J Food Sci Nutr
Vol 14, p 195~200 (2009)
DOI: 10.3746/jfn.2009.14.3.195

J Food Science and Nutrition

Evaluation of the Biological Activity of Extracts from Star-Anise (*Illicium verum*)

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Abstract

This study was carried out to investigate the biological activities of star anise extracts obtained from four different solvents (water, methanol, ethanol and chloroform) by measuring total polyphenol and flavonoid contents, DPPH radical scavenging, hydroxyl radical scavenging, nitrite scavenging activity and antimicrobial activity. The methanol extract showed the highest extraction yield, followed by ethanol, water and chloroform. The properties of the extracting solvents significantly affected the total polyphenol content. Methanol extracts contained more total polyphenols than any other extracts. The highest DPPH radical scavenging activity was found in methanol extract. The hydroxyl radical and nitrite scavenging activity were highest in methanol and ethanol extracts. In antimicrobial activity, water extract showed stronger activities than methanol and ethanol extract against *Micrococcus luteus* and *Bacillus subtilis*, and no inhibitory effects on Gram negative bacteria were found in all extracts at the concentration used.

Key words: star anise, biological activity, polyphenol, flavonoid

INTRODUCTION

There is strong evidence that reactive oxygen species (ROS) are responsible for the damage of lipids, proteins and nucleic acids in cells, leading to aging, cancer and many other degenerative disorders to the human body (1,2). ROS include free radicals, such as superoxide anion radicals (O_2^-), hydroxyl radical species (·OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Spices and herbs have been found to contain a wide variety of free radical scavenging molecules, including polyphenols, flavonoids, vitamins and carotenoids (3,4). The use of these materials as antioxidants is becoming increasingly important in the food industry.

Star anise (*Illicium verum*) is shaped like an eight-pointed star and contains shiny seeds. It is a well-known spice that resembles anise in scent and flavor which comes from the essential oil, anethole (5,6). It is widely used in Chinese, Indian and Malay-Indonesian cuisine and is a part of the Chinese five spice powder, along with cinnamon, cloves, fennel and prickly ash (5). Recently, star anise has come into use in the West in wine, desserts and liquors as a less expensive substitute for anise. Star Anise has been used as a popular folk medicine for rheumatism and colic in the East. It also has been used to control flatulence and nausea and is considered to be an antispasmodic for the gastrointestinal system. It is a common flavoring for medicinal teas and cough mixtures (5).

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Limited research has been done on the biological activities of star anise. Therefore, the objective of this study was to investigate the total polyphenol and flavonoid contents of star anise, as well as the biological activities of the extracts obtained from various solvent systems having diverse polarities.

MATERIALS AND METHODS

Materials and chemicals

Star anise was purchased from Kyungdong market (Seoul, Korea) in a dried form. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), gallic acid, naringin, deoxyribose, ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin–Ciocalteu were purchased from Fluka (Switzerland). All other chemicals and reagents used were of analytical grade and commercially available.

Preparation of star anise extract

The dry star anise were ground to a fine powder and the powder was extracted with 10 volumes of four different solvents (distilled water, 99.9% methanol, 99.9% ethanol, or 99.9% chloroform) in a shaking incubator at 30°C for 3 hr. The extracts were filtered through Whatman No.1 filter paper and the residues were re-extracted in the same condition. The two filtrates of methanol, ethanol and chloroform were combined and ro-

tary-evaporated at 40°C to dryness. Water filtrate was frozen and lyophilized. Each extract was weighed to determine the extraction yield and then stored at -20°C prior to analysis. Each extract sample was evaluated at the final concentration of 1 mg/mL and 3 mg/mL. Ascorbic acid was used as a positive control.

Determination of total polyphenol and flavonoid contents of star anise extract

Total polyphenol content was determined using a modified version of the Folin-Ciocalteu method (7). An aliquot (0.1 mL) of each sample solution was mixed with 1.9 mL of distilled water and 0.2 mL of Folin-Ciocalteu's phenol reagent. The mixture was then allowed to stand at room temperature for 3 min and then 0.4 mL of saturated sodium carbonate solution and 1.9 mL of distilled water were added. After 1 hr at room temperature, the resulting blue complex was measured at 725 nm. The standard curve for total polyphenol content was made using gallic acid standard solution and total polyphenolics were expressed as mg of gallic acid equivalents (GAE)/g star anise extract. The flavonoid content was determined by using a colorimetric method described by Lee and Kim (8) with slight modification. An aliquot (0.2 mL) of sample solution was added to 10 mL of diethyleneglycol and 1 mL of 1 N NaOH. After 1 hr incubation at 37°C, the absorbance was measured at 420 nm. The standard curve was made using naringin and flavonoid were expressed as mg naringin equivalents/g star anise extract.

Determination of DPPH radical scavenging activity of star anise extract

The DPPH radical scavenging activity of star anise was measured according to the method of Blois (9) with modification. An aliquot (0.2 mL) of each sample solution at two different concentrations was added to 2 mL of 0.1 mM DPPH ethanol solution. The reaction mixture was shaken well and allowed to stand for 30 min at room temperature. The absorbance at 517 nm was measured with a UV-Visible spectrophotometer (Smart Plus, Korea). The radical scavenging activity was calculated according to the following formula:

DPPH radical scavenging activity (%)=$(1-A_1/A_0)\times 100$

where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

Determination of hydroxyl radical scavenging activity of star anise extract

The hydroxyl radical scavenging activity of star anise extract was measured according to the method of Nagai et al. (10) with modification. The reacting mixture, which contained 100 μL of a premixed 10 mM $FeSO_4$ and 10 mM EDTA (1:1, v/v), 200 μL of 10 mM deoxyribose, 1.4 mL of 0.1 M phosphate buffer (pH 7.4), 100 μL of sample solution, and 200 μL of 10 mM H_2O_2 was incubated at 37°C for 1 hr. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added to each mixture and heated on a water bath for 15 min at 100°C to develop the pink colored malondialdehyde-thiobarbituric acid and the reaction was stopped by 5 min in an ice-cold water bath. The absorbance of the resulting solution was measured at 532 nm and the scavenging effect of hydroxyl radicals was calculated using the following formula:

Hydroxyl radical scavenging activity (%)=$(1-A_1/A_0)\times 100$

where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

Determination of nitrite scavenging ability of star anise extract

The nitrite scavenging activity was measured according to the method of Kato et al. (11) with modification. An aliquot (0.1 mL) of each sample solution was added to 0.2 mL of 1 mM $NaNO_2$ solution and pH values of the resulting mixtures were adjusted to 1.2, 3.0, and 6.2 and the final volume of each sample was adjusted to 1.0 mL with buffer solution. After 1 hr incubation at 37°C, 5 mL of 2% acetic acid and 0.4 mL of Griess reagent (premixed 1% sulfanilic acid and 1% naphthylamine, 1:1, v/v in 30% acetic acid) was added to the mixture and allowed to stand at room temperature for 15 min. The nitrite scavenging activity was determined based on the following formula:

$$\text{Nitrite scavenging activity (\%)} = \left(1-\frac{A_1-A_2}{A_0}\right)\times 100$$

where A_0 was the absorbance of the control, A_1 was the absorbance of sample and A_2 was the absorbance of the sample blank.

Antimicrobial activity test of star anise extract

Bacillus subtilis (ATTC 9372), *Micrococcus luteus* (ATCC 9341), *Staphylcoccus auresus* (IMSNU 11089), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella* Typhymurium (ATCC 25922) were used for the antimicrobial activity test. The microorganisms were grown overnight at 37°C in nutrient broth. Antimicrobial activity of extract samples were investigated by the disc diffusion method. Forty μL (100 mg/mL concentration) of each extracts were impregnated on sterile paper discs of 8 mm diameter and discs were let to dry to remove any residual solvent which might interfere with the result. Petri plates

were prepared by pouring 20 mL of nutrient agar and allowed to solidify. Plates were dried and 0.2 mL of inoculums was poured and uniformly spread. Discs were then placed on the agar plates, and the plates were incubated at 37°C for 24 hr. After the incubation, the zones of growth inhibition around the discs were measured and all determinations were made in triplicate.

Statistical analysis

The statistical analysis was performed using the SPSS version 15.0. All data were recorded as mean±standard deviation of at least triplicate measurements. Means were compared with Duncan's multiple range test with $p<0.05$.

RESULTS AND DISCUSSION

Extraction yield of star anise extracts

Extraction yield of star anise from different solvents are summarized in Table 1. The extraction yield varied from 6.6% to 21.8% depending on the extraction solvent. The methanol extract showed the highest yield, followed by ethanol, water and chloroform. No significant difference was found between the yield of ethanol and chloroform.

Total polyphenol and flavonoid content of star anise extracts

Total polyphenol and flavonoid content of star anise extracts from different solvents are summarized in Table 2. The methanol extract exhibited the highest contents of total polyphenols of 163.6 mg/g, while water, ethanol and chloroform extracts contained 114.6, 110.4, and 25.5 mg/g, respectively. Total polyphenol content of methanol extract was 1.5 times and 6.4 times higher than those of water and chloroform extract, respectively. Flavonoid contents ranged from 22.8 to 31.9 mg/g and no significant difference was observed among the solvent extracts, although the methanol extracts exhibited the highest contents. Flavonoids are polyphenols with diphenylpropane (C_6-C_3-C_6) skeletone and considered to be the largest group of naturally occurring phenols in plants (1). It is known that polyphenols and flavonoids play an important role in the high antioxidative activity in plants (12,13). Therefore, it was expected that the high polyphenol and flavonoid contents of methanol extract of star anise might be responsible for the higher antioxidative activity than other extracts.

Table 1. The yield of star anise extract by different solvents

Extract	Water	Methanol	Ethanol	Chloroform
Yield (%)	16.4±0.4[1)b]	21.8±1.0[a2)]	8.6±0.7[c]	6.6±0.4[c]

[1)]Each value is mean±SD.
[2)]Means with different letters within a row are significantly different from each other at $p<0.05$ as determined by Duncan's multiple range test.

DPPH radical scavenging activity of star anise extracts

Table 3 shows the DPPH radical scavenging activity of various solvent extracts from star anise. At the concentration of 1 mg/mL, significant differences in DPPH radical scavenging activities were observed between solvent extracts ($p<0.05$). The methanol extract showed the highest radical scavenging activity (89.02%), the ethanol extract next (58.39%), and the chloroform extract had the lowest antioxidant activity (5.75%). As the concentration of extracts increased to 3 mg/mL, the radical scavenging activity increased slightly, showing the activities of 92.73%, 91.99%, 89.09%, 11.33% for the water, methanol, ethanol, chloroform extracts, respectively. Methanol extract, which had higher total polyphenol and flavonoid contents than did other solvent extracts (Table 2), exhibited the best antioxidant activity in all solvent extracts, as expected, and was nearly equal to that of ascorbic acid. A positive correlation was observed be-

Table 2. Total polyphenol and flavonoid contents of star anise extract by different solvents

Extract	Water	Methanol	Ethanol	Chloroform
Total polyphenol (mg/g)	114.6±3.06[b1)]	163.6±1.13[a]	110.4±1.74[b]	25.5±0.45[c]
Flavonoid (mg/g)	24.5±0.24	31.9±0.35	23.4±0.24	22.8±0.26

[1)]Means with different letters within a row are significantly different from each other at $p<0.05$ as determined by Duncan's multiple range test.

Table 3. DPPH radical scavenging activity of star anise extracts by different solvents (unit: %)

Extract	Water	Methanol	Ethanol	Chloroform	Ascorbic acid
1 mg/mL	54.36±0.31[c1)]	89.02±3.14[a]	58.39±1.98[b]	5.75±0.91[d]	95.62±0.33
3 mg/mL	89.09±0.11[b]	92.73±0.34[a]	91.99±0.89[a]	11.33±0.48[c]	97.07±2.52

[1)]Means with different letters within a row are significantly different from each other at $p<0.05$ as determined by Duncan's multiple range test.

tween DPPH radical scavenging activity and contents of antioxidant components (total polyphenols, $r>0.92$; total flavonoids, $r>0.63$) (data not shown). These findings are consistent with the results of Liu et al. (14) who have reported that the contents of antioxidant components such as total phenols and flavonoids of various solvent extracts from lychee flowers were positively correlated with antioxidant activities.

The DPPH radical model is a relatively quick method for evaluating free radical scavenging activity by a visible color change from purple to yellow. The effect of antioxidants on DPPH radical scavenging is believed to occur due to their hydrogen donating ability (15). Many studies have been conducted to evaluate the antioxidant activities of extract obtained by various solvents. For example, Padmashree et al. (16) reported that the ethanol/water extract of star anise exhibited higher DPPH radical antioxygenic activity than water and petroleum ether extract and the scavenging activity increased with increasing concentrations. Kim (17) reported that the most potent DPPH radical scavenging activity was observed in 70% acetone extract, followed by methanol and ethanol extract of *Stantalum album*.

Hydroxyl radical scavenging activity of star anise extracts

Table 4 shows the hydroxyl radical scavenging activity of different solvent extracts from star anise. At the concentration of 1 mg/mL, hydroxyl radical scavenging activity ranged from 28.50 to 73.50% and methanol and ethanol extracts were equally effective in scavenging hydroxyl radicals, followed by chloroform and water extracts. Similar trends were observed at the concentration of 3 mg/mL. Methanol and ethanol extracts had higher activity than other extracts, but the activity tended to decrease in all extracts, exhibiting the range of 24.50 to 66.37%. Ascorbic acid was completely devoid of activity at both 1 and 3 mg/mL concentration. This finding is dissimilar to those of Nagai et al. (10) who reported that 1 mM ascorbic acid possessed hydroxyl radical scavenging activity of 13.2%, and slightly higher activity of 17.6% at 5 mM concentration.

Among the ROS, hydroxyl radicals are known to be the most reactive species and are thought to initiate cell damage by abstracting hydrogen atoms from membranes, which brings about peroxidic reactions of lipids (10,18). Therefore, removal of hydroxyl radicals is one of the most effective defenses in a living body against various diseases (15). From this, it was suggested that methanol and ethanol extract would be an effective hydroxyl radical scavenger compared to other solvent extracts.

Nitrite scavenging activity of star anise extracts

The nitrite scavenging activity of different solvent extracts from star anise are summarized in Table 5. At pH 1.2, methanol and ethanol extracts showed high nitrite scavenging activity by 90.16 and 90.98%, respectively, at a concentration of 1 mg/mL, which was nearly equal to that of 1 mg/mL of ascorbic acid. At pH 3.0, methanol and ethanol extracts showed strong scavenging activity of about 90%, which was superior to ascorbic acid, whereas water and chloroform extracts showed a decrease in activity. At pH 6.2, the activity was drastically decreased in all solvent extracts and the water and chloroform extracts were practically devoid of activity. Similar results were obtained by Jeon et al. (19) who found that the nitrite scavenging activity of water and ethanol extracts of *Torreya nucifera* were over

Table 4. Hydroxyl radical scavenging activity of star anise extracts by different solvents

Extract	Water	Methanol	Ethanol	Chloroform	Ascorbic acid
1 mg/mL	$28.50\pm0.70^{c1)}$	73.50 ± 2.47^{a}	72.62 ± 0.17^{a}	49.25 ± 0.70^{b}	−
3 mg/mL	24.50 ± 3.53^{c}	66.37 ± 7.95^{a}	54.12 ± 1.23^{ab}	47.62 ± 3.71^{b}	−

[1)]Means with different letters within a column are significantly different from each other at $p<0.05$ as determined by Duncan's multiple range test.

Table 5. Nitrite scavenging ability of star anise extracts by different solvents (unit: %)

	Extract	Water	Methanol	Ethanol	Chloroform	Ascorbic acid
pH 1.2	1 mg/mL	$20.08\pm7.53^{c1)}$	90.16 ± 1.16^{a}	90.98 ± 1.16^{a}	38.93 ± 1.74^{b}	92.15 ± 1.01
	3 mg/mL	48.31 ± 3.60^{b}	89.83 ± 8.39^{a}	89.41 ± 1.79^{a}	51.27 ± 4.19^{b}	93.22 ± 0.50
pH 3.0	1 mg/mL	12.88 ± 0.21^{c}	93.19 ± 1.93^{a}	89.54 ± 1.06^{b}	12.57 ± 0.22^{c}	45.16 ± 1.92
	3 mg/mL	25.61 ± 0.64^{b}	92.13 ± 2.14^{a}	88.63 ± 1.93^{a}	10.00 ± 0.42^{c}	71.71 ± 5.48
pH 6.2	1 mg/mL	−	2.75 ± 0.86^{a}	0.76 ± 1.08^{b}	−	−
	3 mg/mL	0.59 ± 0.84^{b}	6.23 ± 1.73^{a}	5.52 ± 1.05^{a}	−	−

[1)]Means with different letters within a column are significantly different from each other at $p<0.05$ as determined by Duncan's multiple range test.

90% at pH 1.2 and 3.0, and were low at a pH of 6.0. Kwon and Park (20) also found that the nitrite scavenging abilities of water and ethanol extract of omija were the highest at pH 1.2 and decreased with increasing pH. It has been reported that phenolic compounds may interact with nitrite to produce *p*-nitrosophenol, and *p*- and *o*-diazoquinone, which may reduce nitrosamine formation, and this reaction is facilitated by the acidic condition of pH 3.0 (21).

Nitrite (NO_2^-) is toxic, and the consumption of excess amounts of nitrite, for example, in sausages and cured meats, results in the oxidization of hemoglobin, which can lead to methemoglobinemia (22). Nitrites can react with degradation products of proteins known as amines to form nitrosamine, which is a carcinogen. In addition, the acidic conditions present in the human gastrointestinal tract promote the formation of nitrosamine (23). The fact that the nitrite scavenging activity was high at pH 1.2 suggests that nitrosamine production can be inhibited *in vivo*. From these findings, it was suggested that methanol or ethanol extracts of star anise would be an effective nitrite scavenger at pH values lower than 3.0.

Antimicrobial activity of star anise extracts

The disc diffusion method was used to determine the inhibition zones of the three Gram positive, and three Gram negative bacterial strains. Among the Gram-positive bacteria, *Micrococcus luteus* and *Bacillus subtilis* were sensitive to star anise extracts as shown in Fig. 1. Water extract was most effective to inhibitory to *Micrococcus luteus* and *Bacillus subtilis*, showing the average zone of inhibition of 27.5 and 18.5 mm, respectively. Methanol extract exhibited the zone of inhibition 22.5 and 14.5 mm for *Micrococcus luteus*, and *Bacillus subtilis*, respectively. Chloroform extract did not show any antimicrobial activity. Studies in the literature have shown that star anise contains a high percentage of shikimic acid which is known as a key material in the manufacture of antiviral drug (6). Shikimic acid is highly soluble in water and insoluble in nonpolar solvents such as chloroform and benzene, and this may partly account for the result of antimicrobial activities shown in Fig. 1. All of star anise extracts had no inhibitory effect on Gram negative bacteria. According to Norajit et al. (24), the resistance of Gram-negative bacteria to some antimicrobial agents may be due to the outer membrane impermeability to hydrophobic substances. Our results show that star anise extracts were more active against Gram-positive than Gram-negative bacteria and water extract had much more powerful antimicrobial activity than other extracts on *Micrococcus luteus* and *B. subtilis*.

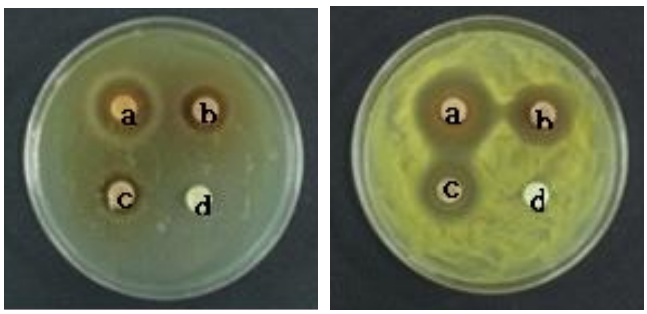

Fig. 1. Inhibitory effect of star anise extract on the growth of microorganisms. left: *Bacillus subtilis*, right: *Micrococcos luteus*. a: water extract, b: methanol extract, c: ethanol extract, d: chloroform extract.

ACKNOWLEDGEMENT

This work was supported by the Daejin University Research Grants in 2009.

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(Received August 31, 2009; Accepted September 14, 2009)