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Antioxidative and antiproliferative activities of isolated compounds from *Prunus domestica* : an *in vitro* study

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Abstract

In this investigation the antioxidant as well as antiproliferative activities of different isolated compounds from ethyl acetate fraction of *Prunus domestica* (peel + flesh) were studied in two human breast cancer cell lines, MCF-7 and MDA-MB-468. Free radical scavenging study as done with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) indicated different degrees of antioxidative activity of the isolated compounds such as chlorogenic acid, protocatechuic acid, vanillic acid, ferulic acid, p-coumaric acid and rutin. However, maximum antioxidative activity was observed in chlorogenic acid with IC_{50} of 0.115 mg/ml. With respect to antiproliferative potential, chlorogenic acid also exhibited the maximum antiproliferative activity on MCF-7 and protocatechuic acid on MDA-MB-468 human breast cancer cell lines. This appears to be the first report that provides a comparative account on the antioxidant and antiproliferative property of some isolated active compounds of the Indian variety of fruit, *Prunus domestica*.

Keywords: *Prunus domestica*, antioxidant, antiproliferative, DPPH, human breast cancer cell lines.

Introduction

Damage imposed by free radicals and other reactive species is involved in numerous chronic diseases including cancer. Our body is usually protected by a natural defence system against these free radicals by antioxidant molecules and enzymes. However, when level of reactive oxygen species (ROS) exceeds, the capacity of antioxidant system declines and this results in induction of various human diseases [1]. Antioxidants such as polyphenols, carotenoids, ascorbic acids, tocopherol and flavonoids from natural sources which interfere with the production of these free radicals and inactivate them, have received much attention of the scientists. Efforts have also been made to identify new natural resources for health promoting antioxidative agents in human diets. Reports suggest that consumption of diets rich in fruits and vegetables provide protection against different health problems such as cardiovascular diseases and certain types of cancer. Currently there is a great deal of research interest in understanding natural antioxidants and anticancer compounds present in different fruits and vegetables [2]. In fact, numbers of medicinal plants or fruits have been evaluated for their antioxidant activities and whole crude extracts or isolated pure compounds from them have been found to work as effective antioxidants [3, 4]. However, on the active compounds of *P. domestica*, nothing much has been studied.

Prunus domestica belongs to family rosacea which is one of the largest families and has immense therapeutic potential [5]. Towards the chemical constituents of *P. domestica,* the main compound that has been isolated is domesticoside (2-*O*-β-D-glucopyranosyl-4-*O*-methylphloracetophenone) from the bark of the tree [6]. But other minor compounds including chlorogenic acid and neochlorogenic acid the two phenolic compounds of *P. domestica* are reported to reduce human low density lipoprotein (LDL) [7]. The high antioxidant activity of *P. domestica* is believed to be associated with its caffeoylquinic acid isomers [8]. It is also reported that, *P. domestica* is very effective in scavenging the peroxyl radicals, in fact, better than Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and propyl gallate [9]. Further, phenolic fractions of *P. domestica* inhibit the growth of the estrogen independent MDA-MB-435 breast cancer cells over the estrogen dependent MCF-7 breast cancer cells or the breast epithelial MCF-10A cells [10]. However, antiproliferative property of isolated compounds from *P. domestica* fruits is still not clear. Therefore, keeping in mind the paucity of information on the isolated components from *P. domestica* pitted fruit and for finding new sources for natural antioxidants and anticancer agents, the present investigation was under taken.

Thus the primary aim of the study was to isolate the different active compounds from *P. domestica* and to study their antioxidative as well as anti-proliferative activity. This appears to be the first attempt to isolate some active compounds from the Indian variety of *P. domestica* with simple and effective warring blender method.

Materials and Methods

General

The optical density was measured with a Bio-Tek ELx 808 (Winooski, VT, USA) and Shimadzu-1700 sphectrophotometer (Kyoto, Japan). The 1H- spectra were recorded on a Bruker Avance Digital 400 spectrometer (Karlsruhe, Germany) at 400 MHz. Chemical shift is given in (ppm) from tetramethylsilane

(TMS). TLC was performed on a precoated silica gel plate (Kiesel gel 60F254, Merck, Darmstadt, Germany). Column chromatography was carried out on silica gel (200–300 mesh), Octadecylsilane (Sigma Aldrich, USA) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). DPPH (2, 2-diphenyl-1-picrylhydrazyl) was purchased from Sigma–Aldrich, U.S.A. All other chemicals were of analytical grade and were purchased from Sigma Aldrich, U.S.A.

Plant material, extraction and isolation

Fruits of *Prunus domestica* were collected from Kullu, Himachal Pradesh and a voucher specimen (PD-10/02) of this collection has been deposited in the School of Life Sciences, Devi Ahilya University, Indore, India. *P. domestica* pitted fruits were extracted and fractionated according to their polarity as shown in Figure 1. In brief, *P. domestica* fruits were pitted and extracted thrice with acetone through warring blender method. Seven kilogram of fruit pulp were extracted with 80% acetone in the ratio 1:10 (w/v) in warring blender for 5 minutes and homogenized through polytron homogenizer for 3 minutes. The left residue is followed with same procedure thrice and solvent were evaporated under reduced pressure at 50 ^{0} C up to 90 %. The remaining liquid was successively partitioned with hexane, ethyl acetate and n-butanol. They were separately pooled and evaporated to dryness under reduced pressure, while the aqueous layer was lyophilised to dryness. The fractions were designated as HF (4 g), EAF (628 g), BUF (824 g) and AQF (1532 g) respectively. The DPPH antioxidant activity of the four fractions was determined using a spectrophotometric method. Highest antioxidant activity was found in EAF. Therefore, EAF were subjected to silica gel column chromatography (SG CC) eluted with increasing polarity of chloroform and methanol for the isolation of components. Total five fractions were collected which were further purified. Fraction 1 was subjected to SG CC using chloroform: methanol as mobile phase to give compound 1 (100 mg). Fraction 2 was subjected to sephadex LH-20 using methanol as eluting phase to give compound 2 (25 mg). Fraction 3 was further purified by ODS column using H_20/CH_3CN as mobile phase and seven fractions were collected which were labelled as fraction 3.1 to fraction 3.7. Fraction 3.3 was further purified with SG CC using $CHCl_3$: Acetone was used as solvent system to obtain compound 3 (12 mg). Fraction 3.4 was purified by using SG CC using Hex: EtOAc to give yellow crystals which was further purified using recrystallization using ethyl-acetate as solvent to give compound 4 (62 mg). Fraction 3.5 was purified using sephadex LH-20 using methanol as mobile phase to give compound 5 (82 mg). Fraction 4 was further purified using SG CC and $CHCl_3$ MeOH as solvent system to give compound 6 (14 mg).

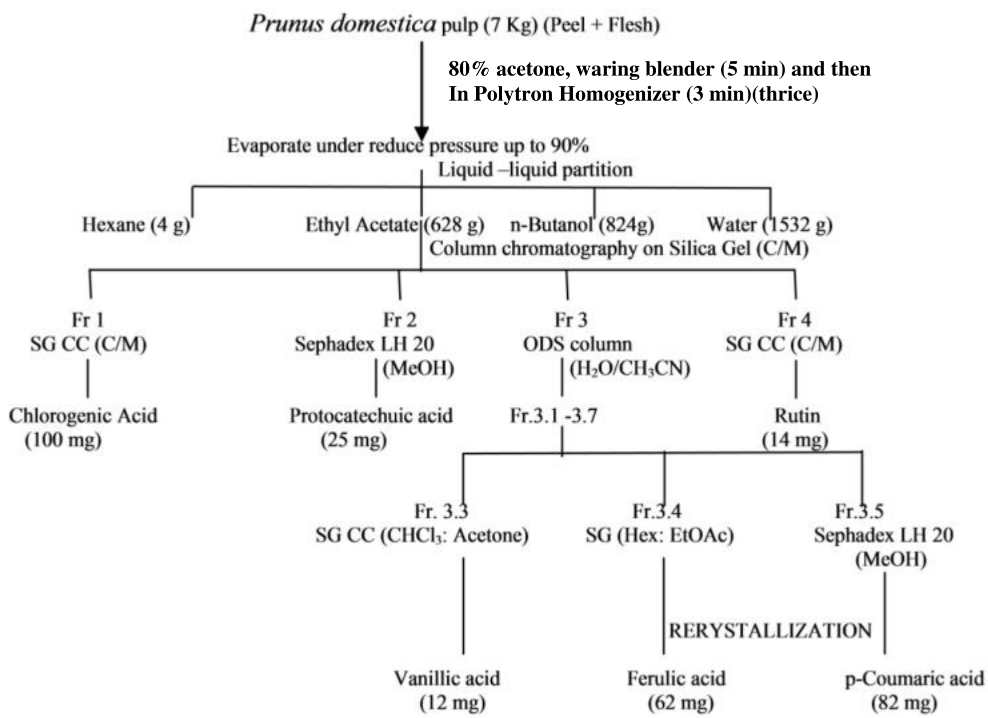


Figure 1. Flow chart of isolation scheme of compounds from the *Prunus domestica* fruits.

Structure elucidation for isolated compounds

Compound 1 was obtained as white powder, melting point (m.p.) 200-205 ºC. The molecular formula was established as $C_6H_6O_3$ by ESI MS *m/z* 354, 1HNMR (400 MHz, DMSO-d6): 9.62 (1H, s, H-4'), 9.19 (1H, s, 3'-OH), 7.42 (1H, d, J = 16.0 Hz, H-7'), 7.03 (1H, brs, H-2'), 6.98 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 6.76 (1H, d, J = 8.0 Hz, H-5'), 6.15 (1H, d, J = 16.0 Hz, H-8'), 5.06 (1H, ddd, J = 10.0, 6.0 Hz, H-3), 3.92 (1H, brs, H-5), 3.42 (1H, brs, H-4), 2.03 - 1.77 (4H, m, H-2/H-6). Compound 1 was identified as chlorogenic acid that agreed with the data reported earlier [11].

Compound 2 was obtained as white powder, m.p. 200-203 ºC. The molecular formula was established as $C_6H_6O_3$ by ESI MS m/z 154 and 1H NMR data were (400 MHz, DMSO-d6): 7.33 (1H, s, H2), 6.77 (1H, d, *J* 8.0 Hz, H5) and 7.28 (1H, d, *J* 8.0 Hz, H6) . This compound was identified as protocatechuic acid that agreed with the data reported earlier [12].

Compound 3 was obtained as white powder, m.p. 200-203 ºC. The molecular formula was established as $C_6H_6O_3$ by ESI MS *m/z* 168. Its 1H NMR data were (400 MHz, DMSO-d6): 7.42 (1H, s, H2), 6.79 (1H, d, *J* 8.0 Hz, H5), 7.41 (1H, d, *J* 9.2 Hz, H6), 3.78 (3H, s, OC*H3*). This compound was identified as vanillic acid that also agreed with the data reported earlier [12].

Compound 4 was obtained as white powder, m.p. 170-172 ºC. The molecular formula was established as $C_6H_6O_3$ by ESI MS m/z 194, 1H NMR data were (400 MHz, DMSO-d6): 7.53 (d, *J* = 15.56 Hz, 1H, H3′), 6.99 (d, *J* = 7.96 Hz, 1H, H6″), 6.91 (s, 1H, H2″), 6.84 (d, *J* = 8.14 Hz, 1H, H5″), 6.36 (s, 2H, OH, NH), 6.29 (d, *J* = 15.54 Hz, 1H, H2′), 4.14 (s, 2H, H1) and 3.81 (s, 3H, CH3) . This compound was identified as ferulic acid that also agreed with the data reported earlier [13].

Compound 5 was obtained as white powder, m.p. 200-205 ºC. The molecular formula was established as $C_6H_6O_3$ by ESI MS m/z 164. Its 1H NMR data were (400 MHz, DMSO-d6): 12.09 (s, broad, 1H), 9.18, (s, broad, 1H), 7.34 (d, 1H, J=15.8 Hz), 7.16 (t, 1H, J=7.7Hz), 6.97 (d, 1H, J=7.7Hz), 6.95 (d, 1H, J=2.2Hz), 6.74 (dd, 1H, J=2.2, 7.7 Hz) and 6.45 (d, 1H, J=15.8 Hz). This compound was identified as also p-coumaric acid as reported earlier [11].

Compound 6 was obtained as white powder, m.p. 225-230 ºC. The molecular formula was established as $C_{27}H_{30}O_{16}$ by ESI MS *m/z* 610. Its 1H NMR data were (400 MHz, DMSO-d6): 7.55 (1H, d, H-6'), 7.54 (1H, dd, H-2'), 6.82 (1H, d, H-5'), 6.36 (1H, d, H-8), 6.17 (1H,d, H-6), 5.32 (1H, d, H-1"), 5.02 (1H, d, H-1'") and 1.01 (3H, d, H-6'"). This compound 6 was identified as rutin similar to the observed data as reported earlier [14].

DPPH scavenging capacity

The DPPH radical-scavenging activity was determined using the previously described method with little modification [3]. Briefly, DPPH (100 μM) solution (1 ml) was added to 1 ml of polyphenol extract with 1 ml of methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 10 min. The decrease in absorbance of the resulting solution was monitored at 517 nm at 10 min. BHT was used as standard control. The % of DPPH discolouration of the sample was calculated according to the following equation:

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

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples or standard.

Cytotoxity assay against breast cancer cell line (MDA-MB-468 and MCF-7)

In vitro cytotoxicity of extracts was determined using sulforhodamine-B (SRB) on estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB-468 human breast cancer cell lines as described previously [15]. Briefly, both the breast cancer cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium). An aliquot of 100 μl of cell suspension (5 X 10^3 cells/well) was transferred to a well of 96-well tissue culture plate and incubated for 24 h. The test materials (100 μl) were then added to the wells and incubated for another 48 h. The cell growth was stopped by 50 μl of 50% trichloroacetic acid and plates were further incubated at 4ºC for an hour. The plates were washed with distilled water and air-dried. Sulforhodamine B (100 μl, 0.4% in 1% acetic acid) was added to each well and plates were incubated at room temperature for 30 min. The unbound SRB was removed by washing with 1% acetic acid and was air-dried. Tris-HCL buffer (100 μl, 0.01 M, pH 10.4) was added to all the wells and stirrer. The optical density was recorded on ELISA reader at wavelength of 540 nm with 690 nm reference wavelength.

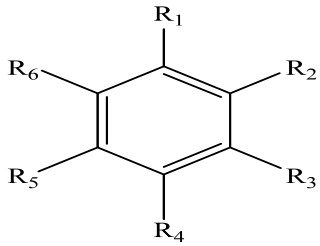
Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells.

$$\% \text{ Growth} = [A_1/ A_0] * 100$$

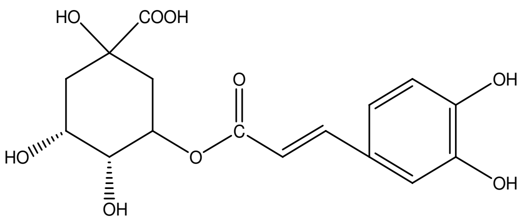
Where A_1 was the average absorbance of the test well and A_0 was the average absorbance of the control well.

Results and Discussion

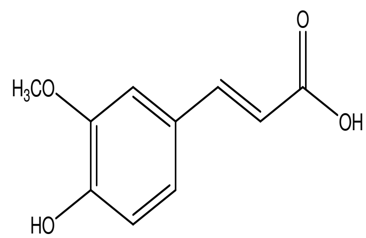
Results indicate that, ethyl acetate fraction of *P. domestica* fruits exhibits the highest antioxidative activity. Repeated column chromatography of this fraction resulted in the isolation of six pure compounds, chlorogenic acid, protocatechuic acid, vanillic acid, ferulic acid, p-coumaric acid and rutin. The chemical structures of the identified compounds are presented in Figure 2.



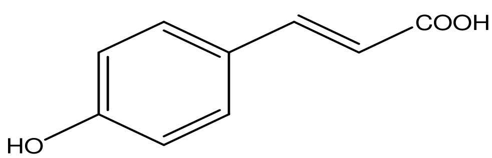
	R_1	R_2	R_3	R_4	R_5		R_6
Protocatechuic acid	COOH	H	OH	OH	H		H
Vanillic acid	COOH	H	OCH_3	OH	H		H



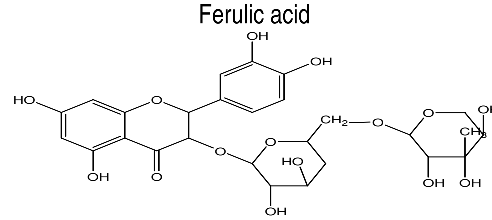
Chlorogenic acid



Ferulic acid



p- coumaric acid



Rutin

Figure 2. Structure of isolated compounds from *P. domestica*

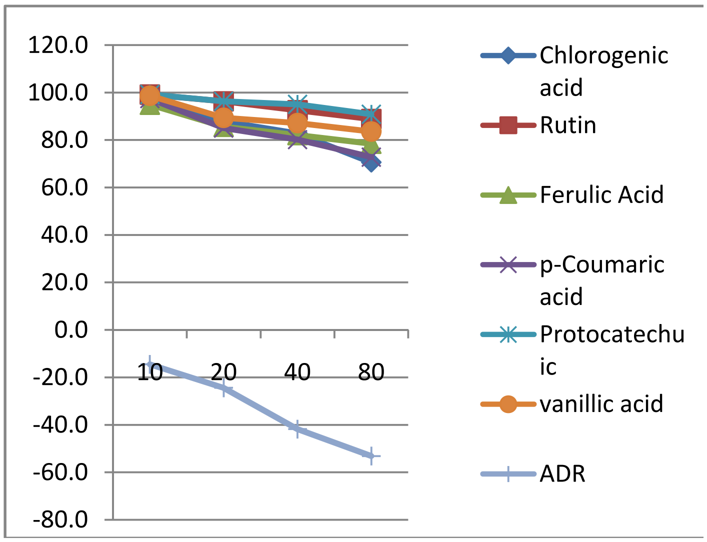
As shown in Table 1, all isolated compounds exhibited potent antioxidant activity. However, chlorogenic acid showed the maximum activity with IC_{50} of 0.115 mg/ml, while p-coumaric acid exhibited the least antioxidant activity with IC_{50} of 0.524±0.002 mg/ml in comparison to standard BHT. Ferulic acid and rutin showed similar antioxidative activity with IC_{50} of 0.121 ± 0.000 and 0.121 ± 0.004 mg/ml respectively, whereas protocatechuic acid and vanillic acid showed the IC_{50} of 0.122 ± 0.003 and 0.444 ± 0.003 mg/ml respectively However, previous report indicated, chlorogenic acid with IC_{50} of 0.167 mg/ml [16] and p-coumaric acid with 1.327 mg/ml [17]. When these isolated compounds were evaluated for their antiproliferative activity; all the compounds with four different concentrations reduced the percent growth in a dose-dependent manner as compared to control value (Table 2 and 3). In comparison to MCF-7 (estrogen positive receptor cell lines), isolated compounds were quite active for MDA-MB-468 (estrogen negative receptor cell lines) (Figure 3). All isolated compounds showed LC_{50} of >80 µg/ml, whereas standard adriamycin had LC_{50} of 61.5 µg/ml (data not shown).

Table 1: DPPH activity of fractions and isolated compounds

COMPOUNDS	IC_{50} mg/ml
HF	1.98 ± 0.120
EAF	0.124 ± 0.001
BUF	0.154 ± 0.002
AQF	0.218 ± 0.005
Chlrogenic acid	0.115±0.000
Protocatechuic acid	0.122±0.003
Vanillic acid	0.444±0.003
Ferulic acid	0.121±0.000
p-Coumaric acid	0.524±0.002
Rutin	0.121±0.004
BHT	0.152±0.001

As shown in table 2 and 3, all isolated compounds significantly inhibited cell proliferation in dose-dependent manner, except, chlorogenic acid for MDA-MB-468 cells. For MCF-7 cells, ferulic acid was most potent than all other compounds at the lowest concentration tested (10 µg/ml), in comparison to other compounds. On the other hand at 40 µg/ml p-coumaric acid showed maximum inhibition of cell proliferation. However, at 80 µg/ml, the highest concentration tested, chlorogenic acid showed a better inhibition of cell proliferation than all other isolated compounds tested with the similar concentration.

(a)



(b)

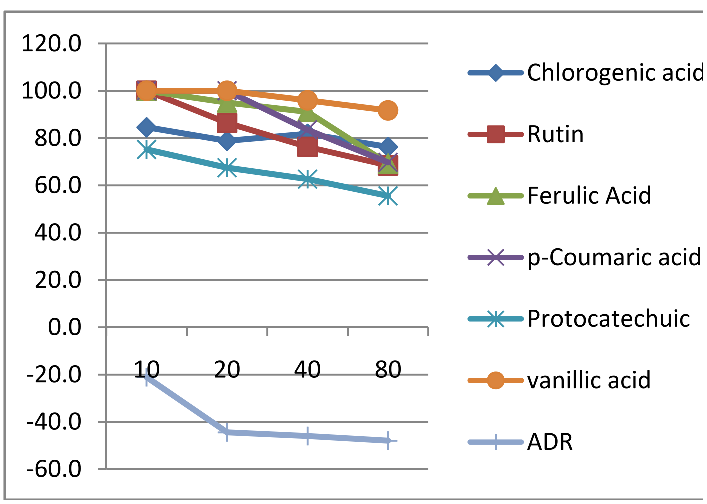


Figure 3. Anti-proliferative effect of isolated compounds and standard adriamycin at different concentration.

Growth between 0% and 50% indicated a cytostatic effect while growth <0% indicated a cytocidal effect. (a) % control growth for MCF-7 cell line (b) % control growth for MDA-MB-468

Table: 2 Percentage control growth on MCF-7 human breast cancer cell line

COMPOUNDS	Drug Concentrations (µg/ml)			
	10	20	40	80
Chlorogenic acid	95.9	88.0	82.8	70.6
Rutin	99.2	96.3	92.6	88.8
Ferulic Acid	94.9	85.6	82.1	78.5
p-Coumaric acid	97.5	85.1	80.2	72.8
Protocatechuic	99.2	96.3	95.0	90.9
vanillic acid	98.7	89.4	87.2	83.6
ADR	-14.5	-24.4	-41.8	-53.1

Drug Concentrations (µg/ml)

Table: 3 Percentage control growth on MDA-MB-468 human breast cancer cell line

COMPOUNDS	Drug Concentrations (µg/ml)			
	10	20	40	80
Chlorogenic acid	84.6	78.8	81.8	76.2
Rutin	100.0	86.4	76.3	68.4
Ferulic Acid	100.0	95.0	91.1	69.2
p-Coumaric acid	100.0	100.0	83.5	69.7
Protocatechuic	75.2	67.4	62.6	55.5
Vanillic acid	100.0	100.0	95.9	91.7
ADR	-21.1	-44.5	-46.0	-48.0

Drug Concentrations (µg/ml)

Furthermore, on MDA-MB-468 cells, protocatechuic acid was found to be most potent antiproliferative compound at all four concentrations tested. Previous report showed that protocatechuic acid was inactive on MCF-7 cells even with the highest dose tested at 50 µg/ml [18]. Excluding prtocatechuic acid, when comparisons were made among other compounds, chlorogenic acid at 20 µg/ml and rutin at 80 µg/ml were most potent inhibitor of cell proliferation. In contrast to the present findings, previous report showed that chlorogenic acid induced growth suppression on estrogen negative MDA-MB-435 cells but without any effect on MCF-7 cells up to the highest dose tested [10]. To our knowledge this is the first report of protocatechuic acid on MDA-MB-468 cell.

The cell proliferation inhibition activities of isolated compounds were compared with their scavenging activity. As it is clear from Table 1, the relative order of DPPH scavenging capacity for the isolated phenolic compounds was found to be as, chlorogenic acid > ferulic acid ~ rutin > protocatechuic acid > BHT > vanillic acid > p-coumaric acid. Surprisingly the antioxidative effects of these isolated compounds did not coincide with the inhibition of cell proliferation indicating that the test compounds might be acting through a different mechanism.

Conclusion

The present study suggests that chlorogenic acid isolated from the ethyl acetate fraction of *P. domestica* pitted fruits with maximum DPPH scavenging activity can serve as potent antioxidant activity as well as antiproliferative agent. However, protocatechuic acid may serve as a potent antiproliferative agent for MDA-MB-468 cells. Though adriamycin is useful in treating various cancers but applications of the drug, it may have side effects in various tissues [19]. Therefore, detailed study of these isolated compounds may prove to be more beneficial for the prevention of cancer.

Author's contribution

This work was carried out in collaboration between all authors. Author Naveen Dhingra performed all experiments and participated in the experimental design, analysis of the data and redaction of the manuscript. Author Prof. Anand Kar and Dr. Rajesh Sharma directed the research and supervised the preparation of the manuscript. All authors have contributed and approved the manuscript.

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