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EVALUATION OF POLYPHENOL COMPOSITION AND BIOLOGICAL ACTIVITIES OF TWO SAMPLES FROM SUMMER AND WINTER SEASONS OF *LIGULARIA FISCHERI* VAR. *SPICIFORMIS* NAKAI

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Phenolic contents, antioxidant and antimicrobial activities were determined by two samples from summer (June) and winter (December) seasons of *Ligularia fischeri* var. *spiciformis* Nakai. A total of 24 phenolic compounds were identified by ultra-performance liquid chromatography (UPLC) analysis. Myricetin (1964.35 and 1829.12 µg/g) was the most dominant flavonol compared to quercetin and kaempferol. Salicylic acid (222.80 and 215.25 µg/g) was the most important phenolic compound compared to pyrogallol, caffeic acid, gentisic acid, o-coumaric acid, gallic acid, protocatechuic acid and ferulic acid in summer (June) and winter (December) seasons. Phenolic contents and antioxidant capacities were estimated for the various solvent extracts (petroleum ether, butanol, ethyl acetate, methanol and water). Ethyl acetate extract exhibited the highest phenolic (332.64 and 299.44 mg/g gallic acid equivalent) and flavonoid contents (5.72 and 5.29 mg/g quercetin equivalent) and also the strongest antioxidant activity in summer and winter seasons. Due to these metabolic variations, the antioxidant and antimicrobial activities were increased with summer seasons compared to winter seasons. Our study shows that the samples collected in June had higher phenolic compounds, stronger antioxidative and antimicrobial activity than the samples of *L. fischeri* leaf extracts collected in December.

Keywords: Biological activities – phenolic compounds – flavonoids – seasonal variations – *Ligularia fischeri*

INTRODUCTION

Ligularia fischeri var. *spiciformis* Nakai is a member of the Compositae family and a well-known wild vegetable in Korea, which is mainly distributed in damp shady regions besides brooks and sloping field. This plant has been eaten as an endemic perennial herbal species in Korea for a long time and southern east part of China [4]. The leaves of this plant have been also used for several medicinal purposes, especially in the treatment of jaundice, scarlet fever, rheumatoid arthritis and hepatic function failure [1]. Previous studies reported that *L. fischeri* leaves contained phenolic constituents of caffeoylquinic acid derivatives [12], norsesquiterpene deriva-

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tives [25], terpenoids, spiciformisins a, b, and monocyclosqualene [13]. *Ligularia fischeri* extracts were used for various biological activities such as a protective effect against hepatotoxic [4], cancer prevention [1], antimutagenic and antigenotoxic activities [7]. Antioxidant activity of these extracts has been reported by several independent methods, indicating that the plant contains high amounts of antioxidant constituents [4, 5, 11]. In recent years, there has been a trend towards the use of natural phytochemicals present in natural resources like vegetables, fruits, oilseeds and herbs which serve as potential antioxidants and functional ingredients [14].

Polyphenolic compounds are a group of low and medium molecular weight secondary metabolites that are widely distributed in plants, which can be divided into two major subgroups: flavonoids and phenolic acids. Flavonoids occur ubiquitously in food plants. *In vitro* and *in vivo* studies have demonstrated that flavonoids exhibit a variety of biological activities including antioxidative effects [2, 14], reduction of cardiovascular disease [9, 18] and reduction of the risk of rheumatoid arthritis [17]. Phenolic acids include mainly hydroxybenzoic acids and hydroxycinnamic acids. The presence of hydroxycinnamic and hydroxybenzoic acids in our diets may also contribute to bolster cellular antioxidant defenses and, to the maintenance a healthy vision [3, 14]. Earlier studies have demonstrated that phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, antibacterial and antiviral effects [22].

Seasonal variation is a changing pattern around a trend line during the specific time period. The important environmental factors are light, temperature and water that change with the season. Korea has distinct four seasons, i.e., winter (Dec – Feb), spring (Mar – May), summer (June – Aug) and autumn (Sep – Nov). Seasonal variation in plant secondary metabolites has been a subject of considerable interest. Researchers over the years are trying to understand the effects of seasonal constraints on variation in the secondary metabolites. In the present investigations were quantified the major individual phenolic compounds by UPLC in *L. fischeri*. In addition, total phenolic, flavonoid contents, antioxidant and antimicrobial activities were determined in the summer (June) and winter (December) seasons. To the best of our knowledge, this is the first report on the phenolic composition and biological activity from the leaves of two different seasons of *L. fischeri*.

MATERIALS AND METHODS

Plant material and preparation of extracts

Ligularia fischeri var. *spiciformis* Nakai plants were collected from National Forest (Pyungchang, Korea) in June 2012 (summer) at 33 °C and December 2012 (winter) at 5 °C. The voucher specimen (KU-12-002 and KU-12-003) is deposited in the

Department of Applied Bioscience. *Ligularia fischeri* leaves were washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water, drained, and dried in a hot air oven at 50 ± 2 °C. The dried leaves were finely powdered. For extract preparation was performed by following our previous described procedures [14, 15, 21]. Briefly, 100 mg of dried leaf powder was extracted twice with 2.0 L of methanol at room temperature for 24 h. The extracts were filtered with Whatman No. 1 filter paper and evaporate to dry the filtrate by using a rotary evaporator. The crude methanol extract was suspended in 0.5 L distilled water and extracted successively with petroleum ether, ethyl acetate and butanol. After removal of solvents *in vacuo*, extracts of ethyl acetate, methanol, butanol and water were obtained, respectively.

Determination of total phenolic (TPC) and flavonoid contents (TFC)

Total phenolic content and flavonoid content of the samples were analyzed by the method of Folin-Ciocalteu reagent and aluminium chloride colorimetric method following the procedure by Thiruvengadam et al. [19–21]. The concentration of the TPC and TFC was determined as mg of gallic acid and quercetin equivalent, respectively.

Extraction of phenolic compounds and analysis by ultra-performance liquid chromatography (UPLC)

One gram of dried plant powder was extracted with the procedure as described by Thiruvengadam et al. [19–21]. The filtrate was used for analysis using a Thermo Accela UPLC (Thermo, New York, USA) system. Separation was achieved using a HALO C18 (2.7 µm, 2.1 × 100 mm) column and the absorbance was measured at 280 nm. The mobile phases were 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The gradient procedure described earlier by Thiruvengadam et al. [19, 20]. Solutions of pure gallic acid, homogentisic acid, protocatechuic acid, gentisic acid, β-resorcylic acid, veratric acid, vanillic acid, caffeic acid, syringic acid, vanillin, ρ-coumaric acid, salicylic acid, ferulic acid, *m*-coumaric acid, pyrogallol, rutin, *o*-coumaric acid, myricetin, resveratrol, quercetin, naringenin, kaempferol, biochanin A and formononetin were used as standards. The individual standards (25, 50, 100, and 150 µg/mL) purchased from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in methanol and analyzed before the samples. Phenolic compounds of plant extracts were identified based on the retention time and UV spectra of authentic standards while the quantitative data were calculated based on the calibration curves of the individual standards. Results were expressed as µg/g of each compound from the total phenolic compounds.

Antioxidant activity

For the antioxidant studies, the 1,1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity, reductive potential and phosphomolybdenum method were analyzed by following the procedure of Thiruvengadam et al. [19–21].

Antimicrobial activity

The pathogenic microorganisms *Staphylococcus aureus* (KACC 13257), *Pseudomonas aeruginosa* (KACC 10259), and *Escherichia coli* (KACC 13821) and the pathogenic fungi *Fusarium oxysporum* and *Aspergillus niger* were used to test for antimicrobial activity. The pure bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, South Korea. The fungal strains were received from Prof. S. C. Chun, Department of Molecular Biotechnology, Konkuk University, Seoul, South Korea. The methanolic extracts of *L. fischeri* leaf extracts (100 mg/mL) were tested for antimicrobial activity. Antimicrobial tests were carried out by the NCCLS disc diffusion method [20]. Briefly, 100 mL of suspension containing approximately 10^8 colony-forming units (CFU)/mL of bacterial cells and 10^4 cells/mL of fungi were spread onto nutrient agar (NA) and potato dextrose agar (PDA) medium, respectively. For the antibacterial and antifungal tests, paper discs (10 mm in diameter) were individually impregnated with 50 µL of 100 mg/mL leaf extract and placed on the inoculated agar. For the positive control, paper discs were impregnated with 50 µL of chloramphenicol or thymol. Plates were incubated at 37 °C for 18–24 h for bacterial strains and for 24–48 h for fungal strains. Antimicrobial activity was assessed by measuring the diameter of growth inhibition zone (IZ) in millimeters.

Experimental design and data analysis

All the experiments were performed in triplicates and each experiment was repeated twice. The data were expressed as means ± standard deviation (SD). One way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \leq 0.05$) differences. All the statistical analyses were done by using SPSS Ver. 20 (SPSS Inc., Chicago, IL, USA) statistical software package.

RESULTS

Total phenolic and flavonoid contents

Ligularia fischeri leaf extracts were analyzed for total phenolic and flavonoid contents (Fig. 1A, B). Among the five extracts, ethyl acetate extract had the highest amount of phenolic compounds in June (332.64 mg/g) and December (299.44 mg/g)

and the lowest were observed in water extracts of June (19.20 mg/g) and December (15.12 mg/g) (Fig. 1A). The total flavonoid content was observed ranging from 0.29 to 5.72 mg QE/g and 0.21 to 5.29 mg QE/g of June and December month, respectively (Fig. 1B).

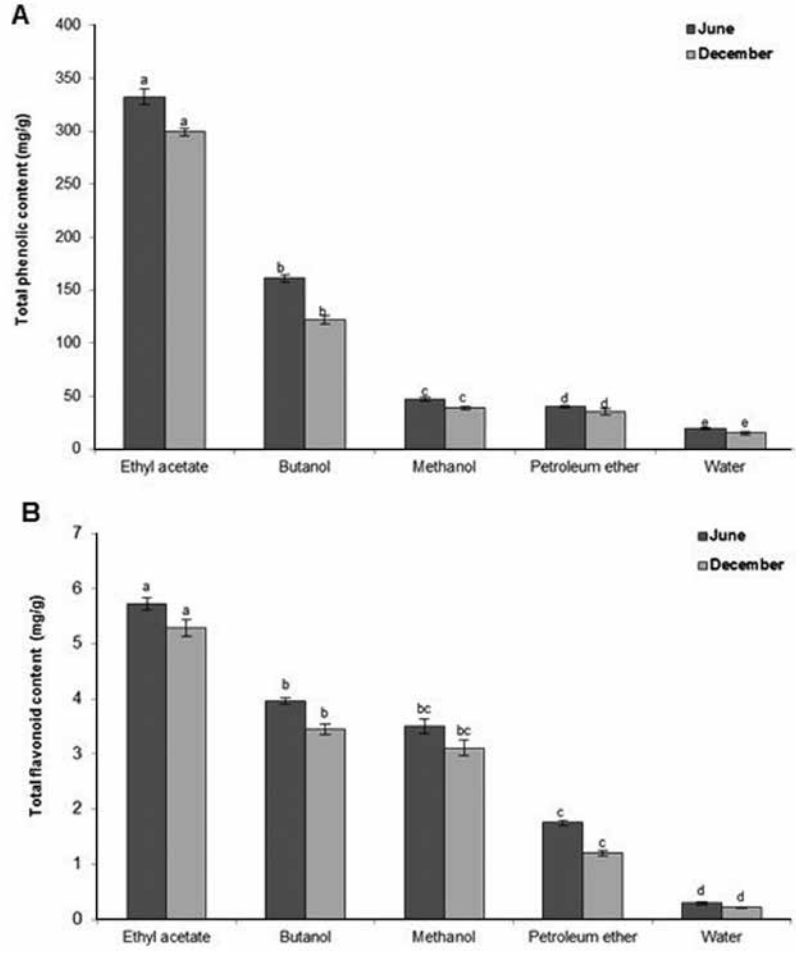

Fig. 1. Total phenolic (TPC expressed as gallic acid equivalents/g) and flavonoid content (TFC expressed as mg quercetin/g) of *L. fischeri* leaf extracts from summer and winter seasons. Data represents mean values ± SD of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

Table 1
Major phenolic compounds identified in the *Ligularia fischeri* leaf extract by UPLC analysis

No	Compounds	Concentration (µg/g)	
	Flavonols	June	December
1	Myricetin	1964.35±2.33^{a}	1829.12±3.52^{a}
2	Quercetin	109.20±0.28^{d}	100.14±0.25^{d}
3	Kaempferol	33.25±0.07^{k}	31.40±0.05^{k}
4	Rutin	57.00±0.42^{hi}	55.22±0.12^{hi}
5	Naringenin	8.95±0.07^{o}	8.10±0.02^{o}
6	Formononetin	11.30±0.42^{no}	11.12±0.11^{no}
7	Biochanin A	4.30±0.14^{p}	4.00±0.00^{p}
	Total	2188.35	2039.1
	Hydroxycinnamic acid		
8	Caffeic acid	99.35±0.21^{e}	97.12±0.15^{e}
9	*p*-Coumaric acid	8.15±0.07^{o}	8.00±0.05^{o}
10	Ferulic acid	31.35±1.06^{k}	30.35±1.00^{k}
11	*m*-Coumaric acid	0.45±0.70^{q}	0.40±0.75^{q}
12	*o*-Coumaric acid	68.95±2.33^{fg}	68.94±2.50^{fg}
	Total	208.25	204.81
	Hydroxybenzoic acid		
13	Gallic acid	58.35±1.34^{h}	55.15±2.25^{h}
14	Protocatechuic acid	36.85±0.35^{j}	35.00±0.30^{j}
15	β-Resorcylic acid	24.35±0.07^{lm}	24.30±0.05^{lm}
16	Vanillic acid	26.60±0.00^{l}	25.20±0.01^{l}
17	Syringic acid	11.45±0.07^{n}	11.25±0.05^{n}
18	Gentisic acid	69.50±0.70^{f}	65.25±1.15^{f}
19	Salicylic acid	222.80±22.20^{b}	215.25±18.00^{b}
	Total	449.90	431.40
	Other phenolic compounds		
20	Pyrogallol	122.00±0.00^{c}	120.15±1.10^{c}
21	Resveratrol	23.05±0.07^{lm}	20.00±0.05^{lm}
22	Veratric acid	22.70±0.42^{lm}	20.10±0.41^{lm}
23	Vanillin	13.15±0.07^{n}	11.10±0.05^{n}
24	Homogentisic acid	8.90±1.27^{o}	8.72±1.10^{o}
	Total	189.8	179.97

Data represents mean values ± SD of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

UPLC separation and determination of phenolic compounds in L. fischeri *leaf extract*

The qualitative and quantitative analysis of phenolic compounds from winter and summer leaf extracts were studied using UPLC (Table 1, Fig. 2A, B). The phenolic compounds in the *L. fischeri* extracts were identified by comparisons of the retention time and UV spectra of authentic standards and the quantitative data were calculated from calibration curves. *L. fischeri* leaf extracts of summer and winter contained flavonols, hydroxybenzoic acid, hydroxycinnamic acid and other phenolic compounds. The seven flavonols identified in the analysis were myricetin, quercetin, kaempferol,

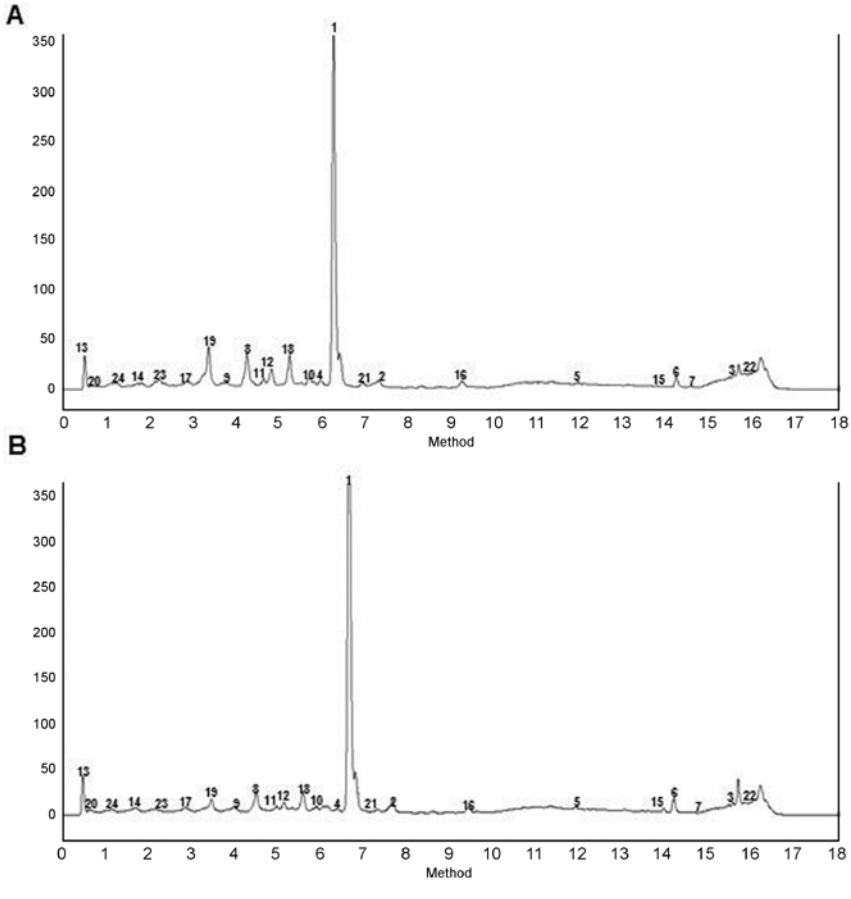

Fig. 2. Chromatograms of phenolic compounds in *Ligularia fischeri*. A) Summer season. B) Winter season. Peaks: (1) Myricetin, (2) Quercetin, (3) Kaempferol, (4) Rutin, (5) Naringenin, (6) Formononetin, (7) Biochanin A, (8) Caffeic acid, (9) *p*-Coumaric acid, (10) Ferulic acid, (11) *m*-Coumaric acid, (12) *o*-Coumaric acid, (13) Gallic acid, (14) Protocatechuic acid, (15) β-Resorcylic acid, (16) Vanillic acid, (17) Syringic acid, (18) Gentisic acid, (19) Salicylic acid, (20) Pyrogallol, (21) Resveratrol, (22) Veratric acid, (23) Vanillin, (24) Homogentisic acid

rutin, naringenin, formononetin and Biochanin A. Myricetin was the most dominant flavonol, it accounted for the largest proportion of the total flavonol content, whereas quercetin and kaempferol were detected in lower amounts in *L. fischeri* leaf extracts of summer and winter seasons (Fig. 2A, B). In the hydroxycinnamic acid group, caffeic acid was the most dominant hydroxycinnamic acid, followed by *o*-coumaric acid, ferulic acid and *p*-coumaric acid present in *L. fischeri* leaf extracts of summer and winter seasons. Salicylic acid was the dominant compound in the hydroxybenzoic acid group followed by gentisic acid, gallic acid, protocatechuic acid, vanillic acid and β-resorcylic acid. *L. fischeri* leaf extracts obtained in summer contained higher amounts of flavonols (2188.35 μg/g), hydroxycinnamic acid (208.25 μg/g), and hydroxybenzoic acid (449.90 μg/g) when compared to the leaf extracts of the winter season, which contained lower amounts of flavonols (2039.1 μg/g), hydroxycinnamic acid (204.81 μg/g), and hydroxybenzoic acid (431.40 μg/g).

DPPH– radical scavenging activity

The free radical scavenging activity of the extracts was tested through DPPH method and the results were compared with BHT (Fig. 3A). The *L. fischeri* leaf extracts were able to reduce the stable radical DPPH– to the yellow colored diphenylpicrylhydrazine. The IC_{50} values of the extracts were ethyl acetate (June – 13.86 μg/mL and December – 14.35 μg/mL), butanol (June – 21.36 μg/mL and December – 22.62 μg/mL), methanol (June – 24.41 μg/mL and December – 25.44 μg/mL), petroleum ether (June – 88.58 μg/mL and December – 102.45 μg/mL) and water (June – 100.48 μg/mL and December – 113.63 μg/mL), respectively. In the present study, the extracts exhibited a concentration dependent antiradical activity by inhibiting DPPH– radical (Fig. 3A). Of the different extracts, ethyl acetate extract exhibited the highest antioxidant activity of June (96.46%) and December (93.85%) at 100 μg/mL concentration, followed by butanol (June, 94.19% and December, 91.29%), methanol (June, 68.71% and December, 62.75%), petroleum ether (June, 45.27% and December, 42.77%) and water (June, 43.27% and December, 40.12%), respectively at the same concentration and it indicates that compounds with strong radical-scavenging capacity are of medium polarity (Fig. 3A). Butylated hydroxytoluene (BHT) showed a higher degree of free radical scavenging activity than that of the extracts at low concentration points. The DPPH activity of BHT was 92.04% at 50 μg/mL concentration.

Assay of reductive potential

In the present study, the extracts exhibited an effective, reducing capacity at all concentration points. Reducing the capacity of the extracts increased with an increase in the concentration (Fig. 3B). The reducing power of the extracts followed the order of ethyl acetate > butanol > methanol > petroleum ether > water extract.

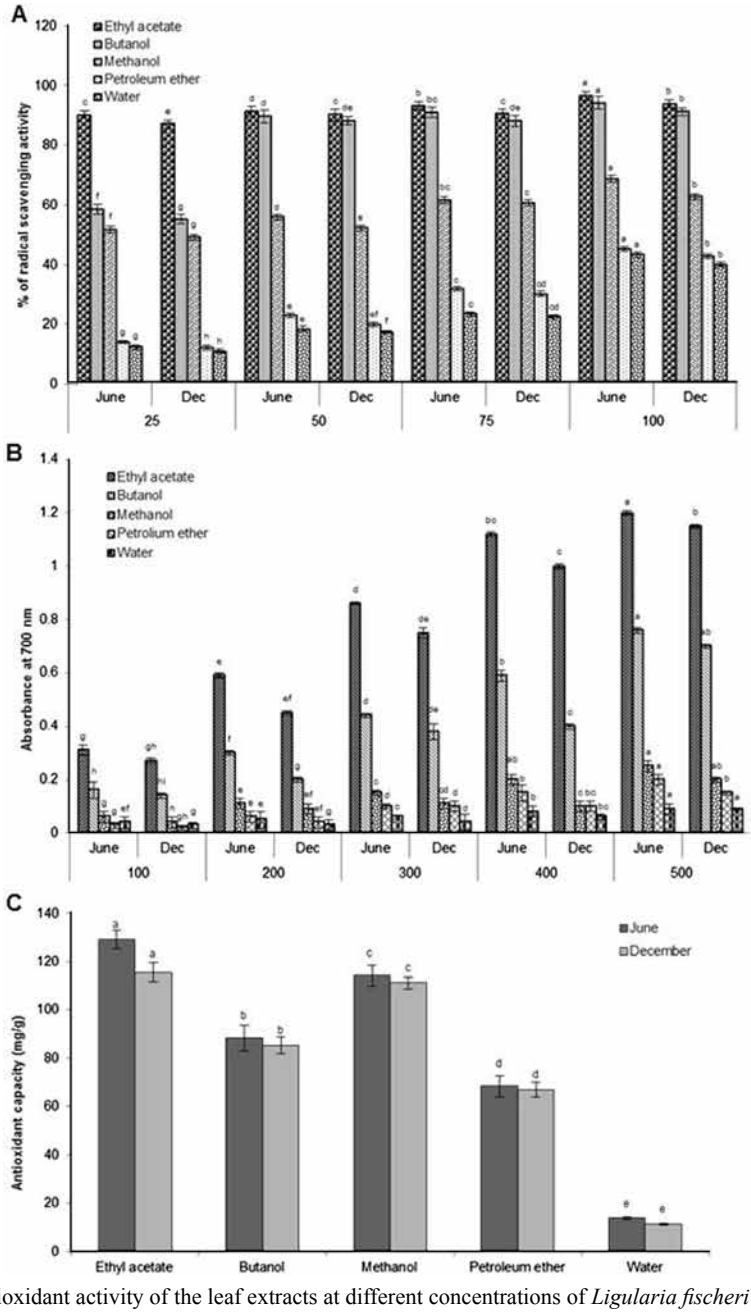

Fig. 3. Antioxidant activity of the leaf extracts at different concentrations of *Ligularia fischeri* from summer and winter seasons. A) DPPH method. B) Reducing power. C) Phosphomolybdenum method (expressed as mg α-tocopherol/g). Data represents mean values ± SD of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of the *L. fischeri* leaf extracts were measured spectrophotometrically by a phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte, and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts was found to decrease in the following order: ethyl acetate > methanol > butanol > petroleum ether > water extract (Fig. 3C).

Antimicrobial activity

Ligularia fischeri leaf extracts exhibited varying antimicrobial activity, as shown by the growth inhibition zones (Table 2). The results from the disc diffusion method indicated that leaf extracts had antibacterial effects against Gram-positive and Gram-negative bacteria. Extracts exhibited good antifungal activity (Table 2). These results were compared with chloramphenicol for bacteria and thymol for fungal activity. The results suggested that leaf extracts of *L. fischeri* can be used for the treatment of bacterial and fungal diseases.

Table 2
Antimicrobial activity of *Ligularia fischeri* leaf extracts against bacteria and fungus

Microorganisms	Zone of inhibition mm at 100 mg/disc			
	Positive control (antibiotics)		Leaf extracts	
	Chloramphenicol	Thymol	June	December
S. aureus	27.1 ± 0.5^{c}		15.5 ± 0.2^{d}	14.1 ± 0.2^{d}
P. aeruginosa	28.8 ± 0.7^{b}		18.0 ± 0.5^{ab}	16.9 ± 0.5^{b}
E. coli	29.0 ± 0.9^{a}		19.5 ± 0.6^{a}	18.4 ± 0.5^{a}
A. niger		26.1 ± 0.6^{a}	18.7 ± 0.4^{b}	17.0 ± 1.0^{b}
F. oxysporum		24.2 ± 0.5^{b}	16.5 ± 0.5^{c}	15.4 ± 0.5^{c}

Data represents mean values ± SD of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

DISCUSSION

It has been previously reported that the ethyl acetate extract had the highest content of phenolics and flavonoids in *Coriandrum sativum* [15] and *Rumex japonicus* [6]. Similarly, our results reported that the ethyl acetate extract was the highest content of phenolics and flavonoids in *Ligularia fischeri.* The total phenolic content was higher in warm months than cooler months of Australian grown tea *(Camellia sinensis)* [24].

Consistently with these previous studies, our results showed that *Ligularia fischeri* summer (June) season of leaf samples had a higher phenolic and flavonoid content than winter (December) seasons.

Myricetin and salicylic acid were the most dominant flavonols and hydroxybenzoic acid compared to other phenolic acids. Correspondingly, myricetin and salicylic acid were presented in higher amount in *Artemisia absinthium* [14] and *Aster scaber* [20]. In our present study exhibited summer (June) season samples had a higher amount of phenolic compounds compared to winter (December) season leaf samples. Similarly, the seasonal factors can affect the phenolic composition of plant tissues during the vegetative cycle [8]. Previously, four major caffeoylquinic acid derivatives were higher in sunlight grown areas, whereas the content of these compounds decreased steadily in shade leaves of *Ligularia fischeri* [12].

In the present study, *Ligularia fischeri* summer (June) season collected leaf samples showed the higher DPPH activity, compared to the winter (December) samples. Similarly, during the winter season DPPH activity was less responsive compared to that found in the summer season in *Nothapodytes nimmoniana* [16]. The trend is similar to that obtained from the phenolic and flavonoid contents of the samples, clearly indicating that samples with higher phenolic content showed higher antioxidant activity. Reducing power indicates that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [10]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing capacity of extracts suggest that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products, and thereby terminate radical chain reactions. The presence of phenolic compounds might contribute to antimicrobial activity in *Ligularia fischeri*. Previous reports demonstrated the antimicrobial activity of phenolics and flavonoids in bitter melon [20] and parsley and cilantro [23]. *Ligularia fischeri* summer (June) samples exhibited good antimicrobial activities compared to winter (December) samples.

CONCLUSION

Our results showed that *Ligularia fischeri* leaf extracts were rich in phenolic constituents and demonstrated good antioxidant and antimicrobial activity. Therefore, qualitative and quantitative analysis of major individual phenolics in *Ligularia fischeri* could be helpful for explaining the relationships between antioxidant capacity and total phenolic contents. The samples of *Ligularia fischeri* collected in the summer season (June) had higher amount of phenolic and flavonoid contents as well as stronger biological activities than the winter season's ones (December). The results obtained can be crucial in understanding the role played by this green leafy vegetable in the prevention and treatment of several diseases.

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