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Full Length Research Paper

Alpha glucosidase inhibitory effect, anti-microbial activity and UPLC analysis of *Rhus verniciflua* under various extract conditions

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In the present study, crude extract of *Rhus verniciflua* stem was screened for its α-glucosidase inhibitory effects and anti-microbial activity using different extraction ratios of water to alcohol. Ultra performance liquid chromatography (UPLC) was used to quantify compounds (fustin, gallic acid, 3', 4', 7-trihydroxyflavone, and fisetin). Ethanolic extract showed higher α-glucosidase inhibitory effect than methanolic extract, whereas distilled water extract did not inhibit α-glucosidase at all. Especially, neat alcoholic (methanolic or ethanolic) extracts exhibited stronger inhibitory activities compared to their corresponding aqueous mixtures. Analysis of the UPLC chromatogram showed that fustin (from 28.5 to 151.7 mg/g extract) was the major compound of *R. verniciflua* stem. One-hundred percent ethanolic extracts produced inhibition zones against *Staphylococcus aureus* with the largest diameters (15 mm). The result from this study provides suitable support for the use of *R. verniciflua* in the treatment of diabetes and for the control of infectious disease caused by *S. aureus*.

Key words: *Rhus verniciflua*, anti-microbial activity, α-glucosidase inhibitory effect.

INTRODUCTION

Rhus verniciflua belongs to the Anacardiaceae family and is mostly grown in the northeast region of Asia, especially in Korea, China and Japan (Kim, 1996). *R. verniciflua* has been used traditionally for the improvement of blood circulation, digestion, high blood pressure, paralysis, aging and vermicides, and also has been traditionally used in Korea as a food additive (Kim et al., 2010). Recently, a number of researchers have reported the various effects of *R. verniciflua* extracts as a livestock feed. For example, Kang et al. (2008a) have reported that egg laying performance and quality in hens was improved by dietary Rhus tree-extract ($p<0.05$). Kang et al. (2008b) reported that beef from Hanwoo cattle fed 4% *R. verniciflua* for 4 to 5 months had improved water-holding capacity, monounsaturated fatty acid content and color stability relative to other beef.

Diabetes mellitus (DM) is a common endocrine system disease that causes metabolic disorders and which leads to multiple organ damage syndrome. Clinical admiral diabetes is divided into two types, with more than 90% of patients having Type II diabetes (Wang and Zhang, 2009). The number of diabetes cases was 171 million in 2000 and is expected to rise to 366 million in 2030 (Si et al., 2010). Acting as a key enzyme for carbohydrate digestion, intestinal α-glucosidase is a glucosidase located at the epithelium of the small intestine. α-glucosidase has been recognized as a therapeutic target for the modulation of postprandial hyperglycemia, which is the earliest metabolic abnormality that occurs in Type II DM (Yao et al., 2010). Several natural α-glucosidase

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Abbreviations: DM, Diabetes mellitus; **6-OHAD,** 6-hydroxydopamine; **pNPG,** 4-Nitrophenyl-α-D-glucopyranoside; **ROS,** reactive oxygen species; **UPLC,** ultra performance liquid chromatography.

inhibitors including acarbose, voglibose and miglitol are clinically used as a treatment (Scott and Spencer, 2000), but their prices are high and clinical side effects occur.

Staphylococcus aureus is an opportunistic pathogen and frequent coloniser of many animal species, including humans. In addition to colonising various hosts, it can cause a wide range of different infections, ranging from dermatitis, pneumonia and septicaemia to osteomyelitis and meningitis in humans (Hasman, 2010).

R. verniciflua contains various flavonoids, which exert a remarkable spectrum of biological activities affecting basic cell functions such as growth, differentiation and apoptosis. Flavonoids are known to have anti-carcinogenic, anti-inflammatory, anti-bacterial, immune-stimulating and anti-viral activities (Jang et al., 2005). The aim of the present study was to analyze the effect of different extraction ratios of water to alcohol on the anti-microbial and α-glucosidase inhibitory activities of crude extract of *R. verniciflua* stem. Several constituent compounds found in *R. verniciflua*, including fustin, gallic acid, 3', 4', 7-trihydroxyflavone and fisetin, were quantified by ultra performance liquid chromatography (UPLC) analysis.

MATERIALS AND METHODS

Chemicals

4-Nitrophenyl-α-D-glucopyranoside (pNPG) and α-glucosidase (E.C. 3.2.1.20) were obtained from Sigma Chemical Co. (St. Louis, MO). Acarbose was obtained from Bayer Schering Pharma. Fustin, gallic acid, 3', 4', 7-trihydroxyflavone and fisetin, isolated from the heartwood of *R. verniciflua* as reported previously, were used as standard (Kim et al., 2010). All reagents were of analytical grade or better.

Plant materials and extraction of *R. verniciflua*

The *R. verniciflua* stem was obtained from Hoengseong-gun, Gangwon-do, Korea. The samples were dried at room temperature and powdered, using a blender. The air-dried, powdered (30 g for each sample) of *R. verniciflua* stem was extracted three times with ethanol (60, 80 and 100%), methanol (60%, 80 and 100%), and distil water, with a 10:1 solvent-sample ratio, for 24 h at room temperature. The solution was filtered, evaporated under reduced pressure and lyophilized to give dried powder extract. Sample was dissolved 80% EtOH (v/v). All the processes were done in triplicates.

Inhibition of α-glucosidase

α-glucosidase (50 µl, 0.5 U/ml) and 0.2 M potassium phosphate buffer (pH 6.8, 50 µl) were mixed with test sample (50 µl, 10, 50 and 100 ppm). After incubation at 37° for 15 min, 3 mM pNPG (100 µl) was added. The reaction was incubated again at 37° for 10 min and then stopped by 0.1 M Na_2CO_3 (750 µl). The absorption of 4-nitrophenol was measured at 405 nm. The reaction mixture without sample was used as a control, and the mixture without substrate was used as a blank. The experiment was performed in triplicate.

The percentage inhibition of α-glucosidase was calculated as follows:

Inhibition rate (%) = {1-(*Abs* sample-*Abs* blank)/ *Abs* control} ×100

Where *Abs* sample is the absorbance of the experimental sample, *Abs* blank is the absorbance of the blank, and *Abs* control is the absorbance of the control.

Quantitative analysis of individual phenolic compounds by UPLC

The obtained sample from the *R. verniciflua* stem was filtered through a 0.2 µm GHP membrane disc filter and analyzed by UPLC. Phenolic compounds analysis was carried out using a Waters acquity UPLC system coupled with an UV-vis wavelength detector (Schwarz et al., 2009). The separation was carried out on a BEH C_{18} column (50 mm × 2.1 mm, 1.7 µm, Waters corp.) maintained at 47℃. The binary system phases were: A (3% ACN, 2% acetic acid, 95% water) and B (85% ACN, 2% acetic acid, 13% water) at a flow rate of 0.7 ml/min. The 6.5 min gradient was as follows: 0 min, 100% A, 3 min, 90% A (curve 6), 4 min, 90% A, 6.5 min, 25% A (curve 6). The injection volume was 2.5 µl and peaks were monitored at 280 nm. Identification of phenolic compounds from *R. verniciflua* was performed by qualitatively and quantitatively comparing peak areas on the chromatograms of samples with those of diluted standard solutions. Analyses were performed in triplicate.

Anti-microbial assay by agar disc diffusion method

The samples were tested for antimicrobial assay by the agar disc diffusion method. Sterile Whatman No. 1 (8 mm) disc papers were individually placed on agar plates, after which 5 mg of samples were applied to the filter paper disk. After incubation at 37℃ for 24 h, plates were determined by observing the zone diameter of areas around the wells.

Statistical analysis

All data were expressed as mean value ± standard deviation (SD) of the number of experiments (n=3). Significance differences for multiple comparisons were determined using one-way analysis of variance (ANOVA). Duncan's multiple range test was used to assess the significant differences with the SPSS statistical analysis. Differences at $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Inhibition of α-glucosidase

Natural products are still the most available source of α-glucosidase inhibitors (Lee et al., 2008). Therefore, we investigated biologically active compounds from *R. verniciflua* stem using different extraction ratios of water to alcohol. Extracts under different conditions of *R. verniciflua* were tested for α-glucosidase inhibitory activity (Figure 1). Ethanolic extract showed higher α-glucosidase inhibitory activity than methanolic extract, whereas distilled water extract did not inhibit α-glucosidase at all. Especially, neat alcoholic (methanolic or ethanolic) extracts exhibited stronger inhibitory effects than their corresponding aqueous mixtures. The percentage inhibition of α-glucosidase by *R. verniciflua* extracts at 2,

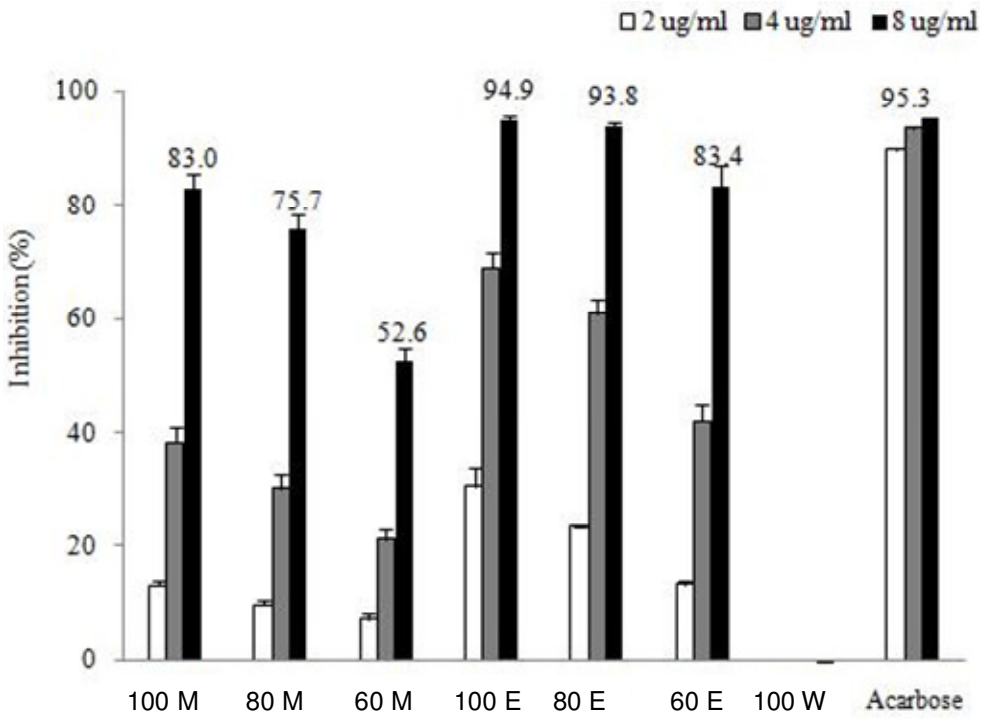

Figure 1. Dose-dependent changes in the α-glucosidase inhibition of *Rhus verniciflua* extracts. 60 M, 60% methanolic extract; 80 M, 80% methanolic extract; 100 M, 100% methanolic extract; 60 E, 60% ethanolic extract; 80E, 80% ethanolic extract; 100 E, 100% ethanolic extract; 100 W, distil water extract.

4 and 8 µg/ml showed dose-dependent acceleration. The α-glucosidase inhibitory effect of acarbose (95.3%, 8 µg/ml) seemed higher than that of ethanolic extract (94.9%, 8 µg/ml). In recent works with traditional plants, polymeric polyphenols were observed to contribute to strong α-glucosidase inhibition (Onal et al., 2005). In our previous paper, 80% ethanolic extract of *R. verniciflua* along with the ethyl acetate and butanol fractions had high phenolic contents and high α-glucosidase inhibitory activities (Kim et al., 2010). Further, 3', 4', 7-trihydroxyflavone exhibited powerful α-glucosidase inhibitory effects (Kim et al., 2010). Therefore, 100% ethanolic extract showed higher α-glucosidase inhibitory activity due to its higher 3', 4', 7-trihydroxyflavone concentration compared to other samples.

Quantitative analysis of individual phenolic compounds

Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids and flavonoids (Kähkönen et al., 1999). Phenolic acids have been repeatedly implicated as natural anti-oxidants in fruits, vegetables, cereals and other plants (Zheng and Wang, 2001). Gallic acid is widely found in nature and is an important ingredient of hydrolysable tannin. This compound has anti-oxidant, anti-bacterial, anti-inflammatory, anti-tumor and anti-mutation activities (She et al., 2005). The most widespread and diverse phenolics are flavonoids having the same C_{15} (C_6-C_3-C_6) skeleton and possessing anti-oxidant capacity toward a variety of easily oxidizable compounds (Robards et al., 1999). Fisetin is a flavonoid that reverses 6-OHDA damage caused by reactive oxygen species (ROS). In addition, fisetin can reduce the calcium concentration as well as the ratio of Bcl-2, Bax and caspase-3 activity; protect neurons (Hu et al., 2009).

The UPLC analyses for the quantitative determination of fustin, gallic acid, 3', 4', 7-trihydroxyflavone and fisetin in *R. verniciflua* were carried out by peak assignment of the retention times and UV-vis spectra. Among these compounds, fustin showed the highest content in *R. verniciflua* (Table 1).

The fustin content of the samples ranged from 28.5 to 151.7 mg/g of extract, whereas the other phenolic compounds (that is, gallic acid, 3', 4', 7-trihydroxyflavone and fisetin) ranged in content from 0 to 13 mg/g of extract. Our results are in agreement with previous reports that found that fustin (18.4 g/100 g of extract) is in the phenolic-rich fraction of *R. verniciflua* (Jung et al., 2007). Using a BEH C_{18} column, gallic acid, fustin, fisetin and 3', 4', 7-trihydroxyflavone were separated with retention times of 0.508, 3.32, 5.046 and 5.27 min,

Table 1. Concentration of phenolic compounds in *Rhus verniciflua* extracts.

Samples	Concentration of phenolic compounds (mg/g extract)			
	Fustin	Gallic acid	3', 4', 7-Trihydroxyflavone	Fisetin
100M[1)]	116.6 b[2)]	1.2 d	9.9 b	7.7 c
80M	106.2 b	0.7 e	8.4 bc	6.6 c
60M	109.7 b	1.5 d	7.7 c	4.8 d
100E	151.7 a	2.0 c	13.0 a	12.7 a
80E	99.7 b	0.8 e	7.6 c	9.8 b
60E	122.6 b	2.8 b	9.4 b	10.7 b
100W	28.5 c	9.5 a	N.D.[3)]	0.1 e

[1)] Abbreviations see Figure 1. [2)] Values with the same superscript are not significantly different by Duncan's multiple range test at $p<0.05$. Values (mg/g) indicate the mean of three replications.[3)] N.D., not detected.

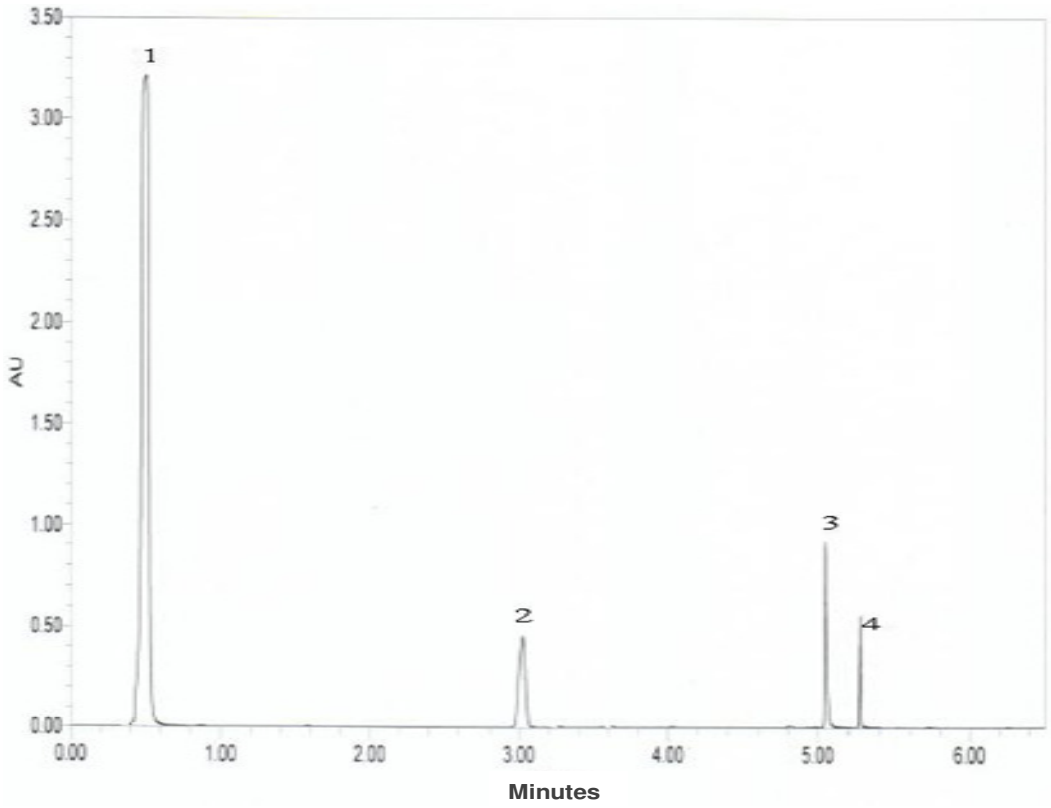

Figure 2. UPLC chromatogram of mixed standards. Detection wavelength was set at 280 nm. Peaks numbers corresponding to: 1, gallic acid; 2, fustin; 3, fisetin; 4, 3', 4', 7-trihydroxyflavone.

respectively. Total time of analysis was less than 6.5 min (Figure 2).

Anti-microbial activity of *Rhus verniciflua* extract

Plant product drugs and herbal remedies have been employed since prehistoric times for the treatment of human and animal diseases, and several countries still rely on plants and herbs as the main sources of drugs (Ogbonnia et al., 2008). Plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth or modulating the development of other vegetables (Karagöz et al., 2009). Extract from *R. verniciflua* reportedly shows anti-oxidant, anti-microbial,

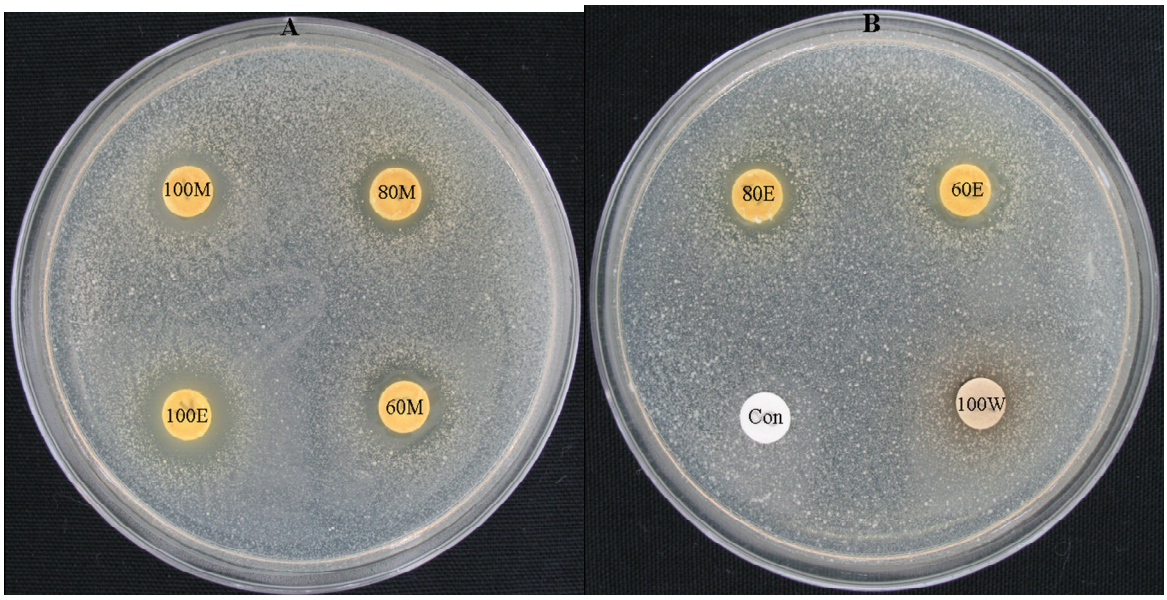

Figure 3. Anti-microbial activity of *Rhus verniciflua* extracts against *Staphylococus aureus* by the disc method. Con, control (without samples). Abbreviations see Figure 1.

anti-mutagenic, anti-arthritic, anti-obesity, anti-platelet and anti-cancer activities (Kim et al., 2010). The anti-microbial activities of the extracts were measured by determining the minimal inhibitory concentration (MIC) and based on the diameter of the inhibition zones using disc agar diffusion assay. 100E and 80M showed the highest anti-microbial activities against the gram-positive bacterium *S. aureus* (MIC = 500 µg/ml), whereas distilled water extract did not show any inhibition (data not shown). The disc agar diffusion assay showed different degrees of anti-microbial activity in the different sample extracts from *S. aureus* (Figure 3). Ethanolic extracts showed more effective anti-bacterial activity than methanolic extracts. Water extracts were fairly weak against *S. aureus*. One-hundred percent ethanolic extracts exhibited the maximum bacterial inhibition zone of 15 mm compared to the other extracts. In our previous work (Kim et al., 2010), 3', 4', 7-trihydroxyflavone exhibited the highest anti-microbial activity against *S. aureus* (MIC = 32 µg/ml) compared to other compounds (MIC = 1000 µg/ml). Therefore, this result suggests that the anti-microbial activity of *R. verniciflua* may protect against disease caused by *S. aureus* infection.

Conclusion

Ethanolic extract exhibited a higher α-glucosidase inhibitory effect than other sample extract. Further, 100% ethanolic extracts from *R. verniciflua* produced inhibition zones against *S. aureus* with the largest diameters. Such results suggest that 3', 4', 7-trihydroxyflavone from *R. verniciflua* may be responsible for the high anti-microbial and α-glucosidase inhibitory activities. Therefore, the results from this study give suitable support to the use of *R. verniciflua* as a treatment for diabetes and for the control of infectious disease caused by *S. aureus*.

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