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Addition of L-Tyrosine to Improve Betalain Production in Red Pitaya Callus

Fadzliana, N. A. F.[1], Rogayah, S.[2], Shaharuddin, N. A.[3,4] and Janna, O. A.[1]*

[1]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Malaysian Agricultural Research and Development Institute (MARDI), 50774 Kuala Lumpur, Malaysia
[3]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4] Institute of Crop Plantations Study, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

ABSTRACT

The aim of this study is to enhance betalain production in red pitaya (*Hylocereus polyrhizus*) callus through supplementation of the precursor L-tyrosine. Red-pigmented calli were produced on Murashige and Skoog (MS) basal salt medium supplemented with 2 mg/L naphthaleneacetic acid (NAA), 4 mg/L thidiazuron (TDZ) and different concentrations of L-tyrosine (20, 40, 60, 80, 100 mg/L). The addition of 20 mg/L L-tyrosine was able to increase betacyanin production by 1.5-fold compared to calli grown on Murashige and Skoog (MS) basal salt medium (MSO). The results also revealed that betalain production (calli cultured with 20 mg/L L-tyrosine) was higher than MS medium with 2 mg/L NAA and 4 mg/L TDZ (without L-tyrosine) up to 1.7-fold. Four compounds, namely betacyanin, betaxanthin and two additional compounds, phenolic and flavonoid compounds, were detected in the red-pigmented calli treated with 20 mg/L L-tyrosine that were not found in the fresh fruit sample. This indicates that the callus system has potential to produce betalain pigment and that red-pigmented calli could be an important antioxidant source.

Keywords: *Hylocereus polyrhizus*, betalains, precursor, L-tyrosine, pitaya callus, antioxidant

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E-mail addresses:
Fadzlian8Faizal@gmail.com (Fadzliana, N. A. F.),
lynn@mardi.gov.my (Rogayah, S.),
noorazmi@upm.edu.my (Shaharuddin, N. A.),
janna@upm.edu.my (Janna, O. A.)
* Corresponding author

INTRODUCTION

Colour additives refer to any dyes, pigments or substances that can impart colour to foods, cosmetics, medicines and other products. They can be classed into natural, synthetic, inorganic and natural-identical depending on their origin.

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Advancements in food technologies have made it feasible to chemically synthesise many synthetic colourants to impart colours to food, making it more appetising and attractive, and most importantly, not easily degraded. However, some synthetic colourants such as tartrazine, carmoisine, brilliant blue and erythrosine are harmful to humans due to their carcinogenic and toxic effects. Based on research carried out by El-Wahab and El-Deen Moram (2012), rats fed with synthetic colourants resulted in liver cells destruction as well as acute kidney dysfunction. They concluded that the administration of synthetic colours resulted in various alterations of the antioxidant system and the use of them in food needs to be limited. Recent studies also showed that tartrazine and carmoisine altered bioelements, especially iron and zinc, in the liver, kidney and brain of rats (Cemek et al., 2014). Consequently, there is growing interest in natural colourants such as plant pigments, namely anthocyanin, chlorophyll, carotenoid and betalain as alternatives to synthetic colourant substitutes. Betalain has gained interest among food scientists as it was found to have a broad pH range, from 3 to 7, a broad range of colours and health-benefit properties (Georgiev et al., 2010; Gandía-Herrero, Escribano, & García-Carmona, 2014).

Betalain is a class of water-soluble nitrogen-containing compounds that can be found in all families belonging to the order of Caryophyllales except the families of Molluginaceae and Caryophyllaceae (Brockington, Walker, Glover, Soltis, & Soltis, 2011). There are two main types of betalain, namely red-violet betacyanin and yellow-orange betaxanthin. Betalain is a secondary metabolite derived from L-tyrosine (Gandía-Herrero & García-Carmona, 2013), one of the amino acids synthesised in the Shikimate pathway. L-tyrosines will undergo hydroxylation and form an intermediate compound called L-dihydroxyphenylalanine (L-DOPA). From L-DOPA, betalamic acid and *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) will be formed. Betacyanin is formed through the condensation of betalamic acid with *cyclo*-DOPA (Sakuta, 2013). Betalamic acid also condenses with amines or amino acids to give betaxanthin (Tanaka, Sasaki, & Ohmiya, 2008). Betacyanin and betaxanthis can be further divided into subclasses depending on the substitution pattern on the betalamic acid.

Beetroot has been the frequently used model of an important betalain source. However, beetroot contains a high nitrate concentration and an unfavourable earthy smell (Lu, Edwards, Fellman, Mattinson, & Navazio, 2003), which led to the search for new betalain sources. Red pitaya is the new focus as an alternative betalain source (Aberoumand, 2011). Red pitaya (*Hylocereus polyhizus*), also known as red dragon fruit, is a species in the family of Cacteceae. Dragon fruit has a thin scaly red peel and red-violet flesh that is interspersed with small digestible black seeds (Lim, 2012). Applications of betalain extracted from red pitaya in food have been

extensively studied. Yogurt added with red pitaya fruit enhanced the rate of milk fermentation, phenolic content, antioxidant activity and produced a high content of lactic acid compared to plain yogurt (Zainoldin & Baba, 2009). In milk, addition of betacyanin from red pitaya improved the colour acceptability and produced better thermal tolerance compared to that produced by red beetroot colourant, called E-162 (Gengatharan, Dykes, & Choo, 2016).

Natural colourants are limited to type and/or part of the plant; the production may be affected by seasonal changes and be susceptible to infection. In order to meet consumer needs and expectations, production of secondary metabolites, particularly natural colourants *in vitro* has been extensively studied. Plant tissue culture is an alternative tool that offers several advantages over field cultivation such as continuous production of desired metabolites in controlled environments. There are many reports on *in vitro* betalain production; however, betalain has been reported to be easily affected by abiotic stresses such as light, temperature and pH (Herbach, Stintzing, & Carle, 2006). Therefore, many strategies have been developed to improve yield such as by precursor feeding and supplementation of biosynthetic precursors in culture medium to increase the production of desired metabolites (Mulabagal & Tsay, 2004). The idea of supplying precursors or intermediate compounds of secondary metabolite routes has been effective in several studies. For example, the addition of L-phenylalanine was able to increase the production of tocopherol and carotene levels in *Rosa damascene* Mill. petal callus culture (Olgunsoy, Ulusoy, & Celikkol-Akcay, 2017). In another study by Mobin, Wu, Tewari and Paek (2015), feeding of L-tryptophan enhanced growth and accumulation of phenolic compounds, flavonoids and caffeic acid in adventitious root cultures of *Echinacea purpurea*. Similarly, phenolic acid concentration in shoot cultures of *Exacum affine* was increased when L-phenylalanine was added (Skrzypczak-Pietraszek, Słota, & Pietraszek, 2014). This study was carried out to enhance betalain production on established red pitaya callus through supplementation of L-tyrosine as a precursor.

MATERIALS AND METHODS

Sample Preparation

Red pitaya (*Hylocereus polyrhizus*) was used throughout this study. The fruit was purchased from a local farm located in Sepang Selangor, Malaysia (Longitude-101.74197; Latitude-2.68803). The fruit was washed in running tap water and detergent (GLO, Malaysia) to remove dirt from the peel and surface and sterilised using 70% (v/v) ethanol for 1 min in a laminar air flow hood. The fruit was halved and the flesh was excised for use as explants. The flesh was cut approximately into 1 cm^3 pieces and all the seeds were removed using forceps prior to culturing.

Callus and Pigment Production

The callus was induced based on the method described by Rogayah et al. (2014). Callus induction was carried out on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with 2 mg/L naphthaleneacetic acid (NAA), 4 mg/L thidiazuron (TDZ), 3% (w/v) sucrose and 0.3% (w/v) phytagel. The results showed that phytohormones at 2 mg/L NAA and 4 mg/L TDZ were found to produce a high amount of calli (bigger in size). This study also used 2 mg/L NAA and 4 mg/L TDZ supplementation to the MS basal medium with the addition of five concentrations of L-tyrosine (20, 40, 60, 80 and 100 mg/L). L-tyrosine was added separately to each sample to examine its effects on callus and pigment (betalain) productions. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All the experiments were performed with five explants in each replicate. The cultures were kept in a dark condition at 25 ± 2ºC.

Analysis of Pigment Production

After one month, the calli produced were harvested for pigment content analysis. The betalain content was extracted by suspending the calli in 10 mL distilled water and filtered (cellulose acetate membrane, 0.45 µM pore size). Betalain content was quantified using a spectrophotometer (Biotek µQuant Microplate Spectrophotometer, Biotek Instrument Inc. Vermont, USA). Two pigments, betacyanin, indicated by red-purple pigmentation, and betaxanthin, indicated by yellow-orange pigmentation, were detected at the wavelength absorbance of 540 nm and 480 nm, respectively. The betalain content was then calculated from the absorbance reading obtained. The betalain content (BC) was calculated as BC (mg/L)=[(A x DF x MW x 1000) / (ε x 1)], where A was absorption, DF was the dilution factor and 1, the width of the spectrophotometer cell (1 cm). For betacyanin, the extinction coefficient (ε) was 60 000 L/(mol cm) and the molecular weight (MW) was 550 g/mol. For betaxanthin, the extinction coefficient (ε) was 48 000 L/(mol cm) and the molecular weight (MW) was 308 g/mol (Stintzing, Schieber, & Carle, 2003; Ravichandran et al., 2013). Betalain was extracted from calli cultured in an MS medium without plant growth regulators (PGRs) and a precursor (MSO), and calli culture in MS medium with 2 mg/L NAA and 4 mg/L TDZ was used as the main control in this study. All analyses were performed in five replicates with the data analysed by Duncan's test using SAS (SAS Institute Inc., Cary, NC) at $p<0.05$ defined as a significant difference. The data were reported as means ± standard deviation.

The betalain content of the calli was then further analysed using high-performance liquid chromatography (Biswas, Das, & Dey, 2013). Calli from each petri dish (10-15 plates) were ground using a mortar and pestle and freeze-dried with liquid nitrogen. The extraction method was adapted from Poh-Hwa, Yoke-Kqueen, Indu Bala and Son (2011), with

slight modification. HPLC Agilent 1200 Series (Agilent Technologies Inc., CA, US) was used for phytochemical profiling. Separation of samples was obtained at room temperature on reverse phase Thermo Hypersil C18 gold (150 x 4.1 mm ID) with guard column, and 20 µL of sample was injected into the column (flow rate at 0.75 ml/min). The mobile phase consisted of 0.5% formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B). The following gradient procedure was used: 0-40 min: 5% to 95% B; 40-45 min: 95% B; and 45-50 min: 5% to 95% B. Monitoring was performed at 280 nm and 360 nm for phenolic compounds and flavonoids, respectively (Francisco & Resurreccion, 2009) at 480 nm for betaxanthin and at 540 nm for betacyanin.

RESULTS AND DISCUSSION

A previous study (unpublished data) was conducted to observe the effects of different combinations and concentrations of plant growth regulators (PGRs) used for callus induction in *H. polyrhizus*. It was found that the best treatment for callus induction was the combination of 2 mg/L naphthaleneacetic acid (NAA) and 4 mg/L thidiazuron (TDZ). Morphological appearance revealed the pigmented calli produced were red with a compact texture and bigger diameter in size (2.5 ± 0.02 cm) when compared to other PGR combinations and concentrations. Absorbance readings showed that a higher content of betalain, about 1.5-fold, was obtained compared to calli cultured on MS basal salt medium (control). Therefore, this study also used 2 mg/L NAA and 4 mg/L TDZ with addition of L-tyrosine, as a precursor, to increase betalain production in the red-pigmented calli.

Two main controls were used in this study to investigate the effects of plant growth regulators (PGRs) and precursors on callus production and betalain production. The first was calli cultured in MS medium without PGRs and a precursor (MSO) and the second control was calli cultured in MS medium with PGRs (2 mg/L NAA and 4 mg/L TDZ), without L-tyrosine. Betalain extracted from fresh fruit was used to compare with betalain extracted from the calli to study the potential of callus culture as a tool for betalain production. The explant, flesh of red pitaya, was cut into approximately 1 cm^3 pieces and cultured in the desired medium (five explants per plate) as shown in Figure 1(i). The results showed that L-tyrosine at 20, 40, 60, 80 and 100 mg/L produced calli of different colour intensities and texture. In general, all the calli produced were reddish and compact as depicted in Figure 1(ii). Calli cultured in MSO were dull and watery. The red colour of calli denoted the presence of betalain, particularly betacyanin. The development of calli could be seen after five to eight days of incubation. The colour intensity of the red-pigmented calli treated with 20 mg/L L-tyrosine was higher compared to other concentrations, where the explant was completely covered with red calli. The size of the callus clumps were also bigger (2.58 ± 0.06 cm) compared to calli grown with PGRs alone.

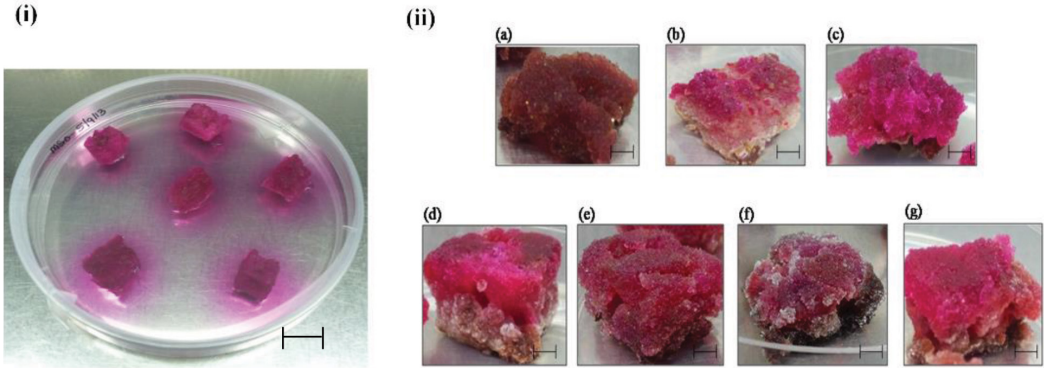

Figure 1. Red-pigmented calli from red pitaya. Five explants of seedless cubed pitaya were cultured per plate (i). After one month of incubation, different morphology appearances of calli were observed (ii), (a) callus induced in MS basal medium without PGRs and precursor (control) was watery (soft) and a dull red. The calli induced in 2 mg/L NAA and 4 mg/L TDZ with (b) 20 mg/L, (c) 40 mg/L, (d) 60 mg/L, (e) 80 mg/L and (f) 100 mg/L L-tyrosine produced different intensities of the colour red and had a friable texture.

Scale: ⊢⊣ indicate 1 cm

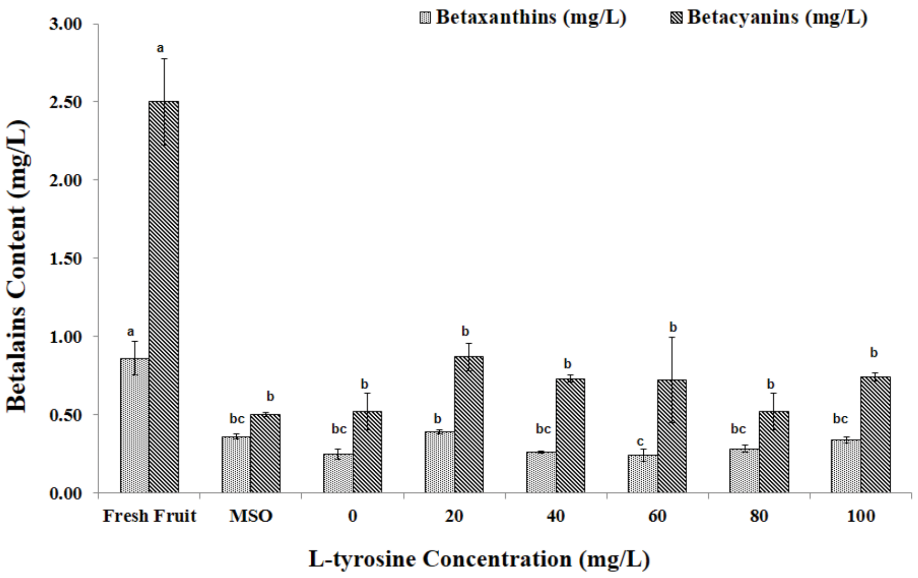

Figure 2. Effects of L-tyrosine concentration on betalain content in red-pigmented callus. Calli were induced in media containing 2 mg/L NAA, 4 mg/L TDZ and five different concentrations of L-tyrosine. Values shown are means of five replicates and error bars indicate standard deviations. Means with the same letter indicate no significant difference (Duncan's Test) at $p<0.05$

Figure 2 summarises the betalain content analysed in each respective red-pigmented callus grown in MS media supplemented with different L-tyrosine concentrations. High betalain content was observed in fresh fruit sample for both betaxanthin and betacyanin content, produced at 0.86 mg/L and 2.50 mg/L, respectively. *H. polyrhizus* is known as being rich in betacyanin content compared to betaxanthin (Wybraniec & Mizrahi, 2002). Betacyanin content in each sample was also higher than betaxanthin content, as predicted from looking at the colour of the calli. Betacyanin in red-pigmented calli showed no significant difference (p>0.05). Total betalain produced in calli cultured with addition of L-tyrosine was higher than in calli cultured in MSO and calli cultured with only PGRs. The highest betalain production was observed in calli cultured in MS medium supplemented with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine, with 1.5-fold increment compared to MSO and 1.7-fold higher than calli cultured with only PGRs. Even though the amount produced in calli showed increment, the betalain content in fresh fruit was 2.7-fold higher. The size of the callus was observed to influence the betalain content as the biggest callus produced the highest betalain content.

The identification of compounds using reverse-phase high performance liquid chromatography (RP-HPLC) was based on absorbance of wavelength, where green represented betacyanin (540 nm), purple represented betaxanthin (480 nm), red represented flavonoid compounds (280 nm) and blue represented phenolic compounds, as shown in Figure 3. The HPLC analysis of betalain content in fresh fruit as shown in Figure 3(a) revealed the presence of two major compounds, betaxanthin (purple) and betacyanin (green), with the highest peaks being detected simultaneously at a retention time (Rt) of 11.83 min. Several peaks of betacyanin were also visible at different Rt, indicating detection of different compounds of betacyanin and betaxanthin. Betacyanin and betaxanthin were also detected in calli grown in MSO (Figure 3(b)) at Rt 8.67 min and 12.07 min. Similarly, HPLC analysis of calli grown in MS medium with 2 mg/L NAA and 4 mg/L TDZ (Figure 3(c)) and calli grown in MS medium with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine (Figure 3(d)) showed the presence of betacyanin and betaxanthin at Rt 8.79 min and 12.04 min. The addition of 2 mg/L NAA and 4 mg/L TDZ did not produce any increment in betalain production compared to calli grown in MSO. However, when 20 mg/L L-tyrosine was added, absorbance of betacyanin in the calli increased by threefold and 1.42-fold compared to calli grown with PGRs only and MSO, respectively. The first *H. polyrhizus* pigments reported consisted of three main components i.e. betanin, hylocerenin and phyllocactin (Wybraniec et al., 2001). Thereafter, eight betacyanins were identified using positive ion electrospray mass spectrometry in *H. polyrhizus* (Stintzing, Schieber, & Carle, 2002). Naderi, Ghazali, Hussin,

Amid and Manap (2012) also reported that phyllocactin and betanin were the prominent betacyanins in the fruit pulp of *H. polyrhizus*.

Two additional compounds, identified as flavonoids (red) and phenolic compounds (blue) were detected in HPLC analysis of calli, which were not found in the fresh fruit sample. The addition of 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine produced several peaks of flavonoids and phenolic acids. Flavonoids and phenolic compounds are known as bioactive compounds, synthesised from the Shikimate pathway, a similar pathway used to synthesise tyrosine, phenylalanine and tryptophan. Phenolic compounds form a diverse group and are characterised by hydroxylated aromatic rings (Mandal, Chakraborty, & Dey, 2010) that include the widely distributed hydroxybenzoic and hydroxycinnamic acids. Flavonoids are synthesised by the phenylpropanoid pathway and the start-up component is phenylalanine. Flavonoids have been classified into six subgroups, namely flavones, flavols, flavanones, flavan-3-ols, isoflavones and anthocyanidin compounds (Ghasemzadeh & Ghasemzadeh, 2011). Phenolic compounds and flavonoids extracted from the treated red-pigmented calli may serve as an important source of antioxidants (Balasundram, Sundram, & Samman, 2006). Phenolic compounds and flavonoids have been extensively exploited for their multiple biological activities such as anti-scavenging, antioxidant, anti-inflammatory and anti-microbial effects (Cushnie & Lamb, 2011; González et al., 2011; Agati, Azzarello, Pollastri, & Tattini, 2012). The presence of phenolic compounds and flavonoids in the calli may be due to enzymatic reactions by the polyphenol oxidase (PPO) and as a response to stress conditions (Winkel-Shirley, 2002; Michalak, 2006). However, some of these compounds can contribute to the development of browning in plant callus cultures (Dong et al., 2016).

L-tyrosine was used in this study because it plays an important role as a precursor for betalain biosynthesis (Gandía-Herrero & García-Carmona, 2013). We postulated that addition of exogenous L-tyrosine in the callus induction medium might induce production of more betalain in the existing red-pigmented calli. A study on application of tyrosine as precursor was conducted by Kleinowski et al. (2014) to investigate its effect in pigment production and growth of Alternanthera plants. The application of 50 µM tyrosine increased betacyanin production to 36.5 mg/100g in the plant shoots. However, the mode of action of L-tyrosine in plant tissue culture is currently not well understood. The lower betalain content in the calli may be due to several factors such as exposure to light, unfavourable temperature or pH, all of which may cause betalain degradation during culturing and incubation (Woo, Ngou, Ngo, Soong, & Tang, 2011). Another possibility is that the precursor itself may not be effective for inducing betalain or the concentration used might not be optimal for betalain induction. Besides

that, the callus culture is only a partial system involving undifferentiated cells for production of secondary metabolites. Secondary metabolites are thought to be produced following long biosynthetic pathways that involve dozens of enzymes (Bourgaud, Gravot, Milesi, & Gontier, 2001). L-tyrosine may not be involved in the activation of specific genes for certain enzymes or it does not interact with membrane receptors that generate signalling compounds.

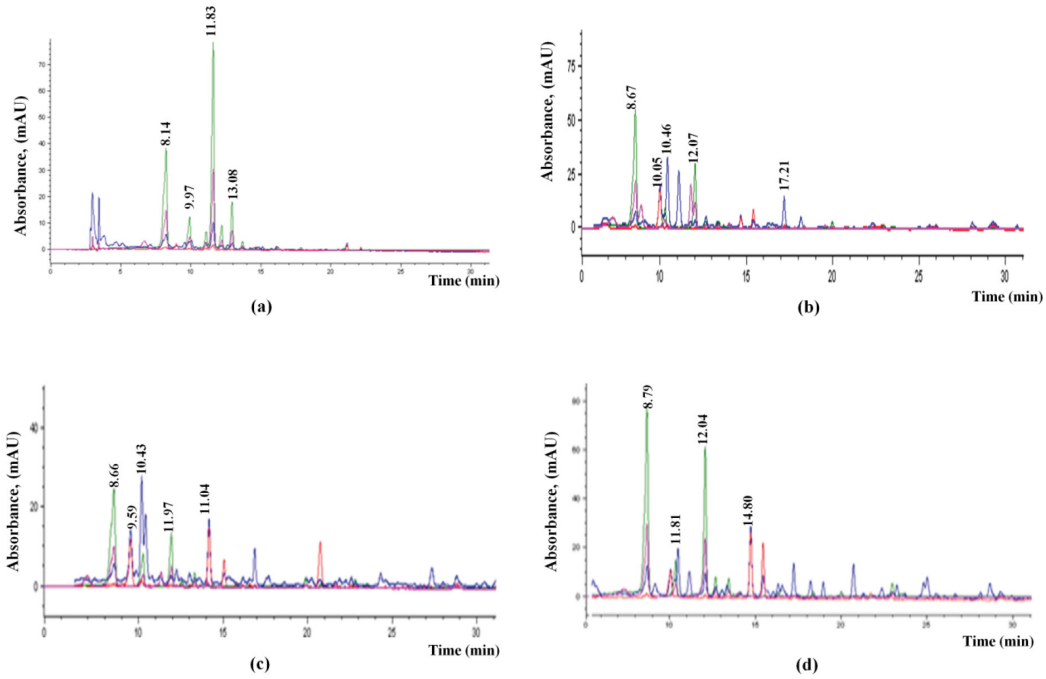

Figure 3. HPLC chromatographs. Fresh fruit sample (a) showed the presence of betacyanin and betaxanthin only, while in calli grown in MSO (b), MS medium with 2 mg/L NAA and 4 mg/L TDZ (c) and MS medium with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine, two additional compounds were detected, phenolic compounds and flavonoids.
*Green represents betacyanins; purple represents betaxanthins; red represents flavonoids; blue represents phenolic compounds

CONCLUSION

Among the different concentrations of L-tyrosine tested as aprecursor, the highest intensity of pigmented calli produced was observed in MS medium containing 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine. Reverse-phase HPLC analysis of extracts obtained from the red-pigmented calli revealed the presence of four different compounds i.e. betacyanin, betaxanthin and phenolic and flavonoid compounds. Further analysis of enhancement of betalain production using other elicitors should be tested as this group of pigments has

beneficial values for the food industry. The callus system established is expected to be useful as a potential *in vitro* system for the production of secondary metabolites.

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