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In-vitro antioxidative potential of different fractions from *Prunus dulcis* seeds: Vis a vis antiproliferative and antibacterial activities of active compounds

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ABSTRACT

In the present investigation, antioxidant activities of different fractions of *Prunus dulcis* seed extract were evaluated. The warring blender method was employed for extraction and total phenol and flavonoid contents were correlated with the diverse antioxidant activities of different fractions that showed significant correlation with the total flavonoid content. Maximum antioxidative activities were exhibited by its ethyl acetate fraction which was selected for isolation of different compounds. Six pure compounds were isolated which were further evaluated for antiradical, antiproliferative and antibacterial activities. Out of six compounds, gallic acid, pyrogallol, ethyl gallate and protocatechuic acid showed potent 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Gallic acid and pyrogallol were also found to be antiproliferative on two breast cancer cell lines (MCF-7 and MDA-MB-468). Further, most of the compounds exhibited an antibacterial activity similar to chloramphenicol. To the best of our knowledge this is the first report on the antioxidant activities of four different fractions of *P. dulcis* seed and also on the antiradical, antiproliferative and antibacterial activities of the isolated compounds.

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1. Introduction

Autoxidation of polyunsaturated fatty acids of cells is associated with ageing and different diseases including cancer (Yin and Porter, 2005; Ackerman and Simon, 2014). Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), widely used in the food industry are known to be carcinogenic in nature (Landete, 2013). Therefore, a great interest has been generated in supplementing them with natural antioxidants from plant sources. These are primarily polyphenols that commonly act as singlet oxygen quenchers and as reducing agents which can donate electron and can block the action of free radicals (Boeing et al., 2012).

It is believed that regular consumption of nuts, including almonds in moderate quantity lowers the risk of cardiovascular diseases (Chen and Blumberg, 2008; David et al., 2008). Nuts contain a good amount of phenolic compounds, terpenoids, pigments, and other natural antioxidants that are helpful in the protection from chronic diseases including cancer, primarily due to their secondary metabolites, fibre, vitamin E contents and some other compounds with antioxidant properties (Craig, 1997). Almond (*Prunus dulcis)* is one of the popular tree nuts that is a good source of phenolic compounds and different secondary metabolites that are considered useful for preventing oxidative stress (Halliwell et al., 1992; Halvorsen et al., 2002). It is further suggested that polyphenols from almond skin may serve as the source of potent antioxidants and as antibiotic against *Helicobacter pylori* (Shengmin et al., 2002; Carlo et al., 2013). In fact, bioactive phenolic and flavonoids have been previously isolated from almond skin (Maria et al., 2007) and antiproliferative terpenoids from almond hulls (Amico et al., 2006) which have been evaluated for their antioxidative properties. Oxidative stress induced by free radicals alters the structure of biological substrates, which was believed to be one of the leading causes of cancer. Antioxidative phytochemicals present in fruits and vegetables can have overlapping mechanisms of action, including modulation of detoxification of enzymes, scavenging of these free radicals etc. and thus could prevent cancer (Dhingra et al., 2014a). It has been reported that polyphenols and flavonoids possess strong binding ability with microbial protein and glycoproteins to form complex through hydrogen bonding and hydrophobic effects, as well as by covalent bond formation and thus could inactivate microbial adhesins, enzymes, cell envelope transport proteins, ion channels etc. One of the mechanisms behind the antibacterial activity of flavonoids is by reducing outer and inner layers of membranes fluidity of bacterial cells. Metabolic perturbation is reported to be one of the major causes for bactericidal effect of flavonoids (Havsteen, 2002; Wagner and Ulrich-Merzenich, 2009).

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Therefore, this investigation primarily aims to investigate total phenols, total flavonoids and diversified antioxidant activities of different fractions of almond seed extract and to correlate total phenols and flavonoids with its antioxidative activities. Further isolation of compounds from the most potent antioxidative fraction was performed to investigate the free radical scavenging, antiproliferative and antibacterial activities. To the best of our knowledge this is the first investigation on the isolation of the compounds from the almond seed extract and on the evaluation of different activities.

2. Materials and methods

2.1. Chemicals

Aluminium chloride, ammonium molybdate, ascorbic acid, butylated hydroxytoluene (BHT) 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride ($FeCl_3$), ferrozine, Folin–Ciocalteu's reagent, gallic acid, methionine, nitroblue tetrazolium (NBT), potassium ferricyanide, quercetin, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium nitrate ($NaNO_2$), sodium phosphate and trichloroacetic acid (TCA) were procured from Sigma Chemicals Co., St. Louis, USA. Ferrous sulphate ($FeSO_4$), sulphuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and all solvents were procured from Merck, Mumbai, India. Riboflavin was purchased from Himedia, Mumbai, India. All chemicals used were of analytical grade.

2.2. Plant materials

Seeds of *Prunus dulcis* were collected from Jammu, India and its voucher specimen (PD-10/03) has been deposited in the School of Life Sciences, Devi Ahilya University, India.

2.3. Sample preparation

Seed powder of *Prunus dulcis* was extracted thrice with acetone by warring blender method. Briefly, seed powder was extracted with chilled 80% acetone in the ratio 1:10 w/v for 5 min and then homogenized through polytron homogenizer for 3 min. The left residue was followed with same procedure and was done thrice. The resulting supernatants were filtered and evaporated under reduced pressure at 50 °C up to 90%. The remaining liquid (aqueous fraction) was successively partitioned with hexane, ethyl acetate and n-butanol and the three respective fractions were separately evaporated to dryness under reduced pressure at 50 °C; while the aqueous layer was lyophilised to dryness. The fractions were designated as HF, EF, BF and AqF for hexane, ethyl acetate, butanol and aqueous fractions respectively.

2.4. Total phenolic content

The total phenolic content was determined by Folin-Ciocalteu method with some modifications (Parmar and Kar, 2009). Briefly, 500 μL of sample was dissolved in 2.5 mL Folin-Cioleauteu's reagent and kept for 2 min. Then 2 mL of Na_2CO_3 was added and adjusted the volume up to 10 mL with distilled water. The solution was well mixed and heated at 45 °C for 15 min and the absorbance was measured after cooling the solution against the blank at 765 nm. Comparisons were made with the standards prepared similarly with known gallic acid concentrations. Results were expressed as gallic acid equivalent per gramme of material (mg GAE/g).

2.5. Total flavonoid content

Total flavonoid content was determined by colorimetric method using aluminium chloride (Dixit and Kar, 2009). Briefly, 500 μL of the sample extract was diluted with 2 mL of distilled water (DW). Then, 0.15 mL of a $NaNO_2$ (5%) solution was added, followed by 0.15 mL of aluminium chloride hexahydrate (10%) solution after 6 min and the mixture was allowed to stand for another 6 min. Two millilitres of 4% NaOH was added, and the total volume was made up to 5 mL with DW. The solution was well mixed and was allowed to stand for 15 min. The absorbance was measured immediately against the blank at 510 nm in comparison with the standards prepared similarly with known quercetin concentrations and results were expressed in quercetin equivalent (mg quercetin/g).

2.6. Antioxidant activities

2.6.1. Total antioxidant assay

Total antioxidant was determined by phosphomolybdate reagent with minor modification (Prieto et al., 1999). Briefly, 0.1 mL of fraction or standard was mixed with 1 mL of phosphomolybdate reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and was incubated at 95 °C for 90 min. After cooling, absorbance was measured against the blank at 695 nm. The antioxidant activity was expressed as μg ascorbic acid equivalent (μg AAE/mL).

2.6.2. DPPH scavenging assay

The DPPH radical-scavenging activity of different fractions of *P. dulcis* extract and of isolated compounds was determined using the method published in our previous paper (Dhingra et al., 2014b). Briefly, DPPH solution was added to test extract, mixture was shaken vigorously and allowed to stand at room temperature in the dark and the decrease in absorbance of the resulting solution was monitored at 517 nm. Butylated hydroxytoluene (BHT) was used as standard control. The percent of DPPH discolouration of the sample was calculated according to the equation:

$$\%\text{Scavenging [DPPH]} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample or standard.

2.6.3. Iron chelating assay

The ferrous ion-chelating ability was determined by our earlier method (Dhingra et al., 2014b). In brief, sample was mixed with DW and $FeSO_4$. The reaction was initiated by the addition of ferrozine and allowed to stand for 10 min at room temperature. The absorbance of the solution was thereafter measured at 562 nm with BHT as a standard. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula as

$$\text{Chelating effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the samples or standard.

2.6.4. Hydrogen peroxide assay

The hydrogen peroxide assay was performed according to the method of Ruch et al. (1989) with minor modifications, in which the concentration of H_2O_2 was determined by absorption at 230 nm using a spectrophotometer. Fractions dissolved in phosphate buffer were added to H_2O_2 and absorbance was measured at 230 nm, after 10 min against a blank solution containing phosphate buffer without H_2O_2. BHT was used for comparison as standard. The percentage of H_2O_2 scavenging is calculated as follows:

$$\%\text{Scavenging } (H_2O_2) = [(A_0 - A_1)/A_0] \times 100$$

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