Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-017-1253-0

ORIGINAL ARTICLE

Antioxidant and cytotoxic activity of bioactive phenolic metabolites isolated from the yeast-extract treated cell culture of apple

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Received: 9 January 2017 / Accepted: 12 June 2017

Abstract Apples are well known for their high nutritional value and health-protective properties. In this study, cell suspension cultures of apple (*Malus domestica* 'florina') were established and maintained in liquid Linsmaier and Skoog (LS) medium supplemented with 2 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 µM naphthaleneacetic acid (NAA). Elicitation of plant cell culture is a promising strategy to enhance and extend production of bioactive metabolites. Apple cell suspension culture in the linear growth phase was treated with yeast-extract elicitor and its effect on the antioxidant properties and accumulation of bioactive phenolic metabolites were characterized. Upon elicitor treatment, the total phenolics and flavonoids were significantly enhanced, preceded by the enhancement of the activity of phenylalanine ammonia lyase (PAL) enzyme. Methanolic extract from the elicited cell culture were analyzed by high performance liquid chromatography (HPLC) for the detection and quantification free phenolic acid and flavonoid. Predominant phenolics detected in the soluble fraction were protocatecuic acid, catechin, chlorogenic acid, vanillic acid, 4-coumaric acid, ferulic acid, benzoic acid and rutin. Out of these phenolics, the content of chlorogenic acid (112 µg/g DW), 4-coumaric acid (122 µg/g DW) ferulic acid (212 µg/g DW), benzoic acid (244 µg/g DW) and rutin (348 µg/g DW) were significantly enhanced upon elicitation. In addition, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) radical scavenging activity was significantly enhanced upon yeast extract treatment, and this was positively correlated with increased accumulation of phenolic metabolites. Finally, cytotoxic effect of elicited cell culture extract was evaluated against human cervical (HeLa cells) and breast (MCF-7 cells) cancer cell lines using MTT assay. Elicited extract exhibited significant apoptosis mediated cell death in both breast (IC_{50}: 31 µg/ml) and cervical (IC_{50}: 38 µg/ml) cancer cell lines. No growth inhibition was observed in normal human embryonic kidney cell line (HEK-293). Our results indicated that yeast-extract treated cell culture of apple provides an promising system for sustainable production of natural antioxidants and anticancer therapeutics.

Keywords Apple · Antioxidant · Cytotoxic · Callus · Florina · Metabolites

Introduction

Apple (*Malus domestica*) is the most preferred deciduous fruit crop grown in India (Ghosh 1999) and in other temperate regions of the world (Velasco 2010). Approximately 76 million tons of apples were produced worldwide in 2012; where Indian contribution was 2.2 million tons (FAO 2015). Apples are consumed for their high nutritional value and health-protective properties. Daily consumption of apples is linked with the reduced risk of cancer and heart disease, which are the top two leading causes of human death (Boyer and Liu 2004; Gerhauser 2008). Apples are one of the main sources of dietary flavonoids and phenolics

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Published online: 17 June 2017

which are associated with lower mortality rate (Vinson et al. 2001; Boyer and Liu 2004). Recent findings showed that apple fruits have excellent anti-proliferative, antioxidant, gastrointestinal protection from drug injury and cholesterol lowering properties (Eberhardt et al. 2000; Leontowicz et al. 2002; Wolfe et al. 2003; Hyson 2011). Major health-protective antioxidant metabolites present in apples include quercetin-derivatives, catechin, epicatechin, procyanidin, cyanidin-3-galactoside, chlorogenic acid, gallic acid, p-coumaric acid and minor amount of phloridzin (Lee et al. 2003).

Plant in vitro cultures offer excellent alternative for large scale production of biologically active secondary metabolites (Sarfaraj-Hussain et al. 2012). Production of value added secondary metabolites through cell cultures would help to save natural plant resources. Moreover, plant cell culture could potentially produce novel secondary metabolites possessing biological activities which can not be produced by chemical synthesis (Siahsar et al. 2011). This approach provides several advantages such as little or no seasonal variation, high yield of metabolites and less production time. The plant cell cultures have been successfully used in the production of many commercially important drugs such as vincristine, vinblastin, camptothecin, taxol, etc (Rao and Ravishankar 2002; Wilson and Roberts 2012), plant-derived pharmaceuticals and food supplements (Sak et al. 2014). However, secondary metabolites production via plant cell culture is often limited by low product yields, unpredictable scale-up and occasional variability in pattern of accumulation among cultures of the same cell lines (Kolewe et al. 2008). To overcome the low production rate of secondary metabolites by cell culture various strategies have been employed, out of which elicitation is the most promising strategy to enhance formation of plant secondary metabolites. Methyl-jasmonate, chitosan, yeast extracts are some of the common elicitors used in the high yield production of plant secondary metabolites (Bourgaud et al. 2001). Elicitor concentrations, cell culture type, age of the cell culture and elicitor dose are important factors necessary to optimize the production of natural products (Murthy et al. 2014). Currently, there is a growing demand of plant-based natural product in food, pharmaceutical and drug industries because of their higher health benefits and little or no side effects. Especially, plants products rich in antioxidants are associated with lowering risk of various age-related diseases and metabolic disorders (Benzie and Choi 2014). Apple extracts have been extensively used as nutritional supplement for various food products and health drinks (Hyson 2011). High antioxidant activity of apple products has been linked with total phenolics and flavonoids content (Boyer and Liu 2004).

In spite of proven role of elicitation for enhancing accumulation of a wide range of bioactive natural products in plant cell cultures, such studies have never been applied to Malus domestica cell cultures. Therefore, this work has been done with the aim of finding whether yeast extract can trigger the enhanced biosynthesis of bioactive secondary metabolites, with focus on anti-cancer activity. For this, we established the cell culture of apple and tested the effect of yeast extract elicitor on phenolic metabolite production and antioxidant activity. Anticancer activity of elicited extract has been tested on human breast (MCF-7 cells) and cervical (HeLa cells) cancer cell lines. We have also investigated the influence of elicitor on the activity of phenylalanine ammonia-lyase (PAL) activity, they key enzyme involved the biosynthesis phenolics metabolites in plants.

Materials and methods

Plant material and chemicals

Apple cultivar 'Florina' (*M. domestica* cv. Florina) was obtained from Central Institute of Temperate Horticulture (CITH), Srinagar, India. Apple plants were maintained under temperate condition in a micro-climate control green house (temperature 20–22 °C and relative humidity of 65–70%). Analytical grade chemicals were used in sample preparation and all solvents used for HPLC analyses were of HPLC grade. All authentic standards were procured from Sigma-Aldrich Chemical Co. Ltd (India). MCF-7, HeLa and HEK-293 cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. DMSO (cell culture grade), MTT (3-(4,5-dimethyl-2-thiazolyl) 2,5diphenyl-2H-tetrazoliumbromide), 5-Fluorouracil (5-FU), plant growth media, plant growth regulators were obtained from Himedia (India).

Induction and maintenance of cell suspension cultures

Primary callus culture was derived from the young leaves. Three to four top young leaves (8–10 days old) were collected and surface sterilized. After surface sterilization, leaf segments were cut into 10 mm sections and put on basal LS medium (Linsmaier and Skoog 1965) supplemented with 30 g l^{-1} sucrose, 7 g l^{-1} agar and various concentrations and combinations of 2,4-D (0.5, 1.0, 2.0, 2.5 µM); NAA (0.5, 1.0 µM) and kinetin (0.5, 1.0 µM) in dark condition for callus induction. Optimum growth regulator combination was selected based on highest callus growth. The pH of the medium was adjusted to 5.8. Aseptic cultures were maintained at 26 °C in dark. Calli were propagated by regular sub-culturing at 4-weeks interval. Medium without plant growth regulators served as control. Friable soft calli were selected for the initiation of cell suspension culture. Cell suspension was initiated in dark by shaking 3 g of calli

at 120 rpm in 50 ml of the liquid LS-medium (in 250 ml Erlenmeyer flasks) containing 2 µM 2,4-D and 1 µM NAA. Cells were harvested at 7- to 9 -day intervals by vacuum filtration.

Elicitor Preparation and treatment

Elicitor stock solution was prepared by dissolving 1.5 g of yeast extract in 10 ml of distilled water followed by filter sterilization. Seven-day-old cell suspension culture from the linear growth phase were elicited with yeast-extract solution at a final concentration of 3 g/l. Flasks were kept at 26° in dark in an orbital shaker at 100 rpm. After onset of elicitation, cell cultures were harvested at defined post-elicitation time points: 0, 12, 24, 48 and 72 h post elicitation (hpe). In the control treatment, similar volume of sterile distilled water was added in lieu of the yeast extract. Three replicates were used for each treatment, and the experiment was repeated two times.

Sample preparation

The cells were harvested at defined post-elicitation time points by vacuum filtration and kept in hot air oven at 60 °C for 4 h. Dried cell mass (2 g) was crushed in liquid nitrogen and subsequently extracted with 5 ml methanol (50%; v/v) at room temperature. The suspension was homogenized for 5 min and then centrifuged at 5000 rpm for 15 min. The resulting supernatant was filtered using a 0.45 µ syringe filter and directly used for HPLC analysis of phenolic metabolites as well as for the evaluation of total soluble phenolics, total flavonoids and antioxidant potential. A separate sample preparation procedure was followed for cytotoxicity assays. Methanolic extract was evaporated in a rotary evaporator to remove methanol and remaining aqueous phase was lyophilized and re-dissolved in 500 µl of dimethyl sulphoxide (DMSO). DMSO extract at various dilutions were used for cytotoxicity assays.

HPLC analysis of phenolic acid

HPLC analysis of metabolites was performed on a Phenomenex™ (Torrance, USA) C_{18} column (RP-Hydro, 4 µm, 250×4.6 mm) using a Shimadzu-HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A controller, LC-20AP pump, SPD-M20A PDA detector. Peaks were identified by comparing their retention time and UV-spectra with those of authentic standards. Data was acquired and processed with LC-Solution software (Shimadzu Corporation, Kyoto, Japan) on Windows 7™ platform. Chromatograms were monitored with a PDA detector on a Windows 7 Professional platform with Lab Solutions Multi LC-PDA software (Shimadzu). An isocratic solvent system 1mM TFA in water: methanol [70:30; (v/v)] with a flow rate of 1.0 ml/min for 60 min was used to elute the phenolic acids and flavonoids.

Determination of total phenolic content (TPC)

Total phenolic content was determined using Folin–Ciocalteau method (Singleton et al. 1999). Briefly, 0.2 ml of 50% methanolic extract was mixed with 0.5 ml Folin–Ciocalteau reagent (dilution 1:9 with water) and incubated at room temperature for 5 min to initiate the reaction. Thereafter, 0.3 ml of 5% sodium carbonate was added to the mixture followed by 20 min incubation in dark at room temperature. The absorbance was measured at 765 nm. Total phenolic content was expressed as micrograms of gallic acid equivalents per gram dry mass.

Determination of total flavonoid content (TFC)

Total flavonoids content was measured as essentially described by (Wang et al. 2008). Methanolic extract (0.5 ml) was added to, 0.5 ml of 2% $AlCl_3$ solution in ethanol and incubated at room temperature. After 1 h incubation, absorbance was measured at 420 nm. Total flavonoid content was expressed as micrograms of quercetin equivalent per gram dry mass.

DPPH radical scavenging activity assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay was performed as essentially described by (Turkoglu et al. 2007). 200 µl of various concentration of methanolic extract was added to 800 µl of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was measured at 517 nm using a blank. Blank reaction consisted of all reagents except the callus extract. Percent (%) inhibition of free radical by DPPH was calculated using following formula.

$$\text{Inhibition}\ (\%) = \left(A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}\right) \times 100$$

The results were expresses as IC_{50} values. The EC50 value is the concentration of an antioxidant required to lower the initial concentration of DPPH by 50%. Ascorbic acid was used as standard antioxidant. All experiments were performed in triplicates.

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) antioxidant potential was determined according to the method described by (Benzie and Strain 1996) with minor modification. FRAP reagent was prepared fresh mixing 1 ml of

10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution with 10 ml of 300 mM acetate buffer in 40 mM hydrochloric acid and 1 ml of 20 mM $FeCl_3 \cdot 6H_2O$. Briefly, 30 µl of methanolic extract, 70 µl of water was added to 1 ml of freshly prepared FRAP reagent and the mixture was incubated at 37 °C for 15 min. Thereafter, the absorbance was measured at 593 nm against a blank. In blank reaction, methanolic extract was replaced by water. A calibration curve was prepared using different concentrations of ascorbic acid. Results were expressed as micromoles of ascorbic acid equivalents per milligram of extract (µmol of AAEs/mg).

Assay of phenylalanine ammonia-lyase (PAL)

Cell-free extract was prepared according to (Sircar and Mitra 2008) at defined post elicitation time points (0, 12, 24, 48 and 72 h) to determine phenylalanine amonia-lyase (PAL) activity. The soluble protein content was determined according to the Bradford method (Bradford 1976) using bovine serum albumin as the standard. Cinnamic acid, the product of PAL-catalyzed reaction was determined by HPLC using Waters Symmetry™ C_{18} reversed-phase column (3.5 µm, 75×4.6 mm). An isocratic linear solvent system comprising 1 mM TFA in water: methanol (55:45; v/v) with a flow rate of 1 ml/min for 10 min was used to separate cinnamic acid. Cinnamic acid was monitored at 280 nm.

Cytotoxicity assay

Elicited cell culture extract (48 hpe) was used to measure the cytotoxicity against human cervical cancer cell line (HeLa cells) and human breast cancer cell line (MCF-7 cells). Human kidney cells (HEK-293) was used as control non-cancer cell line. Cytotoxicity assay was performed as described before (Mosmann 1983). Briefly, 5×10^3 cells in 100 µl of Dulbecco's Modified Eagle Medium were seeded in 96-well plates. Then the serial dilutions of the cell culture extracts (0, 6.25, 12.5, 25, 50, and 100 µg/ml) dissolved in DMSO were added to the monolayer. In all the treatments, the final DMSO concentration was 1% which was also used as negative control. After incubation for 24 h the cultures were analyzed by MTT assay by the addition of 10 µl of 5 mg/ml MTT and further incubating at 37 °C for 4 h. The MTT-containing medium was then removed and precipitated formazone crystals were dissolved in 100 µl of DMSO The absorbance was measured on a Fluostar optima (BMG Labtech, Germany) microplate reader at 570 nm. The percentage inhibition and IC_{50} values were calculated as essentially described (Nikhil et al. 2014). 5-Fluorouracil (5-FU) was used as positive control.

Acridine orange staining to detect apoptosis

Cell apoptosis was checked by monitoring the plasma-membrane permeability, nuclear morphology and the chromatin condensation of MCF-7 and HeLa cells, through Acridine orange (AO)/Ethidium-bromide (EB) dual staining method as essentially described by (Chakraborty et al. 2010). Briefly, 0.5×10^6 cells were seeded for the assay in a 12-well plate and incubated with concentrations equivalent to the IC_{50} value of elicited extract from Florina cell culture and 5-FU (positive control), respectively, for 24 h and then washed properly with PBS (phosphate buffered saline). Thereafter, 500 µg/ml of AO/EB dye mixture (500 µl) was added in each well and cells were observed under fluorescent microscope. (Zeiss, Axiovert 25, Germany).

Sampling and statistical analyses

All the experiments were conducted with three biological replicates and three technical repeats. The data was subjected to statistical analyses following standard procedures. The data are expressed as mean ± SD.

Results and discussion

Callus induction and establishment of cell suspension culture

In the present study, callus induction was initiated by using various combinations of three growth regulators, 2,4-D, NAA and kinetin. The effect of growth regulators on in vitro callus induction and biomass accumulation is presented in (Table 1). The highest callus induction frequency (96%) and biomass accumulation (13.8 g in 4 weeks) was observed in callus growing on LS medium supplemented with 2 µM 2,4-D, 1 µM NAA and 1 µM kinetin (Table 1). Callus induction and growth were observed in response to all levels of growth regulator treatment but the combination of 2,4-D, NAA and kinetin was found to be the most effective for biomass production. The optimum growth regulator combination selected for maintenance of callus was 2 µM 2,4-D, 1 µM NAA and 1 µM kinetin. Callus developed in this optimum combination showed friable light yellow appearance. Minor callus formation (6%) was also observed in control medium. 2,4-D and NAA at higher and lower doses beyond the optimal concentration significantly reduced the callus formation frequency and resulted in marked reduction in the biomass (Table 1). Growth of cell suspension culture in liquid LS-medium showed best result with 2 µM 2,4-D and 1 µM NAA. Addition of kinetin in cell suspension culture showed cell clumping, thereby kinetin was removed. Although there are a few studies on

Table 1 The influence of different concentrations of growth regulators on callus induction in apple cultivar "Florina" after 4 weeks of culture

Treatment			Callus response	
2,4-D (μM)	NAA (μM)	Kinetin (μM)	Callus induction (%)	Callus fresh weight (g)
0	0	0	6	2.5 ± 0.2
0.5	0	0	16	4.2 ± 0.4
0.5	0.5	0.5	42	9.0 ± 0.4
0.5	1.0	1.0	48	9.2 ± 0.4
1.0	0.5	0.5	64	9.2 ± 0.5
1.0	1.0	1.0	69	9.8 ± 0.6
1.5	0.5	0.5	62	9.6 ± 0.8
1.5	1.0	1.0	74	10.8 ± 0.4
2.0	0.5	0	84	12.0 ± 0.7
2.0	1.0	0.5	88	12.2 ± 0.6
2.0	1.0	1.0	96	13.8 ± 0.5
0.0	1.0	1.0	58	9.1 ± 0.4
0.0	0.5	1.0	34	7.6 ± 0.4
2.5	0.5	0.5	65	9.6 ± 0.4
2.5	1.0	1.0	76	10.1 ± 0.5
2.5	1.0	0	72	9.6 ± 0.6

Data represent mean value ± standard deviation (n = 3)

callus induction in apple (Hrazdina et al. 1997; Kumar et al. 2016), no previous report on detailed characterization of callus induction in terms of frequency and biomass are available. (Hrazdina et al. 1997) reported that callus induction in McIntosh and Liberty cultivar requires 2,4-D, indole-3-butyric acid and kinetin in MS medium. The differential response of different cultivars of the same species towards callus induction may be due to their selective physiological and biochemical response potential towards growth regulators. Callus induction and growth usually depends on the plant genotype, nature of explant, in vitro growth conditions and optimal concentration of growth regulators (Mathur and Shekhawat 2013).

Elicitation of bioactive metabolites

Time course analyses of yeast extract treated cell culture showed significant enhancement in the accumulation of total phenolics and total flavonoids as compared to control cultures (Fig. 1). Total phenolics content reached maximum at 48 hpe (842 μg gallic acid equivalent/DW). Total flavonoid also attained peak at 48 hpe (395 μg quercetin equivalent/DW). After 48 hpe, there was slight decline in the content of both total phenolic and flavonoids. Previously (Iqbal et al. 2013) reported a total phenolic content of 157 mg GAE/g dry weight from the ethanolic extracts of apple leaves; whereas as (Mikulic-Petkovšek et al. 2008) reported much less amount of total phenolic accumulation from apple fruits and leaves.

HPLC analyses of soluble phenolics from elicited cell culture showed enhanced accumulation of phenolic acids and flavonoids. Predominant phenolics in soluble fraction was protocatecuic acid, catechin, chlorogenic acid, vanillic acid, 4-coumaric acid, ferulic acid, benzoic acid and rutin (Fig. 2). Amount of phenolics, increased in response to elicitor-treatment. Time-course analyses revealed that chlorogenic acid, 4-coumaric acid, ferulic acid, benzoic acid and rutin contents were uplifted by elicitor treatment (Fig. 3a). No significant enhancement in the amount of protocatecuic acid, catechin, and vanillic acid was observed upon elicitor treatment. Content of chlorogenic acid (112 μg/g DW), 4-coumaric acid (122 μg/g DW) ferulic acid (212 μg/g DW) and benzoic acid (244 μg/g DW) reached peak at 48 hpe. Rutin content (348 μg/g DW) attained maxima at 72 hpe.

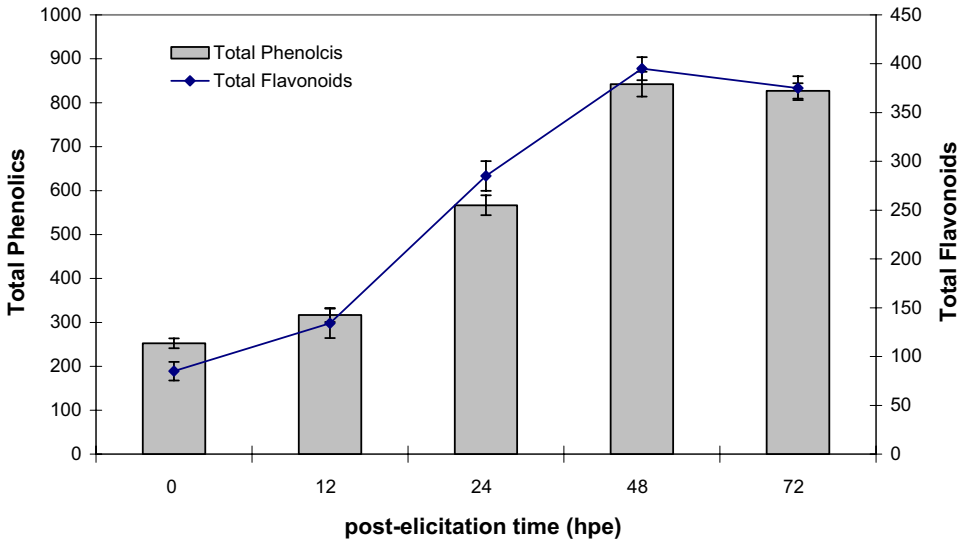

Fig. 1 Time-course analyses of total phenolics and flavonoid accumulation in yeast-extract-treated cell culture of apple cultivar "Florina". Total phenolic was represented as microgram gallic acid equivalent/dry mass; total flavonoid was represented as microgram quercetin equivalent/dry mass. (Mean ± SD, n = 3)

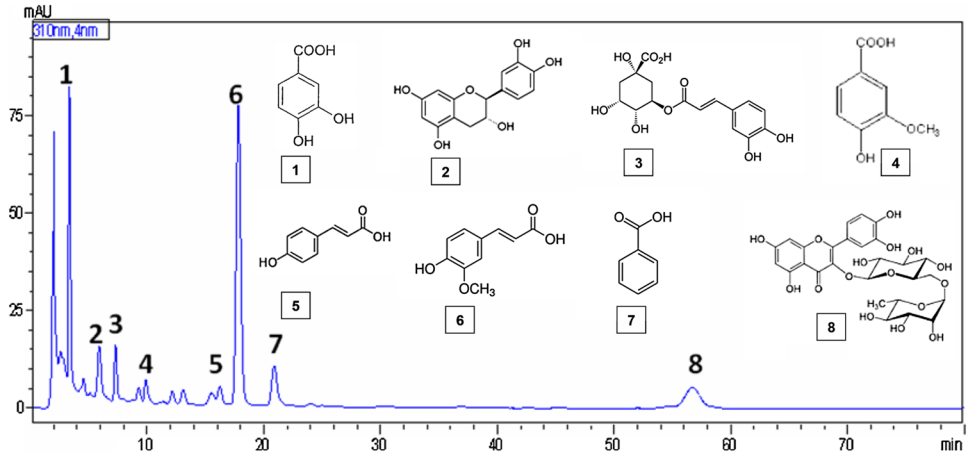

Fig. 2 HPLC-chromatogram showing phenolics metabolite from yeast-extract-treated (48 h post elicitation) cell culture of apple cultivar "Florina". Chromatogram was monitored at 310 nm. Key to peak identity: *1* protocatechuic acid; *2* catechin; *3* chlorogenic acid; *4* vanillic acid; *5* 4-coumaric acid, *6* ferulic acid; *7* benzoic acid; *8* rutin

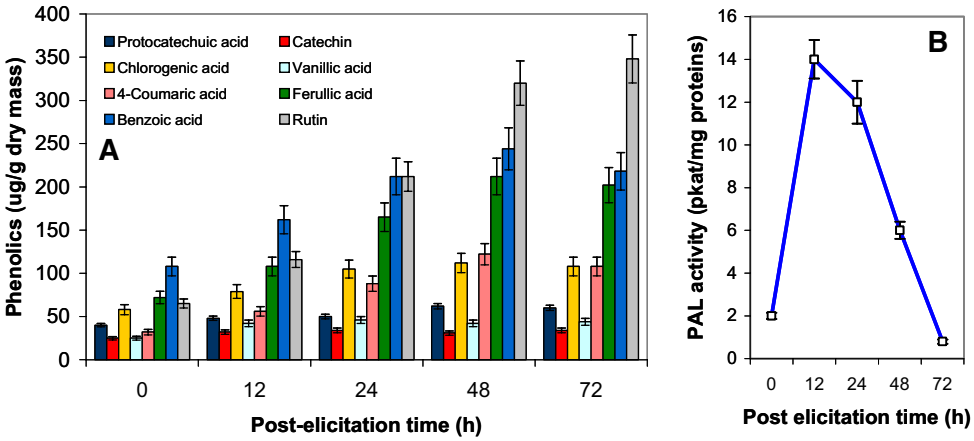

Fig. 3 Yeast-extract induced changes in the accumulation of phenolic metabolites and PAL activity. **a** time-course analyses phenolic metabolites upon yeast extract treatment; **b** time-course analyses of PAL activity. PAL activity was calculated by monitoring the cinnamic acid formation. All results are the mean ± SD, n = 3

The content of protocatecuic acid, catechin and vanillic acid remained nearly unchanged over the time course studied. Non-elicited control cultures showed no change in phenolic acid content over the same time-course studied. Phenolic acids and flavonoids are ubiquitous compounds in plants, which are known to possess several biological properties such as antioxidant, anti-proliferative, anti-cancer activities (Kumar et al. 2016). Phenolic acids, especially benzoic and cinnamic acid derivatives possess various biological activities such as antioxidant, anticancer activities (Sanchez-Maldonado et al. 2011). Phenolic acids also protects against oxidative stress arising from the damaging effect of reactive oxygen species (Khanizadeh et al. 2008). Elicitation of plant cell culture is an excellent strategy for enhanced accumulation of bioactive secondary metabolites in relatively short time (Sarfaraj-Hussain et al. 2012). Among biotic elicitors, yeast extract is well known for triggering phenolic acid biosynthesis (Cai et al. 2014). Presence of chlorogenic acid, catechin, epicatechin, rutin, floridzin, Quercetin-3-rhamnoside, Avicularin was previously reported in the leaves of different apple cultivars (Mikulic-Petkovšek et al. 2010; Liaudanskas et al. 2014). Other studies on the composition and content of phenolic compounds in apple leaves were conducted in relation to scab infections in the apple tree. However, biosynthetic potential of apple cultivar "Florina" has never been tested in terms of bioactive phenolic metabolites. Yeast-extract elicitor used in our study represented effective elicitor for phenolics biosynthesis in the cell culture of "Florina" cultivar of apple. Elicited apple cell culture might be an excellent production platform for high value phenolics such as catechin, rutin, chlorogenic acid etc. The advantages of using yeast extract elicitor, is low price and easy preparation procedure.

Phenylalanine ammonia-lyase (PAL)

The activity of phenylalanine ammonia-lyase (PAL), the first enzyme of phenylpropanoid pathway rapidly increased after yeast-extract treatment. PAL activity attained peak at 12 hpe (14 pkat/mg protein). Thereafter, a slow decrease in PAL activity was noticed up to 48 h (Fig. 3b). Beyond, 48 h, PAL activity decreased rapidly, reaching near to zero at 72 hpe. Enhanced PAL activity precedes phenolic

accumulation suggesting PAL-mediated biosynthesis of phenolics upon elicitor treatment. Being the first enzyme of phenylpropanoid biosynthesis, PAL activity is up-regulated by elicitor treatment, which in turn accelerates phenolics biosynthesis. This is in agreement with the previous finding, where yeast extract elicitor triggered enlistment in PAL activity in *Hypericum calycinum* cell culture directing towards enhanced xanthone biosynthesis (Gaid et al. 2012). The PAL activities remained more or less constant in control treatment (2 pkat/mg protein) over the similar time-course studied. Compared to control treatment, the peak of PAL activity was about sevenfold higher in the elicited cultures (Fig. 4).

Antioxidant activities

Determining the antioxidant activity of the elicited cell culture of apple was important to assess its therapeutic potential. Plant extracts comprises a range of secondary metabolites with antioxidant activity determined by the set of different mechanism of actions, so antioxidant effect cannot be adequately tested using only one method (Prior et al. 2005). For these reasons, we used two different methods (DPPH and FRAP) for the determination of antioxidant potential. Results of the FRAP and DPPH assays from apple callus extracts is presented in (Table 2). Elicited cell culture showed high antioxidant activities as revealed from FRAP and DPPH assays. Highest FRAP activity was observed at 72 hpe (22.14 µg AAE/mg DW). Maximum DPPH activity was observed at 72 hpe (IC_{50} = 45.3 µg/ml). There was a strong positive correlation between total phenolic as well as flavonoid contents and the antioxidant activity assessed by both the methods, which is in agreement with several previous studies (Luximon-Ramma et al. 2002; Liaudanskas et al. 2014). Antioxidant potential of plants has been shown to be correlated to the type and concentration of phenolic acids (Rocha et al. 2012; Khanizadeh et al. 2008; Xu et al. 2016). The difference in the anti-oxidant activities at different post elicitation time points could be explained by the differences in the amount of phenolic acid and flavonoids (Krishnan et al. 2015). Concerning antioxidant potential of apple, so far only one report is available from leaves (Liaudanskas et al. 2014). Our results demonstrated that elicited Florina cell culture is an excellent source of antioxidants.

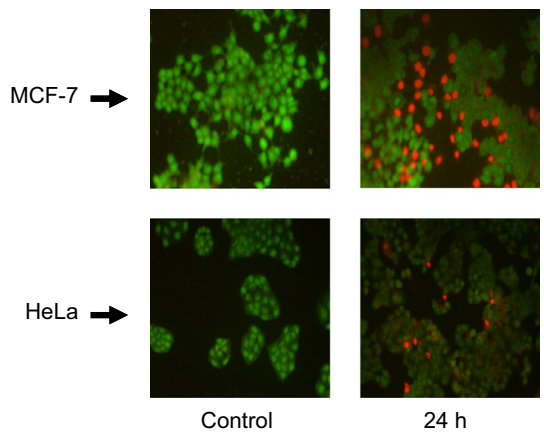

Fig. 4 Apoptosis assay by elicited extracts from apple cell culture on MCF-7 and HeLa cells. The cells were incubated for 24 h with IC_{50} value equivalent of extract and 5-FU (positive control; 55 µM). Nuclear staining was visualized under 200× objectives. Red cells indicate late apoptotic stage

Cytotoxic effect of metabolites from elicited cell culture

The effect of apple callus extract on the growth of human cervical cancer cell line (HeLa cells) and human breast cancer cell line (MCF-7 cells) in vitro is summarized in (Table 3). The results showed that both breast and cervical cancer cell growth was inhibited by elicited extract. The anti-proliferative activity was expressed as IC_{50} values. The lower IC_{50} values represent higher cell growth inhibitory activities. In both the tested cell lines, elicited extract (48 hpe) had almost two-fold lower IC_{50} values as compared to the untreated control

Table 2 Quantitative estimation of antioxidant activities of yeast-extract treated cell culture of apple at various post elicitation time points

Elicited extract (h)	FRAP value (µg AAE/mg extract)	DPPH assay (IC_{50} µg/mL)
0	15.2 ± 1.2	80.6 ± 3.0
12	18.6 ± 1.6	72 ± 4.0
24	20.2 ± 1.9	58.3 ± 4.5
48	19.4 ± 1.3	50.6 ± 3.0
72	22.2 ± 1.8	45.3 ± 4.1

Table 3 IC_{50} Values for the inhibition of cellular proliferation by the cell culture extracts

Cell Line	IC_{50} value (µg/ml)[a]	
	Control extract (0 hpe)	Elicited extract (48 hpe)
HeLa	75.0 ± 5.5[b]	38.0 ± 3.0[b]
MCF-7	62.0 ± 4.8[b]	31.0 ± 3.4[b]
HEK-293	>200	>200

[a]50% Growth inhibition as determined by MTT assay (24 h exposure with cell culture extract)

[b]Experiments were conducted in triplicate, and data expressed as mean value ± SD of three independent experiments

cultures (Table 3). 5-FU was used as positive drug control (MCF-7 $IC_{50} = <0.65$; HeLa $IC_{50} = 5.2 \pm 0.4$). Moreover, when the extract was tested in human non-cancer cell lines HEK-293, it was found to have IC_{50} values above 200 µg/ml concentrations, indicative of poor response as anti-proliferative functions. The enhanced accumulation of phenolics, especially rutin, could be a probable reason for higher cytotoxicity by elicited cells. Further, our results clearly demonstrated that the phenolic metabolites from elicited cell culture of apple cultivar "Florina" had wide in vitro cytotoxicity, i.e., active against both cervical and breast cancer cell lines. Previously, anticancer activities from dried fruit extracts of Red delicious, Fuji, Golden delicious, and Granny smith apple cultivars against MDA-MB-468 human breast cancer cell line was reported (Thompson et al. 2009). This differential activity could be attributed to the activation of cell specific targets by different bioactive metabolites present in the extracts as tested (Sak 2014; Tiwary et al. 2015). When MCF-7 and HeLa cells were treated with elicited extract at a concentration equivalent to IC50 values, clear apoptosis was observed after 24 h of incubation. AO/EB dual staining served the purpose of measuring apoptosis. Since AO permeated all cells, the nuclei appeared green in vehicle treated MCF-7 and HeLa cells (Fig. 4 control cells). EB is taken up by the cells only when the cytoplasmic membrane integrity is lost and served as the marker for late apoptosis (Fig. 4). Further research is needed to delineate the specific metabolites or metabolite combinations (synergistic effect) that are responsible for the cytotoxic effects as shown by elicited extract and analyzing the underlying mechanism of actions.

In conclusion, this study describes for the first time the antioxidant and cytotoxic activity of the bioactive phenolic metabolites isolated from the elicited cell culture of apple. We believe that this elicitation approach offers a starting point to improve the production of natural antioxidants and bioactive secondary metabolites for industrial purposes in future. However, which particular metabolite or metabolite combinations is actually responsible for cytotoxic and antioxidant activities and their underlying detail mechanisms of action need to be investigated further.

Acknowledgements This work was supported by a research grant (SB/YS/LS-192/2013 to D. Sircar) from the Science & Engineering Research Board (SERB), India. "Amol Sarkate" acknowledges the IIT Roorkee for the award of MHRD-research assistantship for perusing doctoral research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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