Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(5): 175-179

Received: 03-07-2017
Accepted: 03-08-2017

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Antioxidant, Ferric Iron Chelation and Antimicrobial Activities of Extracts of *Pseudocydonia sinensis* (Chinese Quince) Fruit

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Abstract
There are accumulated lines of evidence on the importance of plants as natural sources of medicinal agents since most synthetic antioxidants and antibiotics have been implicated in cytotoxicity and microbial resistance. However, biological activities of phytochemicals are partly dependent on extraction solvents. In this study, the antioxidant, ferric ion chelation and antimicrobial activities of the fruit extracts of *Pseudocydonia sinensis* (Chinese quince) were examined. The methanol, aqueous and ethanol extract recorded highest antioxidant, ferric ion chelation and antimicrobial activities, respectively. The ethanol and methanol extracts showed significant antimicrobial activities against all microbes employed in this study. The fruit extract may therefore be a promising source of antioxidant and antimicrobial agents of natural origin.

Keywords: *Pseudocydonia sinensis*, Ferric ion chelation Antioxidant; Antimicrobial activity

1. Introduction
Pseudocydonia sinensis, the Chinese quince is a deciduous or semi-evergreen tree in the family Rosaceae and native to Eastern Asia in China. In China, the species is mostly called 'mugua' while in Korea, it is called 'mogwa'. Chinese quince is consumed as processed food products. The plant extracts have given positive effects on human health due to its diverse groups of bioactive components. The polyphenols content of Chinese quince has various properties such as anti-oxidative, anti-inflammatory and anti-pruritic activities [1, 2]. Plant secondary metabolites may have properties that are beneficial to human health. In addition, these can be used against cancer, bacterial infections, inflammation, and many other diseases [3, 4]. Consuming antioxidants as free radical scavengers may be necessary so as to complement the inherent defense system [5]. Some synthetic antioxidants such as butylated hydroxyanisole (bha) and butylated hydroxytoluene (bht) exhibit potent free radical scavenging potentials but have been implicated in cytotoxicity. This has incited a large body of research on natural sources of antioxidants.
Chinese quince fruits have been used in traditional Chinese medicine to treat rheumatoid arthritis [6]. The biological activities of phytochemicals are known to vary greatly with extraction solvents [7]. However, recent studies have examined antimicrobial activities of ethanolic extracts of Chinese quince pomace [8]. Nevertheless, no reports on the extensive antioxidant and antimicrobial studies, including the use of different solvent extracts of the fruit. Hence, the purpose of this study was to examine the antioxidant, ferric iron chelatory and antimicrobial effects of methanol, ethanol and aqueous extracts of the fruit of *Pseudocydonia sinensis*.

2. Materials and Methods
2.1. Plant material and preparation of extracts
Fresh fruits of the plant were collected around Dongguk University campus, Gyeongju, South korea. Fruits were washed, air dried and ground to fine powder. To 100 g powder, 300 ml of methanol, ethanol or water was added, swell to ensure a uniform mixture and extracted for 24h at room temperature. The extracts were concentrated under reduced pressure using a rotary vacuum evaporator and freeze dried. A reddish brown, yellow and light-yellow residue was obtained for methanol, aqueous and ethanol extracts respectively.

2.2. 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The assay was performed using the method described by Re *et al.* [9] with a slight modification. Stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate were prepared in distilled water. Equal volumes of the stock solutions were mixed in order to generate the ABTS free radical ($ABTS^{\bullet+}$). The resulting solution was incubated in the dark at room temperature for 12 hours until the reaction was completed, by the observation of a constant absorbance. The $ABTS^{\bullet+}$ solution (1 ml) was further diluted in 50 ml of methanol and the absorbance calibrated to 0.70± 0.01 at 734 nm. 30 µl of different concentrations of samples and standard was added to 1ml of $ABTS^{\bullet+}$ solution, mixed and incubated at 30 ºC for 10 minutes. The absorbance was read at 734nm. The test was done three times. BHT was used as a positive control. Percentage inhibition of $ABTS^{\bullet+}$ was calculated as; (%) inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$

Where A_{sample} is the absorbance of $ABTS^{\bullet+}$ + sample extract or standard and $A_{control\ is}$ the absorbance of $ABTS^{\bullet+}$ + methanol (same volume as sample).

2.3. Nitric oxide (NO) radical scavenging assay

The NO scavenging assay was carried out using the method described by Jeon (2010). A 5 µL aliquot of different concentrations of each extract was added to 495 µL of sodium nitroprusside solution (5 mM) and vortexed. After incubation at room temperature for 150 min, 100 µL aliquots were removed from the reaction mixture and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride, 2.5% H3PO4). The absorbance at 540 nm was determined after 5 minutes. Ascorbic acid was used as a positive control.

2.4 Ferrous iron chelation assay

The ferrous iron chelating activity of the plant extracts was carried out as described by Minotti and Aust [10]. The source of Fe^{2+} in this assay is $FeSO_4$. In aqueous solution, Fe^{2+} binds with 1, 10-Phenanthroline to produce a red chromophore which can be quantified spectrophotometrically. Plant extracts and standards compete with 1, 10-Phenanthroline for Fe^{2+} in aqueous solution. The assay was carried out as follows. Plant extracts and standard (0.2 ml) were added to 0.336 ml of Tris HCl (0.1 M, pH7.4), followed by the addition of 0.40 ml of 0.9% NaCl w/v and 100 µl, 500 µM. The mixture was incubated for 5 minutes after which 0.25 ml of 0.25% aqueous 1, 10-Phenanthroline was added. The absorbance was read at 510 nm against a control consisting of the same reaction mixture, except plant solutions. EDTA was used as a positive control.

2.5 Antimicrobial assay

The antimicrobial efficacy of the extracts was examined based on the paper disc diffusion method against pathogenic bacteria; *E. coli*, *S. aureus*, and *B. subtilis*, and pathogenic fungi; *C. albicans* and *S. cerevisiae*. Organisms were grown overnight at 37ºC, after which 500µl aliquots of each organism (1×10^6 cfu/ml) was spread on Mueller Hinton agar using cotton swabs. After 10 minutes of drying, paper discs loaded with 70 µl (3mg/ml) of extracts and reference drug dissolved in DMSO were placed on the surface of cultured plates and incubated at 37ºC for 24 hours. The diameters of inhibition zones were measured. DMSO was used as a negative control.

2.6. Broth dilution assay

Percentage inhibition of microbial growth was evaluated against the test microorganisms. Microbes were grown overnight at 37º C in nutrient broth and optical density adjusted to 5×10^5 cfu/ml. To 10 ml of microbial suspension in test tubes, 200 µl of different concentrations of extracts were added to make final concentrations of (2, 1, 0.5, 0.25, and 0.125) mg in 10ml of suspension. This was incubated at 37º C for 24 hours after which optical densities were measured at 650nm and percentage growth inhibition evaluated.

2.7. Statistical Analysis

All measurements are expressed as mean ± standard error mean (SEM) of independent experiments. IC_{50} values were obtained by interpolations from standard curves. All tests were carried out in triplicate.

3. Results

Table 1 shows the yield of extract from 100 g of plant sample in different solvents. Among the three solvents used in the extraction procedure, ethanol recorded the highest yield, about twice and thrice that of methanol and water respectively.

Table 1: Yield of extracts in different solvents.

Extraction Solvent	Mass of empty bottle/(g)	Mass of bottle + extract/(g)	Mass of extract/(g)	Percentage yield/ (%)
Methanol	161.72	171.38	9.66	9.66
Ethanol	158.40	173.52	15.12	15.12
Water	162.85	168.23	5.38	5.38

The extracts were observed to quench $ABTS^{\bullet+}$ radical in a dose-dependent manner. The methanol extract showed the highest activity, eliciting as much as 88.33% inhibition at 1 mg/ml, comparable to the BHT standard as shown in Figure 1.

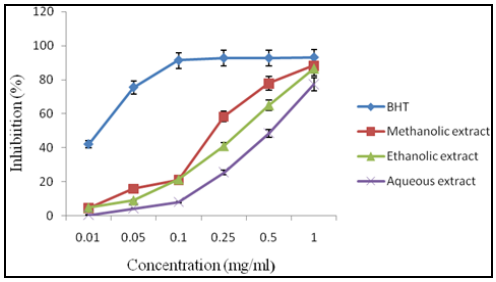

Fig 1: ABTS free radical scavenging activity of different extracts of *Pseudocydonia sinensis* and BHT standard each value is the average of three independent experiments with error bars indicating SEMs.

This was followed by the ethanol and aqueous fractions; the latter recording the least activity (Figure 1). IC_{50} values were 0.02±0.00, 0.22±0.01, 0.36±0.04, and 0.53±0.03 mg/ml respectively for BHT, methanol, ethanol and aqueous fractions (Table 2). The order of NO scavenging can be written as methanol > ethanol> aqueous extract, with IC_{50}s at 0.33±0.16 and 0.87±0.09 mg/ml respectively for methanol and ethanol extracts (Table 2). The aqueous extract recorded the least activity, being unable to inhibit 50% of NO in solution at the maximum concentration used. The methanolic extract, however, demonstrated a significant activity comparable to the ascorbic acid standard.

Table 2: IC_{50} values for extracts of the fruit of *Pseudocydonia sinensis.*

Extract	IC_{50} Values (mg/ml)	
	ABTS Radical Scavenging	Nitric Oxide Scavenging
Methanol	0.22±0.01	0.33±0.16
Ethanol	0.36±0.04	0.87±0.09
Aqueous	0.53±0.03	-
Standard	0.02±0.00	0.02±0.00

Each value is the average of three independent experiments ±SEMs

The extracts were observed to scavenge nitric oxide generated *in vitro* in a dose dependent manner (Figure 2).

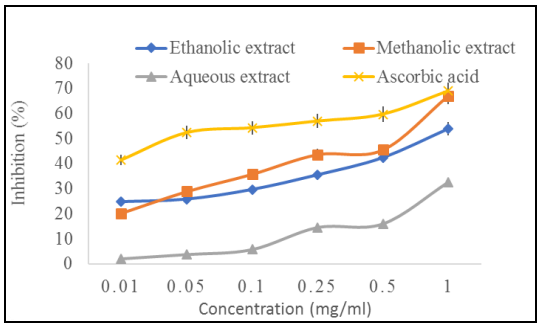

Fig 2: Nitric oxide scavenging activity of extracts of *Pseudocydonia sinensis* fruit and bht standard. Each value is the average of three independent experiments with error bars indicating SEMs.

The chelating activities of the extracts were dose independent. The aqueous extract had the highest chelating activity; at 1 mg/ml it inhibited 60.50±0.06 (%) 1, 10-Phenanthroline-Fe^{2+}complex (Table 3). The order of chelating activity of the extracts was aqueous > ethanol > methanol extract.

Table 3: Ferrous iron chelating activity of fruit extracts of *Pseudocydonia sinensis.*

Concentration (mg/mL)	Percentage inhibition of 1,10-Phenanthroline-Fe^{2+}complex			
	EDTA	Methanol	Ethanol	water
1.00	89.40±0.06	34.45±4.86	40.95±0.10	60.50±0.06
0.50	85.61±0.00	28.92±2.49	37.17±0.19	59.73±0.35
0.25	78.93±0.32	29.86±1.61	31.19±0.28	54.93±0.34
0.10	72.11±1.15	27.85±0.13	32.53±0.24	51.61±0.10
0.05	61.82±0.07	26.02±0.91	29.34±1.44	49.29±0.04
0.01	52.63±0.33	26.27±0.71	34.61±0.43	50.67±1.68

Results are average of three independent experiments ± SEMs.

The ethanol and methanol extracts showed some significant antimicrobial activities against all microbes employed in this study. The aqueous extract recorded the least activity and revealed no toxicity against *C. albicans* and *S. aureus* in the disc diffusion assay (Table 4).

Table 4: Antimicrobial effects of fruit extracts of *Pseudocydonia sinensis.*

Strain	Methanolic extract	Ethanolic extract	Aqueous extract	Penicillin
B. subtilis	11.0± 0.5	13.0±0.3	10.0±0.0	18.0±0.3
E. coli (KCTC 2441)	9.0±0.0	12.0±1.2	9.0±0.5	23.0±0.0
S. aureus (KCTC 1916)	8.0±0.0	12.0±0.0	ND	14.0±0.6
C. albicans	10.0±0.3	14.0±0.6	ND	17.0±0.0
S. cerevisiae	13.0±0.0	13.0±0.0	10.0±0.6	15.0±0.0

Results are the means of diameters values ± SEM. ND: No detected inhibition.

In the broth dilution assay, microbes in inoculums were significantly reduced in a dose-dependent manner by the extracts (Figures 3-5). The trend of activity was however in the same order as in the disc diffusion method.

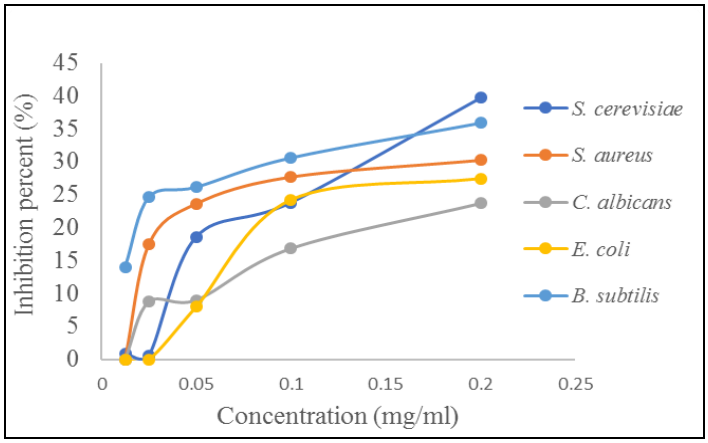

Fig 3: Dose dependent antimicrobial activity of aqueous extracts of *Pseudocydonia sinensis*

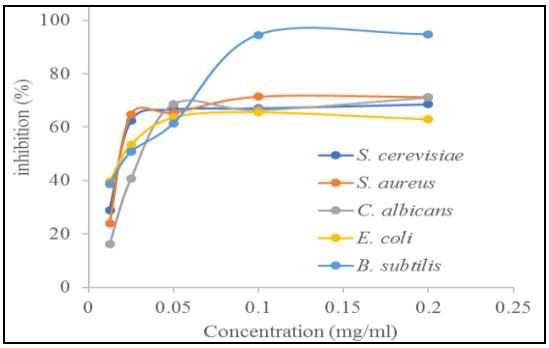

Fig 4: Dose dependent antimicrobial activity of ethanol extracts of *Pseudocydonia sinensis*

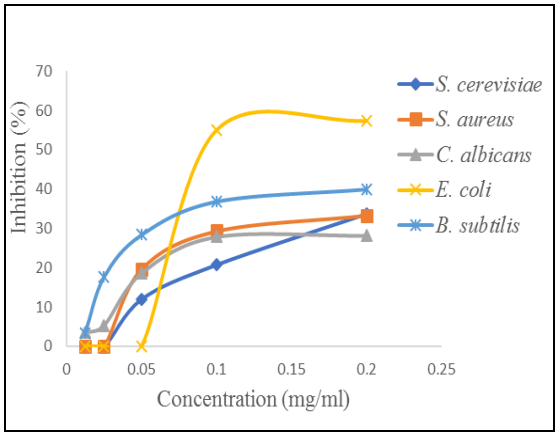

Fig 5: Dose dependent antimicrobial activity of methanol extracts of *Pseudocydonia sinensis*

4. Discussion

The antioxidant activities of the three extracts were investigated using $ABTS^{\bullet+}$ decolonization and nitric oxide scavenging assays. Proton radical scavenging is an important attribute of antioxidants [11]. $ABTS^{\bullet+}$ is a protonated free radical with a maximum absorbance at 734 nm, which decreases on scavenging by antioxidants. The extracts were observed to quench the radicals in a concentration dependent manner and the results are indicative of the fact that the fruit extracts are promising free radical scavengers.

Nitric oxide is a diffusible free radical which act as an effector molecule in a large number of biological processes [12]. Conversely, mass production has been linked to cytotoxicity. Since the deleterious effects of free radicals are well documented as important contributors to several diseases, the observed nitric oxide scavenging activity of the fruit extracts could be exploited as a therapeutic target in tissue damage and cellular injury.

Iron chelating activity is one of the major characteristics of antioxidants [13]. Elevated levels of iron have been implicated in changing hydrogen peroxide, which is less reactive to hydroxyl radical, a highly toxic free radical and thereby inducing oxidative stress. In view of the aforementioned effects of iron overload, the manufacture of drugs to combat this challenge would be a promising strategy to ameliorate the consequences of oxidative stress and as well reduce the concentration of transition metals as catalysts of lipid peroxidation. From the results, the extracts were not as good as EDTA standard, but demonstrated potent chelating abilities due to the inhibition of significant amounts of 1,10-Phenanthroline-Fe^{2+}complex.

Antimicrobial activity of plants is well known to vary by a lot of factors, which include the microbes tested and the solvent used for extraction [14]. Significant susceptibilities were observed for all experimental microbes towards the ethanol and methanol extracts. The aqueous extract elicited mild activity and had no detectable effect on *S. aureus* and *C. albicans* in the disc diffusion test. The extracts were observed to inhibit the growth of microbes in a manner dependent on concentration in nutrient broth. The rate of microbial resistance to conventional antibiotics is quite alarming; hence, studies connected with the activities of plants against infectious agents should be embraced with alacrity. The fruit extracts have demonstrated impressive antimicrobial activities which may be potentiated on further purification.

Solvent extraction is the method most often employed in isolating bioactive compounds from plants. It can therefore be envisaged that the choice of solvent is expedient so as to aggrandize the total yield and activity of isolated compound. Ethanol is well known for its potential to accumulate high levels of plant phenolics; the major antioxidant, phytochemicals, and hence, it is expected that the ethanol fraction displays highest antioxidant activity. Surprisingly, the methanol fraction had highest free radical scavenging activity. According to Re *et al.* [9], the antioxidant potential of plant phenolics depends on the occurrence of unsaturation in the ring structure, the presence of other group of compounds attached to the phenolic hydroxyl and the number and relative positions of hydroxyl groups attached to the phenol ring. It may therefore be speculated that at least one of the aforementioned features may have accounted for the high antioxidant activity observed in the methanol fraction.

4.1 Conclusion

The results of the present study reveal that fruit extracts of *Pseudocydonia sinensis* possess significant antioxidant, iron chelatory and antimicrobial activities, which varies with the extraction solvent. Methanolic, aqueous and ethanolic extracts had highest antioxidant, iron chelatory and antimicrobial activities respectively. The fruit extracts are therefore presented as promising candidates for the production of novel antioxidative and anti-infectious agents. However, structural elucidation and *in vivo* studies on the absorption and metabolism of bioactive in the various extracts are some topics for further research.

5. References

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