Total polyphenolic compounds, total flavonoids, GC-MS analysis of volatile constituents, evaluation of antioxidant and antimicrobial activities of *Prunus dulcis* nuts

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Abstract: *Prunus dulcis* has been known as a source of nutrients in many traditional foods and as a source of important biologically active compounds in traditional medicines. The present study describes the determination of total polyphenolic compounds, total flavonoids and GC-MS analysis of hexane and chloroform fractions of 70% ethanol extract of whole almond shelled seed. Antioxidant and antimicrobial activities of various extracts of the nuts were evaluated. Folin-Ciocalteau procedure resulted in total polyphenolic compounds as 0.008 mg (gallic acid equivalents)/mg of the dried extract and amount of total flavonoids contents yielded were 0.001 mg (quercetin equivalents)/mg of the nuts dried extracts. GC-MS analysis of the hexane and chloroform fractions yielded a number of volatile constituents, resulting in highest amounts of 6-Octadecenoic acid and 1,1,3,3-Tetramethyl cyclopentane as 37.52% and 24.54% in hexane and chloroform fractions respectively. Antioxidant activity using the DPPH radical scavenging assay resulted in very low activity in all the extracts. Only *n*-butanol extract showed mild activity against the gram negative *E. coli* with inhibition zone diameter 8 mm while rest of samples did not show any activity against any microbial strains under study.

Keywords: *Prunus dulcis* nuts, total polyphenolics and flavonoids contents, GC-MS, antioxidant activity, antimicrobial activity.

INTRODUCTION

Nuts, fruits, vegetables and spices have been the fundamental source of naturally occurring pharmacologically active metabolites for human (Alasalvar and Shahidi, 2009; Qureshi *et al.*, 2014a; Qureshi *et al.*, 2014b). Raw plant materials to the pure compounds extracted from these herbs have been used against different diseases since ancient time (Gilani, 2005; Monagas *et al.*, 2007; Qureshi *et al.*, 2014a; Qureshi *et al.*, 2014b). Due to their various proven pharmacological properties, there is an increasing tendency in using the crude extracts of these nuts, fruits, vegetables and spices in the formulation of various foods, cosmetic and medicinal products (Qureshi *et al.*, 2014c; Qureshi *et al.*, 2012). Sweet almond (Botanical name: *Prunus dulcis*) belongs to the family Rosaceae and is one of the most popular tree nuts worldwide. Almond nuts have been used in various forms as from raw nuts to processed foods as a source of nutrients (Esfahlan *et al.*, 2010; Jamshed and Gilani, 2014; Monagas *et al.*, 2007). Various biologically active compounds, such as phenolic compounds such as falvonoids, phenolic acids, tannins etc and vitamin E have been identified which have been shown to be responsible for their antioxidant and other activities promoting the human health (Rao, 2012; Xie *et al.*, 2012).

Regular moderate use of almond nuts can control the blood pressure, cholesterol level, brings about anti-inflammatory and anticarcinogenic effects; and reduce the risk of cardiovascular disease and obesity related problems (Monagas *et al.*, 2007; Rao, 2012). Literature shows that polyphenolic compounds present in almond skin act synergistically with vitamin C and E, thus enhancing the antioxidant protection by safeguarding the low density lipoprotein (LDL) from oxidation (Chen *et al.*, 2005; Esfahlan *et al.*, 2010; Jamshed and Gilani, 2014). Besides other properties such as antioxidant, antiatherogenic, and anticarcinogenic, antihepatotoxic activity has been shown for the procyanadin present in the skin (de Pascual-Teresa *et al.*, 2010; Rao, 2012; Truong *et al.*, 2014; Wijeratne *et al.*, 2006; Xie *et al.*, 2012). Total polyphenolic compounds (TPC), total contents of flavonoids (TCF), GC-MS analysis of hexane and chloroform fractions along with the determination of antioxidant and antimicrobial activities of various

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fractions of 70% ethanol extract of whole almond shelled seed have been described in the present study.

MATERIALS AND METHODS

Chemicals and reagents

Petroleum ether (60-90°C), *n*-hexane, chloroform, ethyl and absolute ethanol were of analytical grade purchased from Tianjinshi Baishi Chemicals Company (pvt) Urumqi, China. Quercetin (98%) and gallic acid (≥97%), aluminium chloride, sodium acetate, Folin-Ciocalteau reagent (2 N), DPPH, EDTA, vitamin C were purchased from Sigma-Aldrich GmbH Steinheim. Helium gas with a purity of 99.9999% was used as a carrier gar. All the chemicals and reagents were of analytical grade and double distilled water was used throughout the experiment.

Extraction

Almond seed powder was defatted with appropriate amount of petroleum ether:*n*-hexane (1:1) mixture in a steel extractor for one day at room temperature thrice a time. The defatted seed powder was dried at room temperature and extracted with 70% ethanol for 24 hours. Suspension of the dried 70% ethanol extract was partitioned successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol producing their fractions. The remaining is the aqueous extract.

Quantification of TPC and TCF

TPC and TCF were determined according to the procedures adopted by Jiang *et al*. 2015 (Jiang *et al.*, 2015).

GC-MS analysis

Gas chromatography mass spectrometric analysis was performed employing a GC coupled to quadrupole mass spectrometer from Agilent, USA. Fused silica column (HP-5MS) with specifications as length: 30 m; i.d: 0.25 mm and film thickness: 0.25 µm was used for separation of analytes. The column temperature gradient was initiated at 50°C and a linear gradient was obtained by raising the temperature from 50 to 300 °C at the rate of 5°C/min and hold there for 5min. The injector was maintained at 250°C. Helium was used as the carrier gas at the flow rate of 1 mL/min. with the split ratio (5:1). The MS conditions were: ionization energy: 70 eV; electronic impact ion source temperature: 230 °C; quadrupole temperature: 150 °C; scan rate: 3.2 scan/s and the mass range was 30-1000 u. The extracts were filtered through syringe filters and 1 µL of each extract was injected into the GC column.

Antioxidant activity

The scavenging activity of all the extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined based on the reduction of purple DPPH to yellow coloured diphenylpicryl-hydrazine (Imam *et al.*, 2012; Zhao *et al.*, 2013). The extracts were dissolved in DMSO in a concentration of 100 ppm. Different concentrations of each extract were prepared in DMSO. Sample solution in different concentrations was added at 2.5 mL in each well of the 96 microwell plate. One mL of 0.3 mM DPPH solution in ethanol was added in each well to produce the test solutions. DMSO (1 mL) was added to produce the blank solutions. Another 1 mL of DPPH solution was mixed with DMSO (2.5 mL) to prepare the negative control. The solutions were kept in the dark at room temperature for 30 min to let them react. The measurement of absorbance and colour changes was done at the wavelength of 517 nm. Ascorbic acid (vitamin C) was used as the standard sample. The following equation was applied to convert the absorbance values into percentage antioxidant activity:

$$\% \text{ Inhibition} = [(A_{control} - A_{test\ sample})/A_{control}] \times 100$$

Whereas:

$A_{control}$: absorption of negative control sample; $A_{test\ sample}$: absorption of test sample (extract or standard). The half maximal inhibitory concentration (IC_{50}) was determined which is the amount of extract in µg per mL that inhibits the formation of DPPH radicals by 50%. Ascorbic acid was used as positive control in this assay.

Antimicrobial activity

Antimicrobial activities of the crude extracts were measured using the agar well diffusion method (Bozorov *et al.*, 2013; Lin *et al.*, 2012). Fungal and bacterial pathogens: *Candida albicans* (CA; ATCC10231), *E. coli* (EC; ATCC11229) and *Staphylococcus aureus* (SA; ATCC6538) were used as indicator strains for this analysis using Ampicillin sodium salt and Amphotericin B as standards (Bozorov *et al.*, 2014). These microorganisms were aseptically inoculated into appropriate liquid media and incubated at 37 °C. After 16 h, cells were centrifuged at 6000 rpm for 10 min and then suspended in sterile water. Different cells (1 mL) were added to appropriate agar media (100 mL) prior to plating, and wells were made using an agar well borer. To these wells, extracts having 100 ppm concentrations were added and subsequently incubated at 37 °C for 24 h. Zone of inhibitions were estimated by measuring the diameter of the microbial growth inhibition zone. Values were averaged from three independent experiments.

RESULTS

Prunus dulcis nuts were chemically analyzed, focusing on the determination of total polyphenolic compounds, total flavonoids contents, GC-MS analysis of volatile constituents, evaluation of antioxidant and antimicrobial activities of various extracts of nuts. Total polyphenolic compounds obtained were 0.008 mg (gallic acid equivalents)/mg of the dried extract and amount of total flavonoids contents resulted were 0.001 mg (quercetin

Table 1: Results of the GC-MS analysis of the *n*-hexane fraction of 70% ethanol extract of *Prunus dulcis* nuts

S. No.	RT(min)	Compound Name	% conc.
1	3.552	Octane	0.52
2	9.422	Cyclohexylmethyl ethylphosphonofluoridate	0.70
3	9.667	Thymine	0.83
4	11.044	Triethyl phosphate	2.30
5	11.469	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	0.30
6	13.394	4-Aminothiophenol	0.61
7	13.819	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	1.23
8	14.733	2-Cyclohexen-1-ol	0.58
9	18.241	Propyl S-2-(dimethylamino)ethyl propylphosphonofluoridate	0.77
10	25.725	Hexadecanal	0.37
11	27.509	Octadecane	0.23
12	29.421	Fumaric acid, cyclohex-3-enylmethyl heptadecyl ester	0.39
13	29.537	Nonadecane	0.22
14	30.715	2-Octene, 2-methyl-6-methylene-	1.28
15	30.889	*n*-Hexadecanoic acid	8.44
16	31.384	Hexadecanoic acid, ethyl ester	1.89
17	33.354	*cis*-13-Octadecenoic acid, methyl ester	0.70
18	34.313	6-Octadecenoic acid	37.52
19	34.467	9,12-Octadecadienoic acid, ethyl ester (Linoleic acid ethyl ester)	2.88
20	34.583	Ethyl oleate	5.09
21	34.680	Oleic Acid	1.51
22	35.021	Heptadecanoic acid, 15-methyl-, ethyl ester	0.28
23	36.566	Fumaric acid, 2-dimethylaminoethyl octadecyl ester	0.50
24	36.759	17-Pentatriacontene	0.54
25	37.068	1,19-Eicosadiene	2.65
26	37.132	Palmidrol	1.43
27	38.896	13-Octadecenal, (Z)-	0.31
28	39.321	2-Propenoic acid, 2-(dimethylamino)ethyl ester	0.66
29	39.398	Fumaric acid, 2-dimethylaminoethyl octyl ester	2.75
30	39.642	*cis*-9-Hexadecenal	2.44
31	39.971	9,12-Octadecadienoic acid (Z,Z)-	1.47
32	40.151	15-Hydroxypentadecanoic acid	1.29
33	42.372	4-(3,4-Dihydroxy-2-oxo-butylamino)-benzonitrile	0.94
34	42.475	1-Nonadecene	0.28
35	42.938	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	13.92
36	46.658	Stigmastan-3,5-diene	1.43
37	47.946	Ergosta-4,6,22-trien-3.beta.-ol	0.74

equivalents)/mg of the nuts dried extracts. GC-MS analysis of the *n*-hexane and chloroform fractions yielded a number of volatile constituents, resulting in highest amounts of 6-Octadecenoic acid and 1,1,3,3-tetramethyl cyclopentane as 37.52% and 24.54% in hexane and chloroform fractions respectively. Total of 37 compounds were identified in hexane fraction and 22 compounds were detected in the chloroform fraction based on the comparison of the obtained spectra with the standard mass spectra from the NIST and Wiley electronic libraries supplied with the instrument. Table 1 shows the results of the GC-MS analysis of *n*-hexane fractions while percentage composition of the volatile components of chloroform fractions are tabulated in table 2. Antioxidant activity using the DPPH radical scavenging assay resulted in very low activity in all the extracts. Only *n*-butanol extract showed mild activity against the gram negative *E. coli* with inhibition zone diameter 8 mm while rest of samples did not show any activity against any microbial strains under study (table 3).

DISCUSSION

Determination of total polyphenolic compounds

Determination of total polyphenolic compounds was based on the formation of blue coloured complex of

Table 2: Results of the GC-MS analysis of the chloroform fraction of 70% ethanol extract of *Prunus dulcis* nuts

S. No.	RT (min)	Compound Name	% conc.
1	2.812	1-Penten-3-one, 4-methyl-	1.80
2	3.497	Octane	13.85
3	6.226	2,5-Furandione, 3-methyl-	1.36
4	6.526	2-Heptenal, (Z)-	5.52
5	7.468	Benzene, 1,2,3-trimethyl-	1.38
6	9.137	2-Octenal, (E)-	1.75
7	9.394	(Z)1-Allyl-2-methylcyclohexanol	0.95
8	10.400	Nonanal	1.07
9	10.871	Benzene, 1,2,3,4-tetramethyl-	1.21
10	11.031	Triethyl phosphate	7.32
11	13.386	Hexyl methyl ethylphosphonate	2.65
12	13.836	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	3.62
13	14.360	4-Pentenoic acid, 2,4-dimethyl-, methyl ester	2.29
14	14.746	Cyclopentane, 1,1,3,3-tetramethyl-	24.54
15	15.602	2,4-Decadienal, (E,E)-	3.03
16	16.201	2,4-Decadienal, (E,E)-	3.15
17	17.432	2-Dodecenal	1.41
18	18.310	Propyl S-2-(dimethylamino)ethyl propylphosphonofluoridate	3.76
19	19.766	Dimethyl phthalate	0.69
20	24.250	Ethyl .alpha.-d-glucopyranoside	7.40
21	24.625	Ethyl citrate	9.36
22	25.385	Heptadecane	1.89

Table 3: Results of inhibitory effect of *Prunus dulcis* extracts on DPPH radical

Sample	DPPH inhibition assay IC_{50} (µg/mL)
70% ethanol extract	>500
n-Hexane extract	>500
Chloroform extract	>500
Ethyl acetate extract	349.20±19.64
n-Butanol extract	397.91±21.98
Water extract	>500
Vitamin C	5.34±0.42

polyphenolic compounds present in the extract after reaction with Folin-Ciocalteau reagent at room temperature. The coloured complex has a wave length maximum at 740 nm. Seven points calibration curve was obtained with a R^2 value 0.9956. This experiment resulted in amount of total polyphenolic compounds in the extract under study as 0.008 mg (gallic acid equivalents)/mg of the dried extract.

Determination of total flavonoids contents

$AlCl_3$ on reaction with flavonoids, present in the extract, produces a complex having a maximum absorption at 415 nm. The calibration curve with an R^2 value 0.9971 was obtained using the absorption values of the seven working standards of quercetin. The straight line equation (y=9.157x) produced from the calibration curve was used to calculate the total flavonoids contents as 0.001 mg (quercetin equivalents)/mg of the dried extract.

GC-MS analysis

n-Hexane fraction

Total of 37 compounds were detected in quantifiable amount and their percentage composition is given in table 1. In the *n*-hexane fraction highest amount of 6-Octadecenoic acid (37.52%) was yielded. Appreciable amounts of other compounds are: 9-Octadecenoic acid (Z)-2,3-dihydroxypropyl ester (13.92%), *n*-Hexadecanoic acid (8.44%), Ethyl oleate (5.09%), 2-Furancarbox-aldehyde, 5-(hydroxymethyl)- (1.23%), 2-Octene, 2-methyl-6-methylene- (1.28%), Hexadecanoic acid, ethyl ester (1.89%), 9,12-Octadecadienoic acid, ethyl ester (2.88%), Oleic acid (1.51%), 1,19-Eicosadiene (2.65%), Palmidrol (1.43%), Fumaric acid, 2-dimethylaminoethyl octyl ester (2.75%), cis-9-Hexadecenal (2.44%), 9,12-Octadecadienoic acid (Z,Z)- (1.47%), 15-Hydroxypentadecanoic acid (1.29%), Stigmastan-3,5-diene (1.43%). Amounts of the rest of the detected compounds were below 1%.

Chloroform fraction

GC-MS analysis of chloroform fraction delivered total of 22 compounds in quantifiable amounts using the area normalization method for quantification. Identification of the compounds was carried out by comparing the mass spectra obtained with those of standard mass spectra from the NIST and Wiley electronic libraries supplied with the instrument. Percentage composition of the volatile compounds detected in chloroform extract is shown in the table 2. Highest amount of 1,1,3,3-tetramethyl cyclopentane (24.54%) was obtained. Other compounds with appreciable amounts were: octance (13.85%), 2-Heptenal, (Z)- (5.52%), Triethyl phosphate (7.32%), Ethyl .alpha.-d-glucopyranoside (7.40%) and Ethyl citrate (9.36%). Concentrations of the remaining identified compounds were less than 5%.

Antioxidant activity

DPPH is a stable radical and mostly employed for the evaluation of antioxidant activity. It has a characteristic absorption at the wave length 517 nm which decreases when exposed to some radical scavengers. Lower absorption at the wave length 517 nm delivers lower IC_{50} value indicating stronger activity. Antioxidant activities of the seven samples/extracts were tested using the DPPH assay procedure. All the samples showed very low anti-radical activity as compared to the standard used as vitamin C with an IC_{50} value 5.34 µg/mL (table 3). Among the samples ethyl acetate extract showed higher activity with IC_{50} value 349.2 µg/mL while *n*-butanol extract delivered IC_{50} value 397.9 µg/mL. IC_{50} values of rest of the four samples were beyond 500 µg/mL. This low antioxidant activity may be because of high sugar content which co-extracted with phenolic compounds present in the brown skin of almond seed. From our experience it has been observed that sugar molecules dominate the whole extraction from almond nuts. It has been shown that sweet almond contains 26% carbohydrates (Rao, 2012), of which sucrose is the major sugars followed by raffinose (Balta *et al.*, 2009; Barreira *et al.*, 2010). Almond has been described as the nuts containing antioxidants and their antioxidant property has been attributed to the presence of polyphenolic compounds (Monagas *et al.*, 2007; Truong *et al.*, 2014). It has been confirmed from the literature that these antioxidant polyphenolic compounds are present in the brown skin of almond (Monagas *et al.*, 2007). These are mostly in conjugated forms with sugars and other polyols through O-glycosidic linkage (Jia *et al.*, 2011; Milbury *et al.*, 2006; Xiao *et al.*, 2014), which may also be one of the causes of low antioxidant activity as it has been shown that flavonoid aglycons are more antioxidant than their glycosidic forms (da Silva *et al.*, 2013).

Antibacterial activity

All the seven samples were screened against the three microbial strains: *Candida albicans* (CA; ATCC10231; fungus), *Escherichia coli* (EC; ATCC11229; gram negative bacteria) and *Staphylococcus aureus* (SA; ATCC6538; gram positive bacteria) using Ampicillin sodium salt and Amphotericin B as standards. Only *n*-butanol extract showed mild activity against the gram negative *E. coli* with inhibition zone diameter 8 mm, while rest of samples did not show any activity against any microbial strains under study.

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