Food Chemistry 122 (2010) 1168–1172

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vitro antibacterial and antioxidant activities of *Orthosiphon stamineus* Benth. extracts against food-borne bacteria

Chun-Hoong Ho [a], Ismail Noryati [a], Shaida-Fariza Sulaiman [b], Ahmad Rosma [a,*]

[a] School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia
[b] School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form 8 February 2010
Accepted 25 March 2010

Keywords:
Orthosiphon stamineus
Cat whisker
Antibacterial
Antioxidant
Rosmarinic acid
Vibrio parahaemolyticus

ABSTRACT

Extracts of the *Orthosiphon stamineus* plant were tested for antimicrobial and antioxidant activities against selected food-borne bacteria *in vitro*. Whole *O. stamineus* plants (powdered) were extracted using various concentrations (0%, 25%, 50%, 75%, and 100%) of methanol. *O. stamineus* extracted with 50% methanol, 75% methanol and fraction 5 of a 50% methanolic extract demonstrated inhibitory activity against *Vibrio parahaemolyticus*. The inhibition observed with these *O. stamineus* extracts was comparable to the inhibition seen with the natural food preservative 5% lactic acid; this is likely due to the high concentration of rosmarinic acid found in the *O. stamineus* extracts. This study showed that the highest concentration of rosmarinic acid had the best antibacterial and free radical scavenging activities. This suggests that rosmarinic acid content is closely associated with antibacterial and free radical scavenging activities of *O. stamineus* extracts.

1. Introduction

Despite the use of various preservation methods in foods, food poisoning is still a concern for both the food industry and consumers. Therefore, the interest in developing new types of effective and nontoxic antimicrobial compounds is increasing due to concerns about the safety of food containing synthetic preservatives (Shan, Cai, Brooks, & Corke, 2007). Consequently, interest in using natural antibacterial compounds, such as extracts of spices and herbs for preserving food, has become increasingly popular, as consumers today ask for natural products, free of synthetic additives (Suhaj, 2006). Plant extracts, especially herbs and spices, are rich in phenolic secondary metabolites, and some have antimicrobial activity (Lin, Labbe, & Shetty, 2005). For example, curcumin isolated from turmeric (*Curcuma longa*) is effective against *Staphylococcus aureus*, *Staphylococcus albus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Jayaprakasha, Rao, & Sakariah, 2005). There were a number of studies carried on the use and effectiveness of herbal extracts for their antimicrobial activity besides turmeric. Among the herbs studied for their antibacterial effects against food-borne pathogens are coriander (*Coriandrum sativum*) (Delaquis, Stanish, Girard, & Mazza, 2002), cinnamon (*Cinnamomum zeylanicum*) (Burt, 2004), clove (*Syzygium aromaticum*) (Daferera, Ziogas, & Polissiou, 2000), tea tree oil (*Melaleuca alternifolia*) (Ponce, Fritz, Valle, & Roura, 2003), oregano (*Origanum vulgare*) as well as sage (*Salvia officinalis*) (Marino, Bersani, & Comi, 2001). However, information on antimicrobial activity by *Orthosiphon stamineus* is not available although antimicrobial activity in plants for medicinal purposes was intensively researched and comprehensively reviewed (Ríos & Recio, 2005).

In this study, extracts of the *O. stamineus* plant were tested for antimicrobial and antioxidant activities. This plant is relatively easy to be cultivated and become one of plantation crops in tropical countries such as Thailand, Indonesia, Phillipines, Brunei and Malaysia, thus its economic potential as food preservative should be explored. *O. stamineus*, locally known as "cat whisker", contains 20 phenolic compounds, including two flavonol glycosides, nine lipophilic flavones and nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (Sumaryono, Proksch, Wray, Witte, & Hartmann, 1991). The main components of *O. stamineus* leaves and extracts are the pharmacologically active polyphenols (the polymethoxylated flavonoids and caffeic acid derivatives) (Olah, Radu, Mogosan, Hanganu, & Gocan, 2003).

In continuation towards the evaluation of *O. stamineus* as a new source of bioactive compounds in food preservation, rosmarinic acid was thought to contribute to this property. This caffeoyl ester is a substance of significant interest that has led to a broad range of applications, from food preservatives to cosmetics (Peterson & Simmonds, 2003). The antioxidant activity of rosmarinic acid was

* Corresponding author. Address: Bioprocess Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia. Tel.: +60 4 6532118; fax: +60 4 6573678.
E-mail address: rosmah@usm.my (A. Rosma).

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doi:10.1016/j.foodchem.2010.03.110

reported to be better than that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E with antioxidant activity similar to vitamin E) (Lu & Foo, 2002) or vitamin E (Lin, Chang, Kuo, & Shiao, 2002). Additionally, natural antioxidants avoid undesired health problems that may arise from the use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which may be possible promoters of carcinogenesis (Ito et al., 1986).

In addition, rosmarinic acid was reported to have a number of biological activities *in vitro*, such as antiviral (Swarup, Ghosh, Ghosh, Saxena, & Basu, 2007), antibacterial, antioxidant, anti-inflammatory, and anti-carcinogenic activities (Huang & Zheng, 2006). Among the different parts of herbal plants studied, the leaves are reported to possess the highest antioxidant property (Chung et al., 1999). The stems and other parts of the herbs are then treated as agricultural wastes, as only the leaves are harvested for its medicinal value. However, in this study, the whole plant is comprised of young shoots and stems in addition to the leaves, all of which were used for the extraction of rosmarinic acid, thus reducing the amount of agricultural waste, increasing farmers' income and maximising the yield of the active compound. Moreover, powder of the whole *O. stamineus* plant is also easily obtained because of their availability as herbal tea. The extracts were later dissolved in distilled water instead of commonly used solvents such as alcohol and dimethyl sulfoxide (DMSO), thus making it more feasible for them to be incorporated into food without compromising the safety of consumers.

The main objectives of this study were (i) to determine the best ratio of solvents and fractions for increasing the yield of rosmarinic acid, and (ii) to determine the antibacterial and antioxidant activities of the various extracts.

2. Materials and methods

2.1. Plant materials

The material used was the powder of the whole *O. stamineus* plant, which included young shoots, stems as well as leaves was obtained from a plantation field in Jeli District, Kelantan, Malaysia. The herbal powder was packed in sealed plastic bags, each holding 50 g of powder, and kept in an opaque container at room temperature until its use.

2.2. Preparation of crude extracts

The powder of the *O. stamineus* plant (50 g) was macerated in 500 ml of methanol at different concentrations (25%, 50%, 75%, 100% methanol) or in 500 ml of 100% distilled water. Maceration was performed in a water bath at 40 °C for 8 h to allow liquid adsorption before transferring to an incubator shaker at 40 °C for another 16 h. The extracts were then filtered through a nylon filter and later centrifuged at 3500 rpm (KUBOTA 5100, Japan) for 10 min. The supernatants were then filtered under vacuum through a filter paper (Whatman GF/C, England). The filtrates were then evaporated using a rotary evaporator (EYELA, Japan) at 40 °C. The concentrated extracts were then transferred into glass Petri dishes and dried in a drying oven at 40 °C.

2.3. Fractionation of methanolic extract

The 50% methanolic extract, which was determined to be the most effective against *Vibrio parahaemolyticus*, was further fractionated using Whatman 3 mm paper chromatographic sheet (23 × 57 cm). The extract was applied onto the chromatography paper using a capillary glass tube repeatedly until a concentrated streak was obtained. The chromatograms were hung inside separation tanks and developed using BAW (n-butanol–acetic acid–water in 4:1:5 ratio, top layer) for 18 h. The chromatograms were left to air-dry in a fume cupboard. The dried chromatograms were later observed under visible and long-wave ultraviolet light. The R_F values and colours for each band were recorded prior to cutting the bands. Separated bands were eluted using 80% methanol, and the fractions were dried in a drying oven at 40 °C.

2.4. Bacterial cultures

The following bacteria were used: *B. subtilis*, *B. cereus*, *S. aureus*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *E. coli*, *V. parahaemolyticus*, *Salmonella* enteritidis and *Salmonella* typhimurium. All the cultures were isolated in our laboratory from fresh food samples, e.g. fish, meat, etc. and maintained on slant nutrient agars (Merck, Germany) (with the addition of 3% sodium chloride for *V. parahaemolyticus*) at 4 °C with monthly subcultures and were kept in the Culture Collection Centre, School of Industrial Technology, Universiti Sains Malaysia.

2.5. Disc diffusion assay

The antimicrobial activity of *O. stamineus* extracts was determined against nine bacterial cultures using a paper disc diffusion assay. The bacterial strains were cultured in a nutrient broth (Merck, Germany) for 24 h and diluted with sterilised peptone water. Then, 100 μl of each culture (10^6 CFU) was spread onto the surface of Mueller–Hinton agar (Oxoid, England) to create a bacterial lawn. Sterile blank filter paper discs of 6 mm in diameter (Oxoid, England) were wetted with 20 μl crude extract of *O. stamineus* (100 mg/ml) and left to dry before being placed on the microbial lawn. The plates were incubated at 37 °C for 24 h. The antibacterial activity was compared with 10 μl of chloramphenicol (1 mg/ml), 20 μl of 5% lactic acid and 20 μl of 10% acetic acid. Antimicrobial activity was determined based on the diameter of the clear zone surrounding the paper discs. Three replicate discs were prepared for each extract in this study.

2.6. Microdilution assays

The minimum inhibition concentration (MIC) values for the extracts and fractions were studied for the microorganism (*V. parahaemolyticus*), which was determined to be sensitive to the extracts in disc diffusion assay. The MIC values of the *O. stamineus* extracts and the 50% methanolic extract fractions against the bacterial strain were based on a micro-well dilution method (Sahin et al., 2003) with some modifications.

The inoculum of *V. parahaemolyticus* was prepared from a 24 h broth culture, and the suspensions were adjusted to 10^6 CFU/ml. All of the extracts of *O. stamineus* were dissolved in distilled water to the highest concentration of 200 and 50 mg/ml for fractions, and eight serial twofold dilutions were made for extracts and fractions.

Then, 100 μl of *O. stamineus* extract with 95 μl of nutrient broth and 5 μl of the inoculum were dispensed into each well of the 96-well flat bottomed, microtiter plate. The plates were then covered with the sterile plate covers and incubated at 37 °C for 24 h. Then, 40 μl of freshly prepared 0.2 mg/ml INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, 95%) (Sigma–Aldrich, England) was added to each well and further incubated for another 30 min before being read. In this case, a change in the colour of the medium to pinkish-red indicated that the bacteria were metabolically active. The MIC was taken at the last well, where no change of colour (no bacterial growth) was observed.

The minimum bactericidal concentrations (MBC) were determined by plating 100 μl of the medium from each well of the

MIC value and above into the nutrient agar (with addition of 3% NaCl). MBC values were taken at the lowest concentration where 99.9% or more of the initial inoculum was killed. All of the experiments were carried out in triplicate.

2.7. Antioxidant activity by DPPH free radical scavenging assay

The measurement of the antioxidant activity of the various methanolic and water extracts of *O. stamineus* using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was adapted from that of Brand-Williams, Cuvelier, and Berset (1995) with some modifications.

Extracts and positive controls (50 μl) at different concentrations as well as dimethylsulfoxide (DMSO) and distilled water as negative controls were pipetted into each well of a 96-well plate with three replicates. Quercetin, BHT and rosmarinic acid, all from Sigma–Aldrich (England), were used as positive controls. All of the samples and the rosmarinic acid were weighed and diluted in distilled water (quercetin and BHT were diluted in DMSO) to an initial concentration of 4 mg/ml, and eight serial twofold dilutions were made for all samples. Each well was then added with 150 μl of a 300 μM ethanolic DPPH solution.

The 96-well plates were then wrapped with aluminium foil and incubated for 30 min at 37 °C. The absorbance values were then determined by optical density with MR Multiskan EX (Thermo Corporation, Finland) at 515 nm. Results were expressed as EC_{50} (substrate concentration to produce a 50% reduction of the DPPH). EC_{50} obtained from the data (three replicates) were calculated using GraphPad Prism 3.0 software (Graphpad, USA).

2.8. Rosmarinic acid concentration determination by High Performance Liquid Chromatography (HPLC) analysis

The HPLC method applied is a modification of that reported by Akuwoah, Zhari, Norhayati, and Sadikun (2005). The five types of dried extracts and six fractions of 10 mg were dissolved in 10 ml of distilled water to yield a concentration of 1 mg/ml and were filtered through 0.45 μm membrane filters prior to HPLC analysis. This analysis was performed using a Shimadzu Corporation (Japan) HPLC system with a ZORBAX SB-C18 column (250 × 4.6 i.d. mm, 5 μm particle size) (Agilent Technologies, USA). The temperature was maintained at 25 °C with 1 ml/min flow rate and 20 μl of injection volume. The marker (rosmarinic acid) (Sigma–Aldrich, England) was separated with methanol–water–tetrahydrofuran (45:50:5 v/v) as the mobile phase, and the peaks were detected at 340 nm.

3. Results and discussion

3.1. Antimicrobial activity of O. stamineus extracts

The methanolic and aqueous extracts were screened at concentration of 2 mg/disc in a disc diffusion test for their antibacterial activities against nine species of bacteria. The diameters of the inhibition zones obtained are presented in Table 1. *V. parahaemolyticus* (a bacterium that causes mild gastroenteritis in humans on the consumption of infected seafood) was found to be more susceptible to 50–100% methanolic extracts of *O. stamineus*. Therefore, further tests of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were done on this culture against all of the extracts with 5% lactic acid and chloramphenicol as positive controls. Lactic acid was chosen over acetic acid as positive control as inhibition by lactic acid was stronger and is commonly used natural food preservative.

Table 2 shows the test results of MIC and MBC. It was found that *V. parahaemolyticus* was most susceptible to 50% and 75% of methanolic extract. The fractionation of 50% methanolic extract yielded six fractions. The further investigation of these fractions revealed that fraction 5 has highest antibacterial activity against *V. parahaemolyticus*. These 50% and 75% methanolic extracts as well as fraction 5 of 50% methanolic extract gave the lowest MIC values of 1.56 mg/ml, which has the same MIC value as 5% lactic acid. Diluted lactic acid (1–2%) has been shown to reduce the surface microflora of raw meat by between 1 and 3.5 log cycles (Adams & Moss, 2003). It was thought that rosmarinic acid, the primary polyphenol in the *O. stamineus* leaf (Akuwoah, Zhari, Norhayati, Sadikun, & Khamsah, 2004) was responsible for the antimicrobial activity; HPLC analysis was thus applied to determine the concentration of rosmarinic acid present in the extracts and fractions.

It is well known that methanol and other alcohols possess bactericidal activity due to their lipophilicity which enhances penetration into target organisms. Because of this fact, we use only pure water to dissolve all of the extracts, thus eliminating the influence of alcohol and DMSO usually used to dissolve extracts before being tested for their bactericidal activities. Therefore, in this experiment, the bactericidal effect could be only attributed to the antimicrobial compound(s) present in the extracts.

3.2. Rosmarinic acid concentration by HPLC analysis

The peak of rosmarinic acid in the extracts was confirmed by the comparison of the retention time to the reference marker (rosmarinic acid). The linearity of the HPLC calibration curve was established for the rosmarinic acid (98% purity); the linear regres-

Table 1
Inhibition zone indicating the antibacterial activity of methanolic extracts of *O. stamineus*.

	Extracts					Chloramphenicol	Lactic acid	Acetic acid
	25%	50%	75%	100%	H_2O			
Gram-positive bacteria								
B. subtilis	–	–	–	+	–	+++	+++	+
B. cereus	+	+	+	+	–	+++	+++	+
S. aureus	+	–	–	–	+	+++	++	+
L. monocytogenes	–	+	–	–	+	+++	+++	+
Gram-negative bacteria								
E. coli	–	–	–	–	–	+++	+++	+++
V. parahaemolyticus	+	++	++	++	+	+++	+++	+
S. enteritidis	–	+	–	+	–	+++	++	–
S. typhimurium	+	+	+	+	+	+++	++	+
K. pneumoniae	–	–	–	–	+	+++	+++	+

Key: (–): no inhibition; (+): weak inhibition (<8 mm); (++): modest inhibition (8 mm $\leqslant x \leqslant$ 0 mm); (+++): strong inhibition ($\geqslant$10 mm); all readings were inclusive of 6 mm disc diameter.

Table 2
Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of different extracts of *O. stamineus*, fractions and controls on *V. parahaemolyticus*.

Crude extracts, fractions and controls	Minimum inhibitory concentrations (mg/ml)	Minimum bactericidal concentrations (mg/ml)
25%	3.13	>100
50%	1.56	>100
75%	1.56	>100
100%	25	>100
H_2O	25	>100
Fraction 1	>25	>25
Fraction 2	6.25	>25
Fraction 3	12.5	>25
Fraction 4	12.5	>25
Fraction 5	1.56	25
Fraction 6	12.5	>25
Chloramphenicol	<0.78	0.78
5% Lactic acid	1.56	0.78

Table 3
R_F value and colour bands developed by paper chromatography chromatograms of fractions of 50% methanolic extract using n-butanol–acetic acid–water solvent system.

Sample	Colour		R_F value (×100)
	Visible	Long UV wavelength	
Fraction 1	Brown	Blue	2
Fraction 2	Brown	Light brown	17
Fraction 3	Light brown	Light yellow	36
Fraction 4	Dark yellow	Dark yellow	56
Fraction 5	Light yellow	Light green	79
Fraction 6	Green	Blue	93

sion coefficient of five points was 0.998. A quantitative HPLC procedure was performed under isocratic conditions to determine the concentration of rosmarinic acid in *O. stamineus* extracts. Among the crude extracts of *O. stamineus*, rosmarinic acid concentration (Table 4) was present in the following order ($P < 0.05$): 50% methanol > 75% methanol > 100% methanol > 25% methanol > water. This supports the findings of Akuwoah et al. (2005) of an increasing amount of rosmarinic acid extracted from *O. stamineus* leaves using different solvent systems (50% methanol > 100% methanol > 70% acetone > water > $CHCl_3$).

Among the fractions in the 50% methanolic *O. stamineus* extract, the rosmarinic concentration was in the following order ($P < 0.05$): fraction 5 > fraction 4 > fraction 6 > fraction 2 > fraction 1 > fraction 3. Harborne (1984) illustrates that the R_F (×100) value of pure rosmarinic acid runs in BAW system is 83. This suggests that rosmarinic acid is present in fraction 5 of the 50% methanolic extract with R_F (×100) value of 79 (Table 3), and HPLC analysis confirmed this result. Other compounds may also be present in this fraction, as the separation is not always complete. It was thought that trace of rosmarinic acid was also present in other fractions. Paper chromatography was used here due to the considerable reproducibility of R_F values obtained in this way and by the convenience of carrying out separations on sheets of filter paper (Harborne, 1984).

3.3. Free radical scavenging activity

This assay is widely used to evaluate the antioxidant capacity of extracts from different plant materials (Alothman, Bhat, & Karim, 2009). Flavonoids and phenolic acids have been known as natural antioxidants in plants, vegetables and fruits (Amzad Hossain, Salehuddin, Kabir, Rahman, & Vasantha Rupasinghe, 2009). The relationship between the antioxidant activity of plant extracts and their phenolic content has been reported previously (Maillard & Berset, 1995). The EC_{50} is a value representing the concentration needed to reach 50% free radical scavenging activity. Thus, a lower value indicates a stronger free radical scavenging activity. The 50% methanolic extract was found to possess the highest free radical scavenging activity among the extracts, suggesting that the presence of rosmarinic acid contributes tremendously to the extracts' free radical scavenging activity (Table 4). After the fractionation of 50% methanolic extract, *O. stamineus* was partially purified, which lead to a higher concentration of rosmarinic acid. Thus, fraction 5 demonstrated the highest free radical scavenging activity. However, it should be noted that the free radical scavenging activity is not limited to rosmarinic acid alone; the presence of other active compounds present in the *O. stamineus* extracts could also contribute to low EC_{50} values. These compounds, even in small quantities, could act synergistically with rosmarinic acid to produce the desired effects. Despite all these, it was reported that there is a strong correlation between the level of rosmarinic acid and the antioxidant activity potential (Tepe, Eminagaoglu, Akpulat, & Aydin, 2007). In addition, Lu and Foo (1999) and Lu, Foo, and Wong (1999) have previously characterised a number of flavonoids and phenolic acids, including rosmarinic acid derivatives, as new potential antioxidant substances.

Table 4
Rosmarinic acid concentration and EC_{50} values of various *O. stamineus* extracts and 50% methanolic fractions.

Extracts	RA concentration (mg/l)	EC_{50} (μg/ml)
25	11.7 ± 1.43^{b}	319
50	33.9 ± 1.25^{c}	231
75	31.2 ± 0.76^{d}	273
100	26.7 ± 0.64^{e}	386
H_2O	1.11 ± 0.02^{a}	307
Fraction 1	0.074 ± 0.00^{a}	–
Fraction 2	1.17 ± 0.01^{a}	205
Fraction 3	–	–
Fraction 4	15.9 ± 0.02^{f}	172
Fraction 5	141 ± 0.81^{g}	147
Fraction 6	5.62 ± 1.27^{h}	–
Quercetin	NA	11.4
BHT	NA	43.1
Rosmarinic acid	NA	41.3

Different superscript letters within a column indicates significantly different values ($P < 0.05$) from triplicate analysis determined by one-way ANOVA and Duncan test. NA – not applicable.

Therefore, we can link the benefits of rosmarinic acid to the antimicrobial and antioxidant properties of *O. stamineus*. This is important as not only the leaves but also the young stems and shoots were used in the extraction of the active compound. This herb and their extracts therefore have the potential to extend the shelf life and improve the safety of different foods.

4. Conclusions

O. stamineus extract demonstrated inhibitory activity against *V. parahaemolyticus*, which was comparable with the natural food preservative lactic acid. The 50% methanolic extract and fraction 5 of the methanolic extract exhibited the highest antibacterial and antioxidant activities due to the high concentration of rosmarinic acid. These findings warrant further studies of *O. stamineus* extract as a preservative in food systems.

Acknowledgements

The authors are grateful to the Ministry of Science, Technology and Innovation (305/PTEKIND/613515) and the Universiti Sains Malaysia Fellowship for their financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2010.03.110.

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