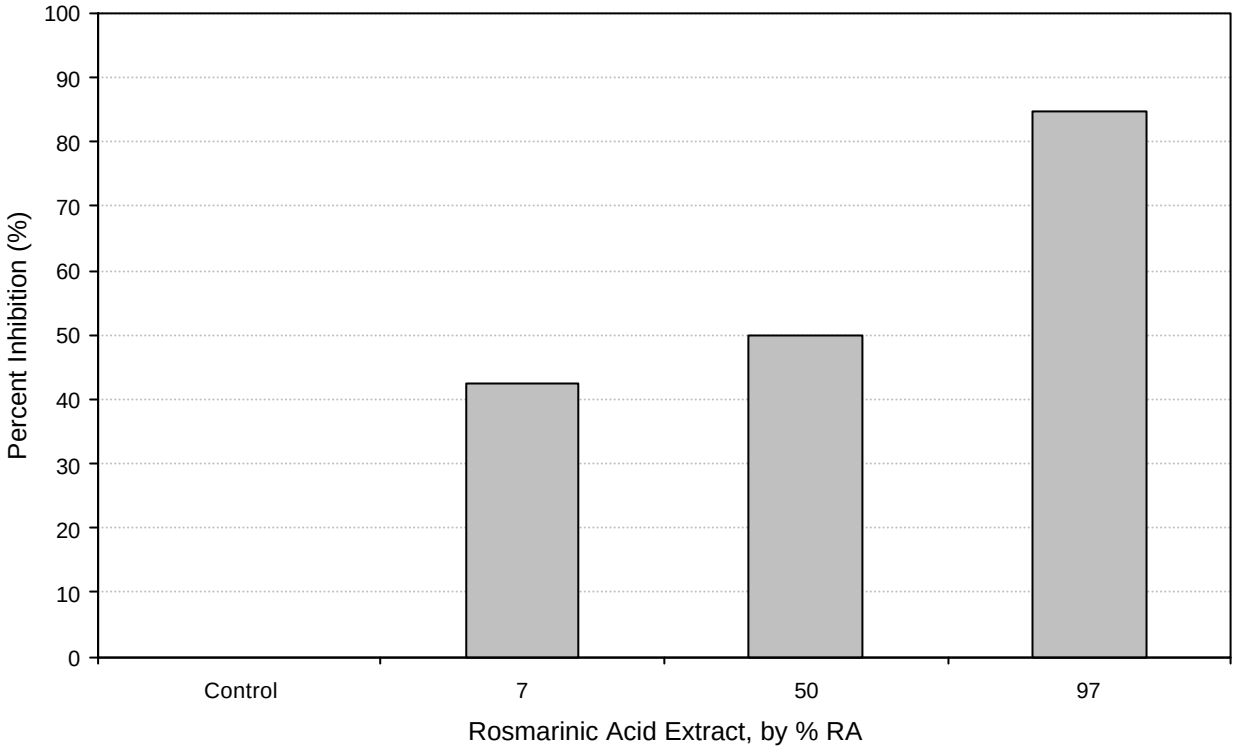

Figure 2. Percent (%) inhibition of amylase derivatives against starch at pH 6.9.

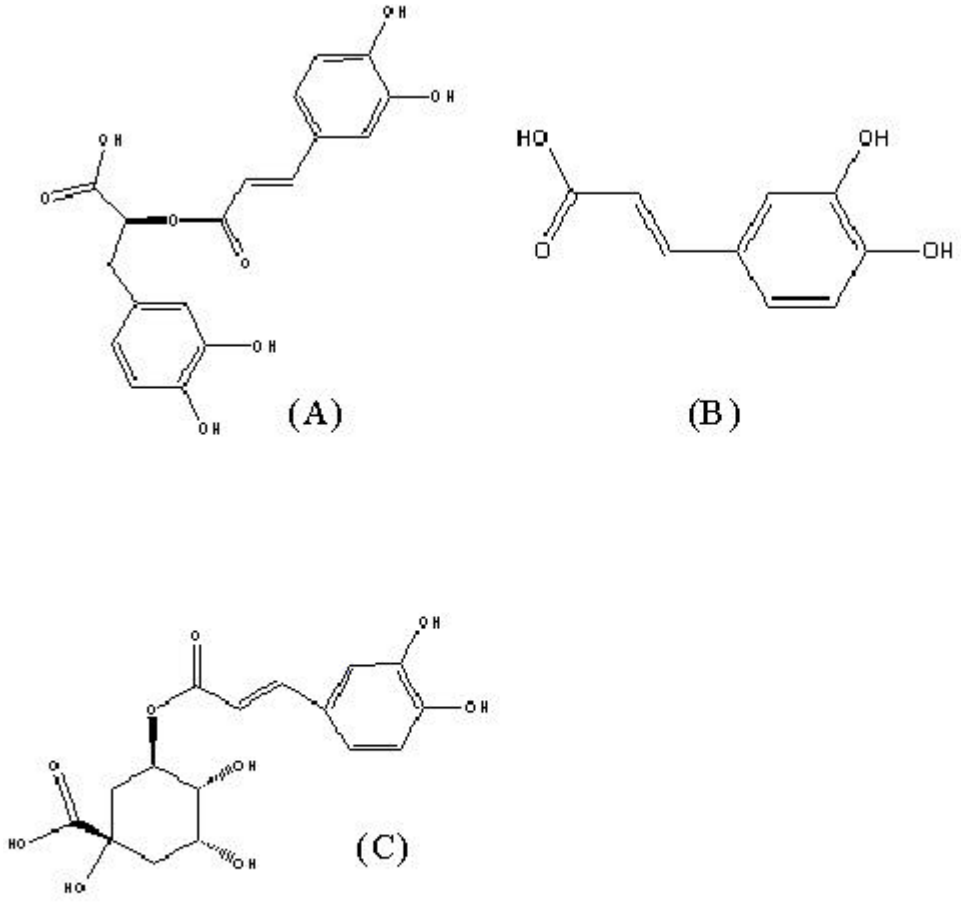

Figure 3. Structures of rosmarinic acid (A), caffeic acid (B), and chlorogenic acid (C).

of rosmarinic acid to both caffeic acid and chlorogenic acid leaves open the possibility that this hypothetical mechanism may occur (Fig. 3).

Previously, Rohn *et al*.,[24] reported that phenolic derivatives of trypsin showed reduced hydrolytic activity. Whether the reduced amylase activity observed in this study was the result of protracted hydrolysis or from complete enzyme inhibition, either result may have beneficial effects on oxidation-linked disease modulation. The dramatic inhibition of amylase activity observed herein occurred at pH 6.9, the peak pH for amylase activity, suggesting that inhibitory activity of these herbal extracts may be even more pronounced in physiological environments that differ in pH, where the amylase activity may be comparatively lower.[25] Further, our results suggest that the health-promoting benefits of RA-containing herbs used in traditional Indian medicine for treatment of diabetes mellitus may involve amylase-inhibiting activity of RA and other phenolics, and that such amylase-inhibiting activity may be promoted by mechanistic synergies among the present phenolic substances.[7]

Natural á-amylase inhibitors from food-grade herbal sources offer an attractive therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch and may have potential for use in the treatment of diabetes mellitus and obesity.[30,31] Powerful synthetic á-glucosidase inhibitors (ie acarbose, voglibose) are available, but cause various negative gastrointestinal symptoms and hepatic disorders.[31] Food-grade phenolic alpha-amylase inhibitors from herbal extracts are potentially safer, and therefore may be a preferred alternative for inhibition of carbohydrate break-down and control of glycemic index of food products. Future research is aimed at investigating the potential amylase-inhibitory activity of other phenolics present in food-grade herbal extracts and at elucidating putative phenolic synergies that may promote inhibition of á-amylase activity.

References

1. Harborne JB. Caffeic acid ester distribution in higher plants. Z Naturforsch 1966; 21b: 604-605.
2. Litvinenko VI, Popova TP, Simonjon AV, Zoz IG, Sokolov VS. “Gerbstoffe” und oxyzimstaureabkommlinge in Labiaten. Planta Med 1975; 27: 372-380.
3. Yang R, Shetty K. Stimulation of rosmarinic acid in shoot cultures of oregano (*Origanum vulgare*) clonal line in response to proline, proline analogue, and proline precursors. J Agric Food Chem 1998; 46 (7): 2888-2893.
4. Malencic DJ, Gasic O, Popovic M, Boza P. Screening for antioxidant properties of *Salvia reflexa* Hornem. Phytother Res 2000; 14: 546-548.
5. Exarchou V, Nenadis N, Tsimidou M, Gerothanassis IP, Troganis A, Boskou D. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. J Agric Food Chem 2000; 50: 5294-5299.
6. Javanmardi J, Khalighi A, Kashi A, Bais HP, Vivanco JM. Chemical characterization of basil (*Ocimum basilicum* L.) found in local accessions and used in traditional medicines in Iran. J Agric Food Chem 2002; 50: 5878-5883.
7. Maroo J, Vasu VT, Aalinkeel R, Gupta S. Glucose lowering effect of aqueous extract of *Enicostemma littorale* Blume in diabetes: a possible mechanism of action. J Ethnopharm 2002; 81: 317-320.
8. Al-Sereiti MR, Abu-Amer KM, Sen P. Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. Indian J Exper Biol 1999; 37: 124-130.
9. Inatani R, Nakatani N, Fuwa H. Antioxidative effect of constituents of rosemary (*Rosmarinus officinalis* L.) and their derivatives. Agric Biol Chem 1983; 47: 521-528.
10. Santamaria L, Tateo F, Bianchi A, Bianchi L. *Rosmarinus officinalis* extract inhibits as antioxidant mutagenesis by 8-methoxypsoralen (8-MOP) and benzo[a]pyrene (BP) in Salmonella typhimurium. Med Biol Environ 1987; 15: 97-101.
11. Zhu BT, Loder DP, Cai MX, Ho CT, Huang MT, Conney AH. Dietary administration of an extract from rosemary leaves enhances the liver microsomal metabolism of endogenous estrogens and decreases their uterotropic action in CD-1 mice. Carcinogenesis 1998; 19 (10): 1821-1827.
12. Kim HK, Lee HK, Shin CG, Huh H. HIV integrase inhibitory activity of *Agastache rugosa*. Arch Pharm Res 1999; 22 (5): 520-523.
13. Makino T, Ono T, Muso E, Yoshida H, Honda G, Sasayama S. Inhibitory effects of rosmarinic acid on the proliferation of cultured murine mesangial cells. Nephrol Dial Transplant 2000; 15: 1140-1145.
14. Kelm MA, Nair MG, Strasburg GM, DeWitt DL. Antioxidant and cyclooxygenase inhibitory phenolic compounds from *Ocimum sanctum* Linn. Phytomedicine 2000; 7 (1): 7-13.
15. Notkins AL. 2002. Immunologic and genetic factors in type 1 diabetes. J Biol Chem 2002; 277 (46): 43545-43548.
16. Fujita H, Yamagami T, Ohshima K. Fermented soybean-derived water-soluble touchi extract inhibits á-glucosidase and is antiglycemic in rats and humans after single oral treatments. J Nutrition 2001; 131: 1211-1213.
17. Ghavami A, Johnston BD, Jensen MT, Svensson B, Pinto BM. Synthesis of nitrogen analogues of salacinol and their evaluation as glycosidase inhibitors. J Am Chem Soc 2001; 123: 6268-6271.
18. Nishioka T, Kawabata J, Aoyama Y. Baicalein, an alpha-glucosidase inhibitor from *Scutellaria baicalensis*. J Nat Prod 1998; 61: 1413-1415.
19. Johnston BD, Ghavami A, Jensen MT, Svensson B, Pinto BM. Synthesis of selenium analogues of the naturally occurring glycosidase inhibitor salacinol and their evaluation as glycosidase inhibitors. J Am Chem Soc 2002; 124: 8245-8250.
20. El-Ashry ESH, Rashed N, Shobier AHS. Glycosidase inhibitors and their chemotherapeutic value, part 3. Pharmazie 2000; 55: 403-415.
21. Kim JS, Kwon CS, Son KH. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. Biosci Biotechnol Biochem 2000; 64 (11): 2458-2461.
22. Matsui T, Ueda T, Oki T, Sugita K, Terahara N, Matsumoto K. -Glucosidase inhibitory action of natural acylated anthocyanins. 1. survey of natural pigments with potent inhibitory activity. J Agric Food Chem 2001; 49: 1948-1951.
23. Kusano G, Takahira M, Shibano M, Kusano A, Okamoto Y, Tsujibo H, Numata A, Inamori Y. Studies on inhibitory activities of fukiic acid esters on germination, á-amylase, and carboxypeptidase A. Biol Pharm Bull 1998; 21 (9): 997-999.

24. Rohn S, Rawel HM, Kroll J. Inhibitory effects of plant phenols on the activity of selected enzymes. J Agric Food Chem 2002; 50: 3566-3571.
25. Worthington V. Alpha amylase. In: Worthington V, ed. Worthington Enzyme Manual; enzymes and related biochemicals. Lakewood, NJ: Worthington Biochemical Company, 1993; 36-41.
26. Vardar-Unlu G, Candan F, Sokmen A, Daferera D, Polissiou M, Sokmen M, Donmez E, Tepe B. Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. Var. *pectinatus* (Lamiaceae). J Agric Food Chem 2003; 51: 63-67.
27. Arts MJ, Haenen GR, Wilms LC, Beetstra SA, Heijnen CG, Voss HP, Bast A. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. J Agric Food Chem 2002; 50: 1184-1187.
28. Griffiths DW. The inhibition of digestive enzymes by polyphenolic compounds. Adv Exp Med Biol 1986; 199: 509-516.
29. Watanabe J, Kawabata J, Kurihara H, Niki R. Isolation and identification of á-glucosidase inhibitors from tochucha (*Eucommia ulmoides*). Biosci Biotech Biochem 1997; 61 (1): 177-178.
30. Gallaher D, Schneeman BO. Nutritional and metabolic response to plant inhibitors of digestive enzymes. Adv Exp Med Biol 1986; 199: 167-184.
31. Murai A, Iwamura K, Takada M, Ogawa K, Usui T, Okumura J. Control of postprandial hyperglycaemia by galactosyl maltobionolactone and its novel anti-amylase effect in mice. Life Sciences 2002; 71: 1405-1415.