Research Paper

Inhibition of Aromatase and Cell Proliferation of Breast Cancer and Human Placenta Choriocarcinoma by *Prunus persica* Extracts

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Dhingra *et al*.: Aromatase inhibition by *Prunus persica* extract

Prunus persica has been used as a source of traditional medicine in many countries for the treatment of different diseases in females. In the present study, cytotoxicity and reactive oxygen species scavenging effects of *Prunus persica* extract on human breast cancer and placenta choriocarcinoma cell lines were evaluated. Besides this, regulations of oestrogen synthesis and antiaromatase activity were observed on human placenta choriocarcinoma cells. Ethyl acetate and butanol fractions had the maximum cytotoxic activity among all the fractions. In comparison to butanol fraction, ethyl acetate fraction was found to scavenge maximum intracellular reactive oxygen species, which was correlated with its total phenolic and flavonoid contents. Ethyl acetate was found to reduce the aromatase activity by 50 % at the concentration of 200 µg/ml. This inhibitory effect on aromatase was accompanied by reduced 17β-estradiol levels in JEG-3 cells. It seemed that the ethyl acetate and n-butanol fractions of *Prunus persica* or its active compound appears to be acting through dual mechanisms, i.e. improvement of antioxidant system and by suppressing aromatase; it may potentially be used as therapeutic agent for the treatment of breast cancer. These findings suggested that *Prunus persica* extract could be an aromatase inhibitor for the treatment of oestrogen-sensitive breast cancer.

Key words: *Prunus persica,* aromatase, oestrogen synthesis, ROS scavenging, breast cancer

There is an overwhelming evidence that consumption of fruits and vegetables is related to their protective efficacy against several chronic diseases, including cancer. Yet nothing much has been studied in *Prunus* fruits. *Prunus persica* belongs to family Rosacea and has been known to exhibit various pharmacological activities such as inhibition of oxidative process, chemotherapy-induced acute nephrotoxicity, hepatotoxicity, fungal infection and allergic reaction[1]. The therapeutic role of its seed is well-known. It is used as a traditional medicine in Japan, China, and other Asian countries mainly for the treatment of different diseases in females, including premenstrual syndrome[2]. However, its role in the regulation of breast cancer was not well understood.

Oxidative stress is known to activate a variety of transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt and Nrf2, which lead to the expression of different genes, including those for growth factors, inflammatory cytokines, chemokines leading to transformation of a normal cell to tumor cell[3]. Polyphenols contribute significantly to the total antioxidant capacity of fruits. Therefore, it is believed that the cellular system can be protected from oxidative stress by these polyphenols[4] and polyphenol containing plant extracts might have potential as anticarcinogenic agents.

Oestrogen plays one of the major roles in breast cancer and aromatase enzyme, a member of cytochrome P450 superfamily, synthesizes oestrogen by converting androstenedione and testosterone to oestrone and oestradiol, respectively. It has also been reported that approximately 60 % of premenopausal and 75 % of postmenopausal patients have oestrogen-dependent carcinomas. But aromatase inhibition reduces the oestrogen levels and thus could be a viable therapeutic target for breast cancer patients. In fact, aromatase

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inhibitors (AIs) are recommended as the first-line therapy for postmenopausal and premenopausal breast cancer[5-8]. However, long-term use of AIs increased the risk of skeletal problems, such as osteoporosis or bone fracture. Therefore, there is an urgent need to develop selective AIs with minimum side effects.

In the present investigation, the cytotoxic activity of different fractions of *P. persica* fruit in two human breast cancer cell lines, oestrogen receptor positive (MCF-7) and oestrogen receptor negative (MDA-MB-468), and in human placenta choriocarcinoma cells (JEG-3) were evaluated. In one of our previous studies, the diversified antioxidant activities of different fractions of *P. persica* fruit[9] were reported. So to understand its antioxidant activity in breast cancer and human placenta choriocarcinoma cells, the intracellular reactive oxygen species (ROS) scavenging activity of the most potent cytotoxic fraction of *P. persica* and aromatase inhibition activity along with its regulatory potential of oestrogen synthesis were investigated. Further, the bioactive compounds from these fractions were isolated and evaluated for cytotoxic activity on human placenta choriocarcinoma cells. In fact, this is the first report on the intracellular ROS scavenging, aromatase and oestrogen synthesis inhibition of *P. persica* fruit fraction.

MATERIAL AND METHODS

Plant collection:

Fruits of *Prunus persica* (peach) were collected from Kullu, Himachal Pradesh, India, identified and a Voucher specimen PP-10/02 of this collection has been deposited in the School of Life Sciences, Devi Ahilya University, Indore, India. Fresh samples were used for the preparation of extract.

Preparation of extracts:

Seeds of *Prunus persica* were removed and flesh part was used for extraction. Fruits were extracted with 80 % acetone in the ratio of 1:10 w/v in a Warring blender for 5 min and then homogenized by polytron homogenizer for 3 min. The residue left was processed thrice with the same procedure. The combined extracts were filtered through Buchner funnel and evaporated under reduced pressure at 50° up to 90 %. Remaining liquid was successively partitioned with hexane, ethyl acetate (EtOAc) and n-butanol. The hexane, EtOAc and n-butanol extracts were separately pooled and evaporated to dryness under reduced pressure, while the aqueous layer was lyophilized to dryness. The fractions were designated as hexane fraction (HF), EtOAc fraction (EF), butanol fraction (BF) and aqueous fraction (AF).

Cell cultures:

The human breast cancer cell lines, MCF-7 and MDA-MB-468 and human placental choriocarcinoma (JEG-3) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco modified Eagle medium (DMEM, GIBCO BRL, Invitrogen, Grand Island, NY, USA) containing 10 % (v/v) fetal calf serum (Thermo Scientific Hyclone, Logan, UT, USA) 2 mM glutamine, and antibiotics (200 U/l of penicillin and 50 mg/l of streptomycin). It was maintained at 37° in a humidified 5 % CO_2 atmosphere.

Cytotoxicity assay:

In vitro cytotoxicity of the extract was determined using sulforhodamine-B (SRB) on MCF-7 and MDA-MB-468 breast cancer cell lines and JEG-3 cells as described earlier[10]. Adriamycin purchased from Sigma-Aldrich, St. Louis, USA, was used as a standard.

Intracellular ROS scavenging assay:

Measurements of intracellular ROS levels in three cell lines were made using Bes-H_2O_2-AC, a highly selective probe for H_2O_2. Bes-H_2O_2-AC can permeate through the cell membrane and is deacetylated inside the cells to give an impermeable product, Bes-H_2O_2. Further intracellular ROS oxidizes Bes-H_2O_2 to give a fluorescent product difluorofluorescein. The intensity of fluorescence decides the oxidative stress of the cells. Decrease in fluorescence is expected to be produced by compounds which can scavenge ROS. This assay was performed using a previously described method[11].

Aromatase enzyme activity:

In vitro enzyme activity assays were performed on JEG-3 cells using both a direct aromatase and indirect aromatase assay systems as described previously with minor modifications[12]. In the direct aromatase assay, the EF and BF were mixed with 54 nM (1β-3H)androstenedione; the cells were treated with this mixture for 2 h.

In the indirect aromatase assay, cells were pretreated with the EF and BF for 18 h, and then exposed to 54 nM (1β-3H)androstenedione for another 1 h. After incubation at 37° the supernatant was collected and extracted with chloroform. The aqueous supernatant

was mixed with 5 % charcoal/0.5 % dextran and incubated for 15 min. The mixture was centrifuged at 14 000 g for 5 min, and the aromatase activity of the supernatant was measured using a liquid scintillation counter (LS-6500, Beckman counter, CA, USA) with a liquid scintillation cocktail (PerkinElmer®, MA, USA).

Determination of 17β-oestradiol synthesis:

JEG-3 cells were seeded in a 24-well plate and were cultured overnight. Next day, EF and BF were added to the cells in phenol red-free DMEM with 5 % charcoal-dextran-treated fetal bovine serum for 12 h. After this incubation, testosterone (10 nM) was added to each well, and the cells were incubated for 12 h. The culture media were then collected and stored at –20°. Levels of 17 β-oestradiol in the media were quantified using a competitive enzyme-immunoassay, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The enzymatic reaction was measured at 412 nm using a plate reader (BioTek, Winooski, US).

Isolation of components:

EF was subjected to silica gel column chromatography eluted with increasing polarity of hexane and EtOAc for the isolation of components. Total 6 fractions were collected and were further purified. Chemical structures

of all the isolated compounds was confirmed by nuclear magnetic resonance (NMR) and mass spectroscopy (MS). While fraction 1 was purified using Sephadex LH 20 using 90 % ethanol as mobile phase to obtain protocatechuic acid (37 mg), fraction 2 was further purified using silica gel column chromatography and hexane:EtOAc as mobile phase to give p-coumaric acid (84 mg). Fraction 3 was purified on an octadecylsilane (ODS) column using 50 % methanol (MeOH) as the eluting phase to give ferulic acid (62 mg). Sephadex LH-20 was used to purify fraction 4 using 100 % MeOH to give gallic acid (28 mg). Pure compounds were obtained from fraction 5 using preparative thin-layer chromatography, using chloroform:MeOH:H$_2$O (8:1:0.3) that yielded vanillic acid (11 mg) and p-hydroxybenzoic acid (14 mg). Fraction 6 was eluted through Sephadex LH-20 using MeOH:H$_2$O, which yielded three fractions viz. fraction 6.1, fraction 6.2 and fraction 6.3. Out of which fraction 6.2 was further purified using ODS column using CH$_3$CN:H$_2$O that yielded chlorogenic acid (100 mg; fig. 1).

RESULTS AND DISCUSSION

Results of cytotoxic activity evaluation performed on 3 cell lines at 4 different concentrations (10, 20, 40 and 80 µg/ml) revealed that on MCF-7 cells, EF and BF were cytotoxic at 20 µg/ml and cytocidal at 40 and
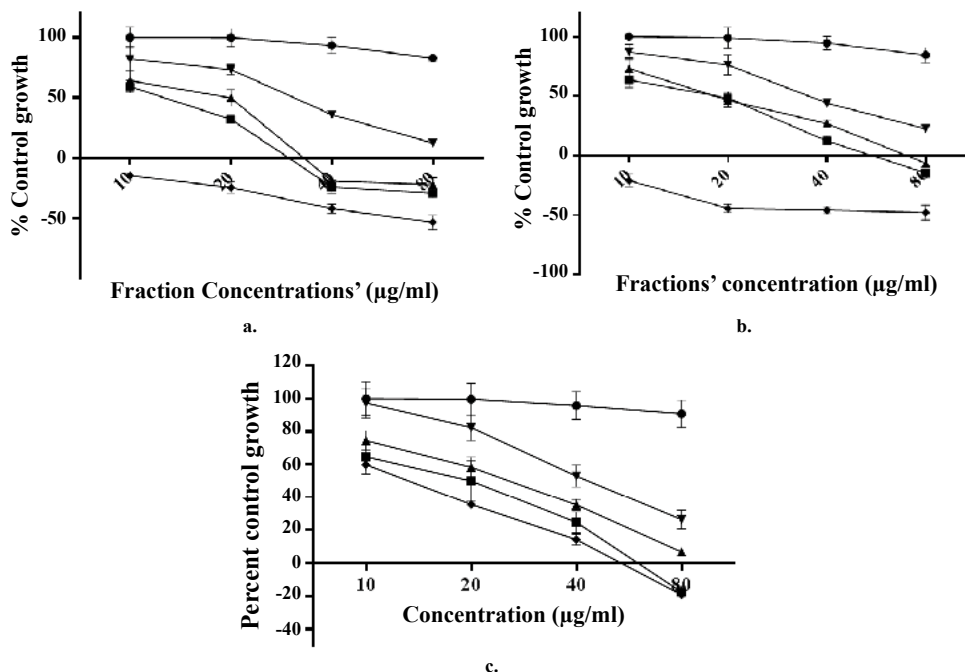

Fig 1: Cytotoxicity of different fractions of *P. persica* crude extract on three cell lines
—●— Hexane fraction; —■— ethyl acetate fraction; —▲— butanol fraction; —▼— aqueous fraction, (a) Oestrogen receptor positive (MCF-7), —♦— adriamycin (b) oestrogen receptor negative (MDA-MB-468), —♦— adriamycin and (c) Human placenta choriocarcinoma cell lines, —♦— exemastane. Results were expressed as mean±SD (n=3) for each group

80 µg/ml; whereas, on MDA-MB-468, EF and BF were cytocidal at 80 µg/ml and cytostatic at >20 µg/ml. Adriamycin was used as a standard for both cell lines. On JEG-3 cell line, EF showed cytostatic effect at 20 and 40 µg/ml and cytocidal effect at 80 µg/ml. BF on JEG-3 cells showed cytostatic effect at 40 µg/ml and cytocidal effect at 80 µg/ml. Moderate activity was exhibited by AF with a cytostatic effect on all three cell lines at 40 and 80 µg/ml of concentrations, whereas HF showed least cytotoxicity activity on all three cell lines (fig. 1). Exemestane was used as a standard for JEG-3 cells as these cells express the aromatase in more quantity than MCF-7 cell line. These cytotoxic effects of EF and BF on different cell lines could be due to the presence of high amount of total phenolic and flavonoid contents as reported earlier[13]. Present findings agree with the reported work, which indicated that different fractions of peach exhibited potent antiproliferative activity against oestrogen-independent MDA-MB-435 cells, but weak activity against oestrogen-dependent MCF-7 cells[14].

Natural polyphenols have long been attributed for their antioxidant activities. In fact, plant extracts contain a mixture of various phenolic and nonphenolic compounds. It has also been reported that these extracts, which are antioxidant in nature also show anticancer properties due to the presence of their polyphenol compounds[15]. In one of our previous investigations, observed that EF and BF of *P. persica* contain very high amount of total phenol and total flavonoid, whereas, HF and AF had less amount. Cytotoxicity activity of four test fractions and reported content of total phenol and flavonoid concord with each other[9].

Excessive production of ROS by dysfunctioning cell organelles would lead to the activation of several pathways, which might further cause cancer[16]. Therefore, a need existed to inhibit the intracellular ROS to prevent breast cancer. ROS-induced pathways persistently get expressed in many types of cancers, in which these would result in cell proliferation, differentiation, and inflammation[17]. Therefore, it is expected that inhibiting the intracellular ROS might prevent breast cancer.

Intracellular ROS scavenging activity of the test fractions in different breast cancer and human placenta choriocarcinoma cells was evaluated. On all the three cell lines, EF and BF scavenged intracellular ROS at concentrations >50 µg/ml (fig. 2). Both the fractions reduced the mean fluorescence intensity significantly

in a dose-dependent manner. On MCF-7 cell line, at 200 µg/ml, EF reduced the fluorescence intensity (MFI) to 0.75, whereas, BF reduced it to 1.01 in comparison to control+H_2O_2 cells. AF and BF on MDA-MB-468 cells reduced the MFI to 1.05 and 1.48, respectively. On JEG-3 cells, EF reduced the MFI to 0.87 and BF reduced it to 1.19. A previous study reported that *P. persica* extract scavenged intracellular ROS in pheochromocytoma cells[18]. Kang *et al.* reported that the ethanol extract of *P. mume* scavenged intracellular ROS in myeloblast cells at a concentration of 500 µg/ml[19]. However, there was no report on the ROS scavenging potential of *P. persica* extract in breast cancer and JEG-3 cells.

Aromatase is an enzyme that is responsible for synthesizing oestrogen from androgen[20]. It is found that aromatase activity is higher in breast tumours. In fact, there is a significant correlation between aromatase and the presence of tumours in breast tissues. A very high level of aromatase expression is also found in placenta during pregnancy. Therefore, JEG-3 cells are often used in the breast cancer study[2]. In the present investigation also inhibition of aromatase activity was considered as a potential target mechanism to prevent breast cancer.

EF and BF were further evaluated for aromatase inhibition activity as these fractions were found potentially more cytotoxic in comparison to the other two fractions. In the direct method it was observed that EF significantly inhibited the aromatase activity at >50 µg/ml; whereas, BF was equally effective at the concentration of >100 µg/ml. At 200 µg/ml concentration EF produced 51.20±1.98 % inhibition, while at the same concentration, BF inhibited the enzyme by 60.25±2.87 % as compared to that of cells exposed to the vehicle control (fig. 3a).

With respect to indirect method, at 200 µg/ml, EF inhibited 48±1.85 % and BF inhibited 59.21±2.97 % of aromatase activity, respectively (fig. 3b). Similar to these findings, some of the flavonoids isolated from heartwood and resin of *P. avium* also showed inhibitory activity on aromatase[21]. In addition, *P. africana* bark extract has also shown potent antiaromatase activity[22]. One of the reports showed that the extract of *Ginkgo biloba,* which contained flavones inhibited aromatase and exerted antitumor effects on breast cancer cells[23].

At the highest concentration of 200 µg/ml tested, EF inhibited oestrogen synthesis to 43.95 % and BF inhibited to 65.25 % as compared to the control (fig. 3c). Statistically 60 % of premenopausal and
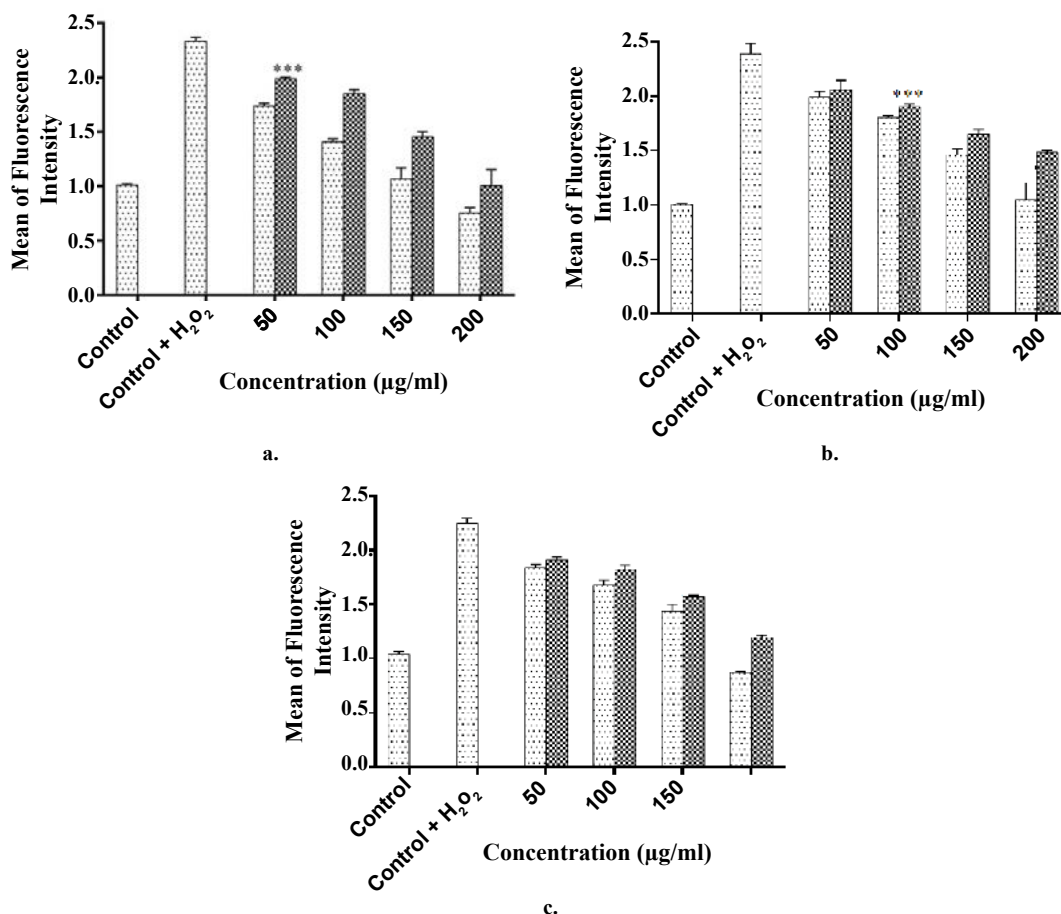
Fig. 2: Fluorescence intensity of three cell lines treated with different concentrations of sample and with H_2O_2
▒ Ethyl acetate fraction; ▨ butanol fraction, (a) oestrogen receptor positive (MCF-7), (b) oestrogen receptor negative (MDA-MB-468) and (c) human placenta choriocarcinoma cell lines. All values are expressed as means±SDs (n=3). Significant difference from control+H_2O_2 was observed by ANOVA where, ***p<0.001

75 % of postmenopausal patients are believed to have oestrogen-dependent carcinomas[24]. Aromatase converted androstenedione to oestradiol and the level of aromatase has a direct correlation with oestrogen synthesis. It has been reported that level of aromatase is expressed more in breast tumor and placenta of pregnant women[25,26]. In this study significant aromatase inhibitory activity of EF and BF was observed. This is similar to a compound found in *P. laurocerasus* that is known to show antiaromatase activity and play an important role in the depletion of oestrogen synthesis[27,28]. Kim *et al.* reported antioestrogenic activity of many medicinal plants and showed that *P. persica* exhibited solvent-dependent antioestrogenic activity[29].

In this study, compounds isolated from EF and identified through MS and NMR analyses as protocatechuic acid, p-coumaric acid, ferulic acid, gallic acid, vanillic acid, p-hydroxybenzoic acid and chlorogenic acid. This appears to be the first report that indicates the presence of all these 7 compounds in EF of *P. persica* whole fruit. Of course, these seven compounds are already reported in literature, but from some other plant extracts[30-34].

The p-coumaric acid was obtained as white powder and the melting point (MP) was 203-205°. Its ^{1}HNMR data were (400 MHz, DMSO-d_6): 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). MS (ESI) calculated for $C_6H_6O_3$ was m/z: 164[30]. Chlorogenic acid was obtained as a white powder and the MP was found to be 200-205°. ^{1}HNMR data were (400 MHz, DMSO-d_6): 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). MS (ESI) calculated for $C_6H_6O_3$ was m/z: 354[30].
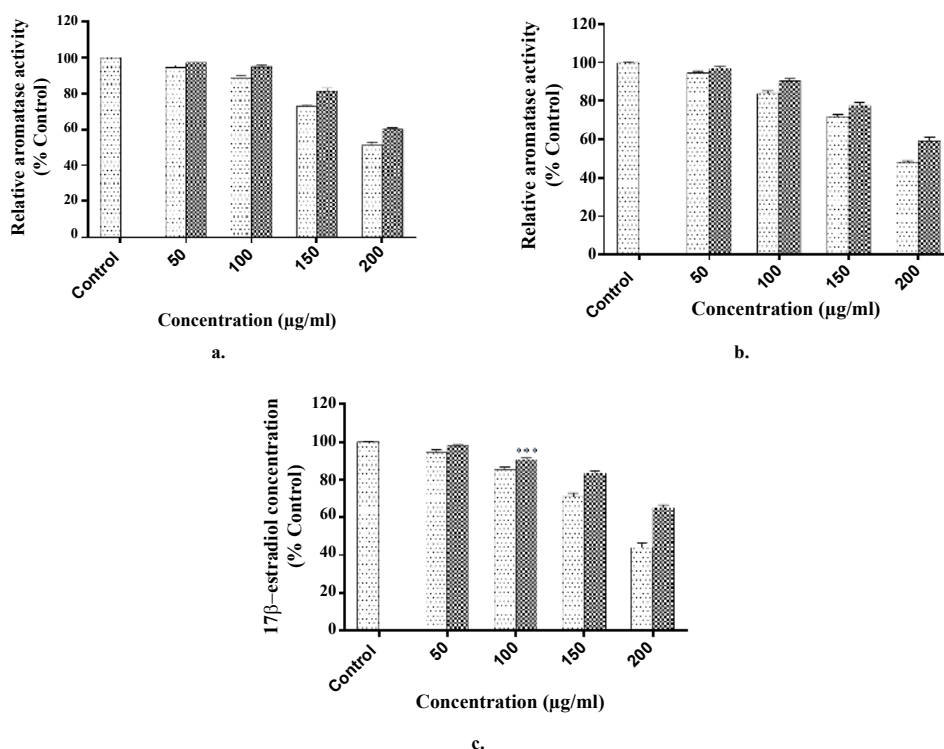
Fig. 3: Aromatase activity of EF and BF on JEG-3 cells

Ethyl acetate fraction (EF); butanol fraction (BF), (a) direct, (b) indirect effects and (c) inhibitory effects on 17β-oestradiol biosynthesis. The results are expressed as the mean±SDs (n=3). Significant difference from control was observed by ANOVA where, ***p<0.001

p-Hydroxybenzoic acid was obtained as a white amorphous powder and the MP was 203-205°. Its ^{1}HNMR data were (400 MHz, DMSO-d$_6$): 7.86 (2H, d, J=8.8 Hz), 6.80 (2H, d, J=8.8 Hz). The molecular formula was established as $C_7H_6O_3$ by MS (ESI) m/z: 137.2 [M-H]$^-$.

The data agreed well with the reported compound, p-hydroxybenzoic acid[31]. Gallic acid was re-crystallized from MeOH as white needles with MP of 253-255°. Its ^{1}H NMR data were, (400 MHz, DMSO-d$_6$): 6.91 (1H, s, H-2, 6). The MS (ESI) showed ion at m/z 169.3 [M-H]$^-$, corresponding to the molecular formula of $C_7H_6O_5$. Based on these data, compound 4 was identified as gallic acid consistent with the data reported[32]. Ferulic acid was obtained as a white powder with an MP of 170-172°. ^{1}H NMR data were (400 MHz, DMSO-d$_6$): 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). MS (ESI) calculated for $C_6H_6O_3$ was m/z: 194. This compound was identified as ferulic acid sine the data agreed with the data reported earlier[33].

Protocatechuic acid obtained as a white powder showed MP at 200-202°. ^{1}H NMR data were (400 MHz, DMSO-d$_6$): 7.33 (1H, s, H2), 6.77 (1H, d, J=8.0 Hz, H5) and 7.28 (1H, d, J=8.0 Hz, H6). MS (ESI) calculated for $C_6H_6O_3$ was m/z: 154. This compound was identified as protocatechuic acid as the data agreed with the data reported earlier[34]. Vanillic acid obtained as a white powder had an MP at 201-203°. ^{1}H NMR data was (400 MHz, DMSO-d$_6$): 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, OCH3). MS (ESI) calculated for $C_6H_6O_3$ was m/z: 168. This compound was identified as vanillic acid that also agreed with the data reported earlier[34].

As shown in fig. 4, gallic acid was found to be most effective in inhibiting the growth of JEG-3 cells (inhibited more than 50 % cells at 20 µg/ml) which also showed cytocidal effect at 80 µg/ml, which was similar to the cytotoxic effect of the standard exemastane. Chlorogenic acid also showed cytostatic effect and was able to inhibit 50 % cell growth at 40 µg/ml. This finding on the cytotoxicity of gallic acid and chlorogenic acid agreed with our previous findings on breast cancer cell lines, MCF-7 and MDA-MB-468[35].

In conclusion, the present study revealed that EF and BF of *P. persica* showed a wide range of cytotoxic and antioxidant activities through aromatase inhibition and scavenging intracellular ROS in breast cancer and
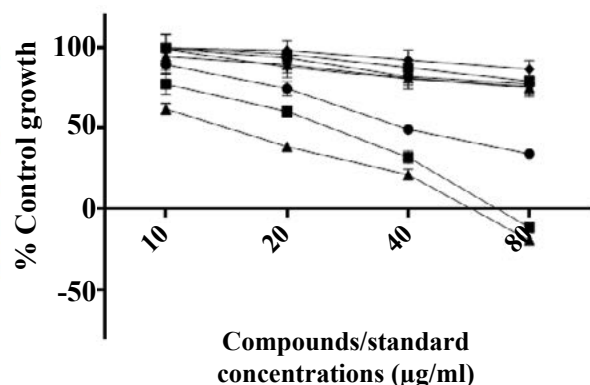
Fig 4: Cytotoxicity study of isolated compounds on JEG-3 cells
━•━ Chlorogenic acid; ━■━ gallic acid; ━▲━ ferulic acid;
━▼━ p-coumaric acid; ━♦━ p-hydroxybenzoic acid; ━•━
protocatechuic acid; ━■━ vanillic acid; ━▲━ exemastane.
The results were expressed as the mean±SD (n=3)

human placenta choriocarcinoma cell lines. Present study further indicated that the EF also regulated oestrogen synthesis in human placenta choriocarcinoma cells. Further, gallic acid isolated from EF showed better cytotoxic activity in comparison to adriamycin. The compounds isolated from these fractions could be further studied to evaluate their potential to prevent breast cancer. As the *P. persica* fruit extract or its active compounds appeared to be acting through dual mechanisms of improving antioxidant system and inhibiting aromatase, it could potentially be used as a therapeutic agent for the treatment of breast cancer.

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Conflict of interest:

There is no conflict of interest among authors.

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