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Full Length Research Paper

Phytochemical contents and biological activities of *Rosa canina* fruit from Iran

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This study was done to evaluate the amount of phytochemicals (phenols and flavonoids) content, antioxidant and antibacterial characteristics of the various extracts of the fruit of *Rosa canina* from Iran through various *in vitro* methods. Among the extracts, the methanol fraction with 11.58 µg/ml and 88.7% antioxidant activities in terms of IC50 values using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), respectively, was the most powerful ones. A study was done on the relationship between antioxidant capacity and total phenolic and flavonoid content, indicating that extracts containing these compounds are the major contributors to the antioxidant properties. According to the antibacterial activity of the various extracts, the methanol fraction was the most impressive extract against the available microorganism. Based on the strong antioxidant and antibacterial activities, the methanol fraction of *R. canina* fruit appears to be a potential herb and can be further explored as a functional medicinal plant for isolating the active ingredient(s), especially among flavonoids, along with animal studies *in vivo.*

Key words: Antibacterial, antioxidant, *Rosa canina*, total flavonoid content, total phenolic content.

INTRODUCTION

The pseudo-fruit of *Rosa canina* L. (Rosaceae), consisting of a u-shaped receptacle with numerous achenes inside, is rich in Vitamin C. Their fresh and dried products are frequently used as an herbal tea (Ausgabe et al., 2005). Rose pseudo-fruit is traditionally used for the prevention and therapy of common cold, prevention of inflammation of the gastric mucosa and gastric ulcer, and for gallstones and biliary complaints. It is also used as a laxative, for disorders of the kidney and the lower urinary tract. In addition, it is used as a diuretic for dropsy. Likewise, it is used as an astringent (Deliorman Orhan et al., 2007; Wenzig et al., 2009; Fecka, 2009) and in the treatment of various inflammatory diseases as a Vitamin C source (Chrubasik et al., 2006). A rose pseudo-fruit, which is marketed as a food supplement, has been shown to reduce osteoarthritis symptoms in clinical trials (Rein et al., 2004). Many works have been published on chemical composition of some rose species fruits, especially Rosa canina. It was previously reported that *R. canina* fruit, containing high phenolic and flavonoid contents, have antioxidant, antimutagenic and anticarcinogenic effects. Passing a different biochemical assay, including the ability to reduce the hydroxyl radical south ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), Trolox equivalent antioxidant capacity, metal ion chelating and free radical scavenging activity, makes it a natural antioxidant to replace the synthetic additives (Kilicgun and Altiner, 2010; Egea et al., 2010). The antioxidant activity of leaf extracts of *R. canina* were also reported by ABTS and DPPH methods (Ghazghazi et al., 2010).

Recent studies revealed that *R. canina* extracts were effective on the inhibition of growth and biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA) (Serteser et al., 2008; Quave et al., 2008).

The increased free-radical production and reduced antioxidant defense may partially mediate the initiation and progression of many diseases complications (Yao et al., 2010). It has been recognized that there is an inverse association between the consumption of some fruits and vegetables and mortality from age related diseases, which could be partly attributed to the presence of

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antioxidant compounds, phenolic acids, flavonoids, coumarin derivatives, etc., and which are the most abundant hydrophilic antioxidants in diet and the most active antioxidant compounds (Phillipson, 1991). On the other hand, among food industries are a widespread agreement that some synthetic antioxidants, such as butyl hydroxyl anisole and butyl hydroxyl toluene (BHA and BHT, respectively), need to be replaced with natural antioxidants because of their potential health risks and toxicity (Scalbert et al., 2005)

Therefore, the search for antioxidants from natural sources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds. In addition, these naturally occurring antioxidants can be formulated to give nutraceuticals, which can help to prevent oxidative damage from occurring in the body.

Due to increased resistance of many microorganisms towards established antibiotics, investigation of the chemical compounds within traditional plants has become desirable (Evans et al., 2002). There are many published reports on the effectiveness of traditional herbs against gram-positive and gram-negative microorganisms, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases (Phillipson, 1991). In the last century, the search for new anti-microbial agents from plants was mainly focused on tropical or subtropical plants, while less attention was paid to temperate plant species.

MATERIALS AND METHODS

Plant material

The fruit of *R. canina*, which is growing wild in Iran, was collected on March 2009 from Kandeloos village, Noshahr Mountains, Mazandaran province, Iran.

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxytoluene (BHT), iron (III) chloride hexa-hydrate, resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide sodium salt), Folin Ciocalteu reagent and quercetin were purchased from Sigma-Aldrich (Germany). Sodium acetatetrihydrate was obtained from Merck (Germany), gallic acid and DMSO were obtained from Acros Organics (USA), and hydrochlorid acid and sodium carbonate were obtained from Sigma-Aldrich (Germany). More so, methanol (M) was obtained from Caledon Laboratories LTD (Canada), while ethyl acetate (EA), acetone (A), chloroform (C) and n-hexane (nH) were purchased from Panreac Co. (Spain). However, MilliQ HPLC grade water (W) was used for the study.

Preparation of plant extracts

An aliquot (50 g) of dried and ground fruit was extracted with 500 ml of n-hexane, ethyl acetate, chloroform, acetone, water and methanol for 24 h at room temperature in a dark place under 750 rpm stirring. Each extract was filtered and dried using a rotary evaporator at 35°C. Extracts were re-suspended in DMSO using vortex to achieve the stock concentration of 10 mg/ml and were kept in a dark place at 4°C till they were used for the study.

Antibacterial test

In vitro antimicrobial activity of extracts was assessed against the following bacterial and yeast strains: *S. aureus* (PTCC1431), *Bacillus cereus* (PTCC 1015), *Bacillus subtilis* (isolated and biochemically characterized in our laboratory), *Escherichia coli* (PTCC1399) and *Candida albicans* (PTCC 5027). Micro-broth dilution susceptibility tests were used according to the standard protocols of NCCLS with some modifications to determine the minimum concentration of each antimicrobial agent required for inhibition (MIC) or killing (MBC) of the test microorganism (National Committee of Clinical Laboratory Standards, 1993a).

The inoculants of the microbial strains were prepared from freshly cultured bacteria and yeast strains that were adjusted to 0.5 McFarland standard turbidity using sterile normal saline, and then were further diluted (1:100) by sterile Mueller-Hinton broth just before they were added to the trays. Extracts solutions on DMSO serial dilutions were made in a concentration range from 30 to 0.125 mg/ml in sterile 96-well plastic micro-dilution trays containing Mueller-Hinton broth. MICs were recorded after 22 h incubation at 37°C.

Minimum bactericidal concentrations were determined by sub-culturing of 100 µl from each negative well and from the positive growth control onto a nutrient agar plate. MBCs were defined as the lowest concentration that could kill 99.9% of the test strains. All tests were performed in triplicate. Streptomycin and chloramphenicol, with well-known broad-spectrum antibiotics, were used as a positive control and the results were checked using 0.22 µm filtered resazurin aqueous solution (10%) as cell viability assays for bacteria.

Antioxidant activity

Free radical scavenging by the use of the DPPH radical

The DPPH radical scavenging capacity of each extract was determined according to the method of Brand-Williams modified by (Miliauskas et al., 2004 Re et al., 1999). DPPH radicals have an absorption that is maximal at 515 nm, and which disappears with reduction by an antioxidant compound. The DPPH$^{\bullet}$ solution in methanol (6×10^{-5} M) was prepared daily, and 200 µl of this solution was mixed with 100 µl of the solutions of plant extracts in a 96-well plastic tray. The samples were incubated for 20 min at 25°C under 450 rpm stirring, and then the decrease in absorbance at 515 nm was measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated using:

$$\% \text{ inhibition} = [1-(A_S - A_B)/A_C] \times 100$$

Where A_S, A_B and A_C were the absorbance of the samples, blank absorbance (extract solution) and absorbance of the control (DPPH solution), respectively.

Free radical scavenging by the use of the ABTS radical

Antioxidant activity based on the decolourization of the preformed radical monocation of ABTS$^{\bullet+}$ through reduction in the presence of hydrogen-donating antioxidants in plant extracts were performed according to the previous reports with some modifications

Table 1. Antibacterial activity of the different extracts of *R. canina*.

Extracts	*S. aureus*		*E. coli*		*B. cereus*		*B. subtilis*		*C. albicans*	
	MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Methanol	2.0	10.5	5.0	15.5	4.5	12.5	4.0	8.5	2.0	5.5
Water	3.5	18	25.0	>30	-	-	12	23.5	7.0	15.5
Acetone	5.0	25	9.0	20	-	-	-	-	15.0	>30
Chloroform**	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	6.5	25	-	-	-	-	-	-	-	-
n-Hexane**	-	-	-	-	-	-	-	-	-	-
Streptomycin	1.0	>2.0	>1.0	2.5	1.0	>2.2	1.0	3.0	1.0	3.0
Chloramphenicol	1.0	3.0	1.0	4.3	0.13	0.8	0.15	>1.0	1.0	3.2

*mg/ml, **no activity on the concentration of 10 mg/ml.

(Brand-Williams et al., 1995). Stock solution of plant extracts were diluted more in DMSO to a concentration of 1 mg/ml. The plant extracts were diluted to a yield concentration range of 7.8 to 1000 µg/ml in a total volume of 75 µl. $ABTS^{*+}$ (225 µl) was added to these extracts and the mixture was incubated for 10 min at room temperature under 450 rpm stirring. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as a positive control. Absorbance at 734 nm was determined using a power wave reader. The background (absorbance of plant extracts without $ABTS^{*+}$) was subtracted from reaction mixture readings. Antioxidant activity was calculated as a percentage of the negative controls. Controls without plant extracts and trolox were assumed to contain 100% oxidized ABTS. The readings were calculated based on the amount of ABTS reduced as obtained by a comparison with the controls. %ABTS oxidized=A_{734} of test×100/A_{734} of negative controls. Antioxidant activity (amount of ABTS reduced) %= 100%–amount of ABTS oxidized. Finally, the ABTS antioxidant activity was compared to trolox based on the calibration curve with the equation of y = 9.030x - 1.526 and 0.992 coefficient of determination.

Phytochemicals content

To evaluate the total phenolic content, diluted extracts solutions on DMSO or standard solution of gallic acid (2.5 µl) were introduced in 96-well plastic micro-dilution trays and mixed with 196 µl Folin-ciocalteu reagent (1.5:100 dilution with deionized water), and were left to stand for 3 min at room temperature under 450 rpm stirring. Then 2.5 µl of sodium carbonate solution (7% in deionized water) was added.

After incubation for 2 h at room temperature in a dark place, the absorbances were measured at 756 nm. Total phenolic content was expressed as mg/g gallic acid equivalent using the following equation based on the calibration curve with the linearity equation: y = 0.006x + 0.0515, and a coefficient of determination value (R^2) 0.998, where x was the absorbance and *y* was the gallic acid equivalent (mg/g).

For the total flavonoid content, the diluted extract in DMSO 100 µl was mixed with 100 µl of $AlCl_3$ in methanol (0.1%). A yellow color indicated the presence of flavonoids. After incubation at room temperature under 450 rpm stirring for 10 min, the absorbance of the reaction mixture was measured at 415 nm. Total flavonoid contents were calculated as quercetin (mg/g) using the following equation based on the calibration curve with the linearity equation: y = 0.009x + 0.229, and a coefficient of determination value (R^2): 0.997, where x was the absorbance and y was the quercetin equivalent (mg/g).

RESULTS AND DISCUSSION

Antibacterial activity

The results of the antibacterial assay of the different extracts of *R. canina* are presented in Table 1. The methanol extract with MIC = 2.0 mg/ml against *S. aureus* and *C. albicans* was the most potent extract. Also, with the exception of *B. cereus*, the water extract demonstrated an activity against both gram-positive and gram-negative bacteria tested in this study, in which the highest MIC was 3.5 mg/ml. The antibacterial activity of the acetone extract was detected against *E. coli, S. aureus* and *C.albicans*. Furthermore, the chloroform and n-hexan extracts, even in the concentration of 10 mg/ml, had no inhibition activity against all tested microorganisms. Although it belongs to the normal flora of humans, an enterohemorrhagic strain of *E. coli* caused serious food poisoning; as such, preservatives used to eliminate its growth are needed (Gulcin et al., 2003). However, some extracts of *R. canina* might therefore be of use. In this study, the antibacterial properties of *R. canina* were not as effective as the commercial drugs, but microorganisms became resistant to antibiotics over time (Table 1).

Antioxidant activity

DPPH scavenging activities of all the extracts were concentration dependent (Figure 1 -blue); however, the highest DPPH scavenging activity was shown by the methanol extract (11.58 µg/ml, IC50) of this species, and the second highest activity was observed in the aqueous extract (15.14 µg/ml, IC50), whereas n-hexane extract recorded the least activity (224.6 µg/ml, IC50). It was also observed that the scavenging activity of the methanol extract was comparable with that of BHT (8.3 µg/ml, IC50), which was a known synthetic antioxidant.

It is well established that the radical system used for antioxidant evaluation might influence the experimental

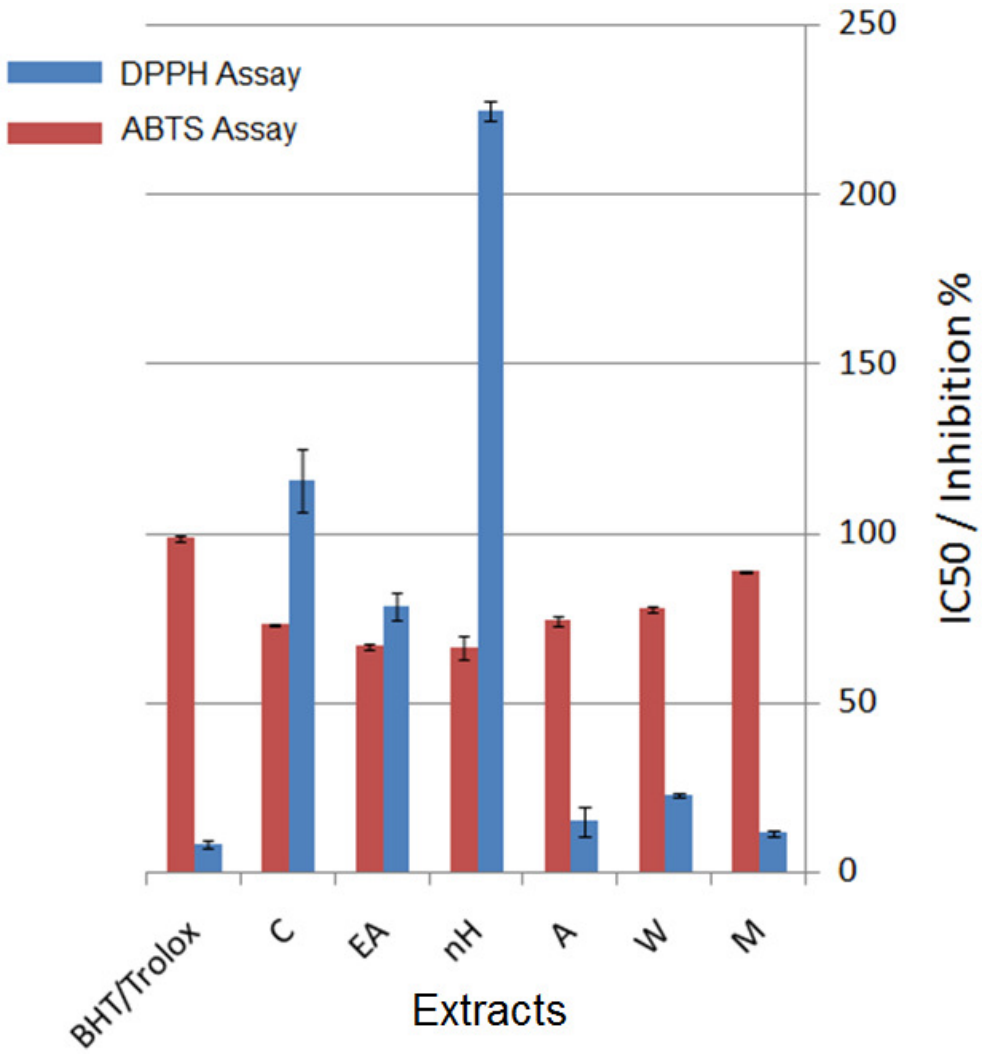

Figure 1. The antioxidant activity of *R. canina* extracts; DPPH assay; µg/ml, IC50 values in comparison with BHT (blue), and the ABTS assay, the inhibition percentage in comparison with Trolox (red).

results, and two or more radical systems are required to investigate the radical scavenging capacities of a selected antioxidant (Yu et al., 2002). To better examine their antioxidant capacities, the samples extracted were also analyzed for free radical scavenging activity against ABTS. Methanol, aqueous, and acetone extracts of *R. canina* were evaluated and compared for their ABTS scavenging capacities with trolox at the same concentrations. All the tested extracts showed significant ABTS scavenging capacity (Figure 1 - Red), which was comparable with that of trolox.

Phytochemical contents

Contents of phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or prevent decomposition of hydroperoxides into free radicals (Pokorny et al., 2001). The total phenols and flavonoids content of the different extracts were determined in this study (Table 2).

The concentration of phenolic compounds was higher in the methanol and acetone extracts when compared with the hexane extract. Differences in polarity (and thus different extractability) of the antioxidative components are obviously the reasons why phenolic compounds and antioxidant activity of the extracts differ especially in the case of n-hexane extract. Thus, the correlation between total phenol and total flavonoids was 0.972. This implies that extracts with the highest concentration of determination of total flavonoids also had higher concentrations with the different classes of phenolic compounds examined (Figure 2). Correlations between the amount of phenolic compounds and percent DPPH /

Table 2. Composition of phenolics and flavonoids of *R. canina* (± SD; n = 4).

Extract	Phenolic (mg GA*/g extract)	Flavonoids (mg Qu**/g extract)
Hexane extract (nH)	63.76 ±2.6	1.2 ± 3.2
Ethyl acetate extract (EA)	173.3 ± 2.8	8.1 ± 2.8
Methanol extract (M)	424.6 ± 1.8	23.6 ± 4.2
Acetone extract (A)	295.8 ± 4.2	18.4 ± 2.2
Water extract (W)	220.2 ± 5.9	10.4 ± 3.3
Chloroform extract (C)	145.8 ± 3.3	4.5 ± 1.2

* Galic Acid, ** Quercetin.

ABTS scavenging activity of the extract were studied. Correlation studies on the contribution of the different classes of these phenolics compounds to DPPH scavenging activity showed that the phenolic compounds were involved with the coefficient of determination (R^2) value of 0.723, whereas flavonoids were 0.813. Thus, the results were not included. Indeed, it is worth mentioning that the common notion which stipulates that antiradical activity strictly correlates with total polyphenol concentration does not always hold true (Chinnici et al., 2004; Maisuthisakul et al., 2007).

Similar finding of low correlations between DPPH radical scavenging activity and phenolic compounds have also been reported (Agbor et al., 2005; Maisuthisakul et al., 2007)

The moderate correlation between ABTS radical scavenging activity of the extract and total phenolic content (R^2 = 0.713) implies that it is not only phenolic compounds that contributed to the radical scavenging action, but same observation was made in the DPPH assay. For the fact that radical scavenging involves donation of a hydrogen atom or electron, there might be some other chemical compounds responsible for this action other than phenolics in this species. This will be interesting for further studies. Since major attention has recently been devoted to natural sources of antioxidant and antibacterial materials, the data obtained in this study suggest a possible use of the methanolic extract of *R. canina* as a source of natural antioxidant and antimicrobial agents.

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