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Silymarin Accumulation in *Silybum marianum* Suspension Culture via Precursor Feeding

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ABSTRACT

Silymarin and phenolic compounds have great pharmaceutical applications, particularly as hepatoprotective agents. Silymarin content in *Silybum marianum* wild populations is low with significant differences between the individuals and the areas of these populations decreased due to the increasing interest in the plant. Therefore, cell suspension culture technique is an alternative and sustainable procedure for the bioproduction and conservation of the plant. The aim of the present study was the production of silymarin and certain phenolic compounds from *S. marianum* cell suspension cultures by the supplementation of the precursor amino acid; L-phenylalanine. Seeds were collected from wild plants at Alexandria desert road and germinated *in vitro*. Seedlings root segments were cultured on Murashige and Skoog basal medium supplemented with 4.55 µM 2,4-dichlorophenoxy acetic acid and 4.44 µM 6-benzyladenine for the induction and proliferation of callus. Cell suspension cultures were established in the same medium supplemented with L-phenylalanine at different concentrations (302.68, 605.36 and 908.05 µM). L-Phenylalanine elevated the cells biomass and the accumulation of silymarin and some phenolic compounds. The significantly highest silymarin accumulation reached 66.33% increase compared to the control. This study highlights the potential of L-phenylalanine for the *in vitro* production of silymarin-rich extracts and protecting the environment by preventing the overcollection of the plant.

Keywords: Flavonolignans; *in vitro* cell culture; Milk thistle; Phenolic compounds; Phenylalanine.

INTRODUCTION

Silybum marianum (L.) Gaertn. is commonly known as milk thistle and is one of the most essential medicinal plants from the family Asteraceae. The plant is native to the Mediterranean region. In Egypt, it occurs naturally in Oasis of Libyan desert, Western Mediterranean coastal region, the Ithemic desert; El-Tih and North of Wadi Tulimat, Nile region, along canal banks, roadsides and waste lands (Boulos, 2002). *S. marianum* has a strong hepatoprotective activity (Abenavoli *et al.*, 2018), it contains active constituents with significant antioxidant and anti-inflammatory potentials, having important pharmaceutical and cosmetic applications (Shah *et al.*, 2019).

The main active constituents in *S. marianum* are flavonolignans and phenolic compounds (Bhattacharya, 2020). Flavonolignans are natural phenolics that include a flavonoid and a lignan. Silymarin is the prominent bioactive compound in the plant and is a mixture of flavonolignans of which the most abundant components are silybin A and B, isosilybin A and B, silychristin and silydianin together with the flavonoid taxifolin (Csupor *et al.*, 2016; Shah *et al.*, 2019). Silymarin is a commonly used plant-derived hepato-protective agent (Vargas-Mendoza *et al.*, 2014). It is optimum for the treatment of liver and gallbladder diseases such as cirrhosis, jaundice and hepatitis (Colica *et al.*, 2017). It protects liver and kidney tissues against toxins including the toxic effect of drugs such as chemo– and radiotherapy (Csupor *et al.*, 2016). Due to the high free radical scavenging activity of silymarin, it has antioxidant activities and neutralizes the effect of oxidative damage and protects the tissues (Soto *et al.*, 2010). Among the other most desirable medicinal properties of silymarin are its anti-cancer, anti-inflammatory, anti-arthritic and anti-viral activities (Bijak, 2017; Bijak *et al.*, 2017). The phenolic compounds produced by the plant are natural protective antioxidant agents (Abbasi *et al.*, 2010; Hano and Tungmunnithum, 2020), hence antioxidant activity is strongly associated to the polyphenolic content of a plant (Fidranny *et al.*, 2015).

The increasing demand for the active constituents from *S. marianum* severely affecting the availability of the plant populations (Ahmad *et al.*, 2008). Moreover, successful trials for its domestication and breeding are scarce (Ram *et al.*, 2005). Seed germination is poor and seedling growth rate is low, depending on numerous environmental and biological factors (Abbasi *et al.*, 2010). In general, conventional cultivation of *S. marianum* is vulnerable for some reasons, especially because of the spiny leaves and flowers, which makes the handling of the plants very difficult and causes the damage of the harvesting machines leading to the reduction of yield. Also, field-grown plants are affected by environmental, seasonal and genetic variations as well as pollutants and herbicides that negatively affect the medicinal value of the plants (Shah *et al.*, 2021).

Due to the increasing interests in *S. marianum*, alternative and sustainable bioproduction procedures other than conventional field cultivation of the plant are required. Tissue and cell culture techniques could be extremely useful for solving these complications. The *in vitro* production of plant secondary metabolites is achieved under controlled conditions and could be modified for the optimum growth and production even outside the growth season of the plant; therefore, it is

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not affected by climatic changes or soil conditions and would be free of infections by microorganisms or insects (Geng *et al.,* 2001). Cell suspension culture offers advantages over chemical synthesis of bioactive compounds because they are difficult to be synthesized with chemical procedures and have harmful side effects on the human health rather than natural biproducts (Zhao and Verpoorte, 2007).

Precursor feeding strategy stimulates bioactive compounds accumulation effectively in callus and cell cultures of many plants; including *S. marianum* (Tůmová *et al.,* 2006; Rahimi *et al.,* 2011; Firouzi *et al.,* 2013; Elhaak *et al.,* 2016). Flavonoids biosynthetically are derived from phenylalanine and malonyl-CoA through the phenylpropanoid pathway (Matkowski, 2008). Elhaak *et al.* (2016) studied the effect of phenylalanine on the accumulation of silymarin in callus tissue of *S. marianum* and obtained the maximum accumulation of 31.4%, compared to control, with 25 µM phenylalanine. Also, Rahimi *et al*. (2011) found that *S. marianum* hairy root culture supplemented with phenylalanine at 100 µM was optimal to produce silymarin (1.84-fold higher than that of the control) and 10 µM increased the root biomass (0.43 g) 1.89-fold compared to the control. Firouzi *et al.* (2013) found that silymarin was 8.6 times higher than control in cell suspension culture from hypocotyl in medium enriched with phenylalanine, methyl jasmonate and yeast extract together. However, Tůmová *et al. (*2006) used the precursor coniferyl alcohol for feeding *S. marianum* cell suspension cultures and it enhanced the content of some components of silymarin complex *in vitro*.

Silymarin occurs in the entire *S. marianum* plant, but it is concentrated in the fruit and seed (Valková *et al.,* 2020). Fruit extract contains 70–80% silymarin representing 1.5-3% of the dry weight (Bijak, 2017; Valková *et al.,* 2020). In Egypt, AbouZid *et al.* (2016) reported that silymarin content in *S. marianum* fruits of wild populations is low and varies between 0.06-2.19% with significant differences in the yield between populations. Therefore, the present study aimed to stimulate the increase of silymarin and phenolic compounds biosynthesis in the Egyptian *S. marianum* cell suspension culture, using the precursor amino acid phenylalanine, for the mass production of the bioactive compounds and consequently conserving the plant from overexploitation.

MATERIALS AND METHODS

The present study was conducted in Tissue Culture Laboratories, Desert Research Center, El-Matareya, Cairo, Egypt.

Plant material

Seeds of *S. marianum* were collected from wild plants grown naturally in Alexandria desert road. Plant specimens were identified by Dr. Omran Ghaly, Head of Plant Taxonomy Unit, Desert Research Center, Egypt. Voucher Herbarium specimen was given the number CAIH-1029-R and deposited in the Herbarium of Desert Research Center (CAIH).

Seed surface sterilization and germination

Seeds were washed under running tap water with liquid detergent (Pril) and Dettol (4.8% w/v chloroxylenol) for 30 min, then surface sterilized in 80% (v/v) commercial bleach solution (Clorox containing 5.25% sodium hypochlorite) for 15 min and finally rinsed five times with sterile distilled water. Seeds were soaked in 500 mg l^{-1} gibberellic acid ($GA_{3;}$ filter sterilized) for 24 h, then cultured on ready-made Murashige and Skoog (MS; Murashing and Skoog, 1962) basal medium (Duchefa, Haarlem, the Netherlands) supplemented with 3% (w/v) sucrose and 0.01% (w/v) myo-inositol (Fluka AG, Switzerland) for *in vitro* germination. The pH of the medium was adjusted to 5.7–5.8 and solidified with 0.275% (w/v) phytagel (Duchefa), before autoclaving at a pressure of 1.06 kg cm^{-2} and 121°C for 20 min. Seeds were incubated in the dark for two weeks, and then transferred to a 16-h photoperiod under white fluorescent light (2500–3000 lux) at 24±2°C. After 30 days of incubation, the seeds were germinated, and root segments from seedlings were used as explants for callus induction.

Induction and proliferation of callus cultures

Root segments (1 cm) were cultured on MS basal medium supplemented with 3% (w/v) sucrose, 0.01% (w/v) myo-inositol, 4.55 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 4.44 µM 6-benzyladenine (BA) for the induction of callus, as reported by Hassanen and Khalil (2013) and Elsawy (2017). All plant growth regulators used in the present study was purchased from Sigma (minimum 90%, St. Louis, USA). The pH of the medium was adjusted to 5.7–5.8 and solidified with 0.275% (w/v) phytagel, before autoclaving. The obtained callus was proliferated by subculturing every 21 days for five times on a fresh medium of the same composition to provide a sufficient source for establishing the cell suspension culture. Callus cultures were incubated under white fluorescent light (2500–3000 lux) at a 16-h photoperiod and 24±2°C.

Establishment of cell suspension cultures

Preparation of the cell suspension

For the preparation of inoculum, homogeneous and friable root-derived callus (1.5 g FW) were cultured in 250 ml Erlenmeyer flasks containing 50 ml of liquid MS basal medium supplemented with 3% (w/v) sucrose, 0.01% (w/v) myo-inositol, 4.55 µM 2,4-D and 4.44 µM BA. The pH of the medium was adjusted before autoclaving as mentioned previously. The cell suspension culture was incubated on a rotary shaking incubator (DAIHAN Scientific, Korea) at a constant agitation of 120 rpm and 24±2°C. After seven days of incubation, the cells were filtered using stainless steel sieves and the filtrate containing cells were used to establish the cell suspension culture experiment.

Characterization of cell suspension culture

The fresh weight (FW) and the percentage of viable cells of suspended cell cultures were recorded at four-day intervals during 36 days of incubation in the described medium and conditions. Fresh weight, as

gram per 50 ml, was determined by weighing the freshly harvested cells after filtration from the medium. Cell number was monitored using the traditional cell counting method by a hemocytometer (Stoddart, 2011). The hemocytometer and its coverslip were prepared by washing with 70% (v/v) ethanol and allowed to dry. Trypan blue dye was prepared at a concentration of 4% in 0.81% sodium chloride and 0.06% dibasic potassium phosphate. A mixture of 1:1 (v/v) of the suspended cells and a 0.4% trypan blue solution was prepared. The mixture was gently mixed and let to stand for 5 min at room temperature, then was loaded into the counting chamber of the hemocytometer. The total number of cells, both viable (unstained) and nonviable (stained) were recorded considering any dilutions made to the cell suspension. The viability percentage of cells was represented by the percentage of unstained cells in the suspension according to the following equation:

$$\% \text{ of viable cells} = \frac{Number\ of\ viable\ cells}{Total\ number\ of\ cells} X100$$

Shake-flask culture experiment and precursor feeding

Cell suspension cultures were established in 250 ml Erlenmeyer flasks containing a total of 50 ml of the liquid-optimized MS basal medium supplemented with 3% (w/v) sucrose, 0.01% (w/v) myo-inositol, 4.55 µM 2,4-D and 4.44 µM BA. The precursor amino acid; L-phenylalanine (Sigma, min. 90%, St. Louis, USA) was added to the medium at different concentrations (302.68, 605.36 and 908.05 µM) for elevating the accumulation of silymarin and some phenolic compounds in the *in vitro* cell suspension culture. Each flask contained 5 ml of the prepared cell suspension in addition to 45 ml of the culture medium. The control treatment was made without phenylalanine. The medium was adjusted at pH 5.7–5.8, then autoclaved. Flasks were closed with cotton plugs and two loosely placed layers of aluminum foil to permit gas exchange. The flasks were incubated on the rotary shaker as mentioned above. Cells were collected after 30 days of incubation, filtered using a filter paper and the fresh weight was recorded as g per 50 ml. Dry weight (DW; as g per 50 ml) was taken after drying the cells in an oven at 45°C until a constant weight was obtained. The content of silymarin and phenolic compounds were determined in the dry matter and calculated as percentage (%) in relation to the dry weight of cells for each treatment.

Determination of secondary metabolites

Determination of silymarin content

Cells of *S. marianum* from each treatment were harvested, separated from the medium and dried. Silymarin was extracted according to the method of Cacho *et al.* (1999) from 0.05 g of the dry matter with 0.5 ml of 80% methanol (MeOH; analytical grade) for 30 min in a water bath at 40°C. This step was repeated five times, and then the methanolic solutions were collected and evaporated. The dry residue was re-dissolved in 2 ml MeOH and kept at 4°C in the dark. Silymarin content was measured spectrophotometrically using UV/VIS spectrophotometer (Chrom-Tech, model CT-2400, Taiwan). Six dilutions of standard silymarin (Sedico, Egypt) were prepared in 80% MeOH for the standard curve. Standard concentrations and sample solutions were scanned in the range of 200-400 nm to determine the maximum absorbance against 80% MeOH as a blank. The maximum absorbance was observed at 255.7 nm and silymarin concentration was calculated based on the calibration curve for the standards and expressed in percentage (%).

Determination of phenolic compounds content

Samples (0.5 g) of the cell cultures were extracted twice with boiling MeOH (50 ml for 2 h). Determination of certain phenolic compounds was performed by a modified high-performance liquid chromategraphy (HPLC) with a diode-array detector (DAD) method of Ellnain-Wojtaszek and Zgórka (1999), using HPLC–DAD (Merck-Hitachi) and RP-18e column (49250 mm, 5 µm; Merck). The mobile phase consisted of A- MeOH: 0.5% acetic acid (1:4 v/v); B- MeOH, at gradient program B from 0 to 100%, at 25ºC. The injection volume was 10 µl at a flow rate of 1 ml min^{-1} and detection wavelength of 254 nm. Quantification was carried out based on the calibration curves for the standards of the following phenolic compounds: gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringic acid, pyro catechol, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, taxifolin, cinnamic acid and kaempferol (Sigma-Aldrich Co., USA). Phenolic compounds concentration was recorded as percentage (%).

Experimental design and statistical analysis

The experiments were set up in completely randomized design with at least three replicates per treatment. The significance of differences among means was carried out by Duncan's multiple range test at the 0.05 level. The results were subjected to one-way analysis of variance (ANOVA) using SPSS v.16 (SPSS, Chicago, USA).

RESULTS

Establishment and characterization of cell suspension culture

Friable callus was obtained from root explants of seedlings, from *in vitro* germinated seeds of *S. marianum*, on MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA (Fig. 1A). Cell suspension cultures were prepared by the same medium composition (Fig. 1B and C). Fresh weight and viability of suspended cells were significantly affected by the duration of the suspension culture. The curves of cells fresh weight and viability percentage are represented in Fig. (2) and (3). Both curves show a gradual increase in the growth parameter of suspended cells by time. The cells fresh weight reached the maximum value of 3.36 g 50 ml^{-1} after 32 days of incubation, then significantly decreased, while cell viability reached the maximum of 87.24% at the 16th day, then significantly decreased gradually by further increasing of the duration.

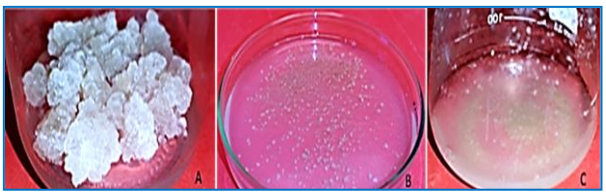

Figure (1): Establishment of the cell suspension cultures of *S. marianum* . A, Callus from root explants on MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA; B, Cell suspension on a plate; C, A well-established cell suspension culture in a flask.

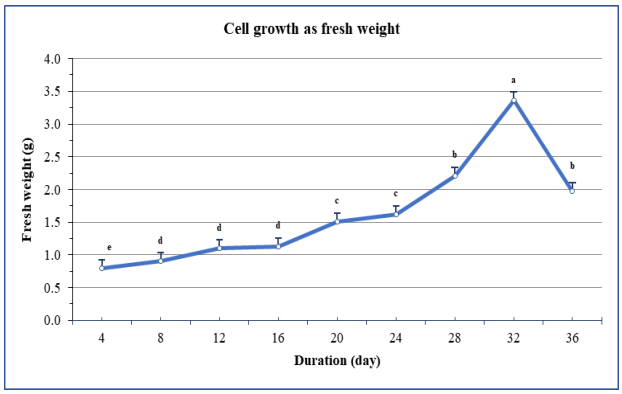

Figure (2): *S. marianum* cell growth in suspension culture during 36 days on MS basal medium supplemented with 4.55M 2,4-D and 4.44M BA. Data are represented in mean ±SE, values with same letter are not significantly different, at the 0.05 level, according to Duncan's multiple range test.

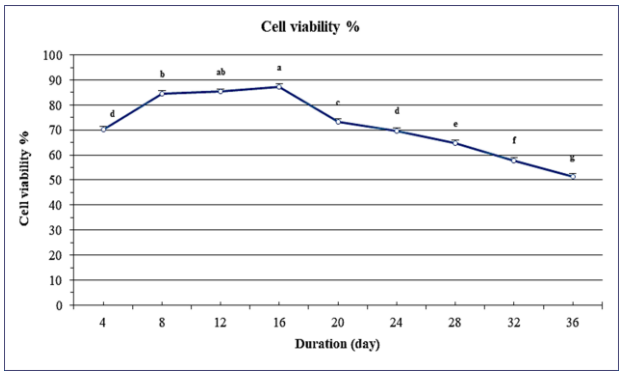

Figure (3): Cell viability in suspension culture of *S. marianum* during 36 days of incubation in MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA. Data are represented in mean ±SE, values with same letter are not significantly different, at the 0.05 level, according to Duncan's multiple range test.

The impact of precursor feeding on biomass and secondary metabolite accumulation

The effect of MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA, in addition to the different concentrations of the amino acid precursor; phenylalanine, on the fresh and dry weight of the cells of *S. marianum* cell suspension cultures is represented in Fig. (4). The fresh and dry weight of cells significantly increased by adding phenylalanine in the medium at 302.68 µM, compared to the control treatment without phenylalanine either in the solid or liquid medium. Higher concentrations of phenylalanine signifycantly decreased the fresh and dry weight of cells. Comparing the two control treatments without phenylalanine, it was obvious that the liquid medium produced significantly higher fresh and dry weight of cells than that of the solid medium. By calculating the water content in the cells according to the FW and DW, it ranged between 93.33% in the cells on the control solid medium and 96.36% in the suspended cells in the medium supplemented with 908.05 µM phenylalanine.

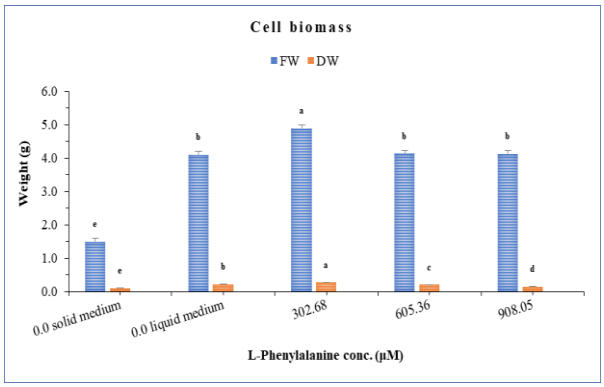

Figure (4): Effect of L-phenylalanine supplement on cell biomass of *S. marianum* suspension cultures. Data are represented in mean ±SE, values with same letter are not significantly different, at the 0.05 level, according to Duncan's multiple range test.

Table (1) shows the content of several phenolic compounds in *S. marianum* suspended cells in MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA, in addition to different concentrations of phenylalanine. Phenylalanine application had a positive effect on the accumulation of some phenolic compounds. Catechin, coumaric acid and taxifolin attained their maximum accumulation on the lowest phenylalanine concentration (302.68 µM), while chlorogenic acid and rutin were maximum on the highest concentration (908.05 µM). Methyl gallate was not detected in all treatments, while very few amounts of coumaric acid, vanillin, cinnamic acid and kaempferol were detected in some treatments. Gallic acid, caffeic acid, syringic acid, ellagic acid and naringenin gained their maximum accumulation in the cells on the control solid medium, while pyrocatechol, vanillin and ferulic acid were the maximum in the suspended cells of the control liquid medium.

DISCUSSION

Callus of *S. marianum* was initiated from root segments of seedlings of *in vitro* germinated seeds. Friable callus was induced on MS basal medium supplemented with 4.55 µM 2,4-D and 4.44 µM BA as reported in previous works of Hassanen and Khalil (2013) and Elsawy (2017). Callus induction is one of the most essential steps in initiating an *in vitro* system for the production of bioactive secondary metabolites from plants for industrial applications (Chandran *et al.*, 2020). Cell suspension cultures are commonly used for industrial purposes due to their rapid growth, stable production, uniformity and the ease of scale-up the biomass production (Chandran *et al.*, 2020).

Cell suspension cultures of *S. marianum* were established in the present study and the growth of cells was estimated using cell fresh weight and viability methods over a 36-day period. The growth of suspended cells displays a normal growth curve and cells started their

Table (1): HPLC analysis of phenolic compounds exist in cells of *S. marianum* cell suspension cultures supplemented with different concentrations of L-phenylalanine as a supplement. Basal MS medium, in solid and liquid state were used as control.

Concentration of detected Phenolic compounds (%)	L-phenylalanine conc. (µM)				
	Control solid medium	Liquid medium			
	0	0	302.68	605.36	908.05
Gallic acid	0.143	0.138	0.139	0.068	0.136
Chlorogenic acid	0.430	0.513	0.451	0.260	0.615
Catechin	0.003	0.000	0.087	0.010	0.058
Methyl gallate	0.000	0.000	0.000	0.000	0.000
Coffeic acid	0.024	0.020	0.022	0.012	0.024
Syringic acid	0.014	0.009	0.007	0.004	0.008
Pyrocatechol	0.010	0.014	0.009	0.004	0.011
Rutin	0.000	0.000	0.149	0.081	0.160
Ellagic acid	0.246	0.114	0.081	0.047	0.085
Coumaric acid	0.008	0.007	0.008	0.003	0.006
Vanillin	0.004	0.005	0.003	0.002	0.003
Ferulic acid	0.016	0.026	0.019	0.010	0.025
Naringenin	1.638	1.428	1.157	0.770	1.624
Taxifolin	0.004	0.000	0.017	0.006	0.004
Cinnamic acid	0.001	0.001	0.000	0.000	0.001
Kaempferol	0.004	0.000	0.000	0.004	0.000

death phase after 16 days of culture. This may be explained by the death of cells that could be occurred by time due to nutrients depletion. Ramulifho *et al. (*2019) reported that the cell viability in cell suspension cultures increased until the cells reach the stationary phase, then the viability gradually decreased, which may indicate a change in the cellular metabolism as cells stop proliferating and the nutrients in the medium become limiting. It was reported that the growth of cells in suspension cultures can be assessed by several methods, including fresh and dry weight of cells, total cell number, packed cell number, cell viability and medium conductivity. It is recommended to perform any two methods to monitor the culture growth characterization (Evans *et al.,* 2003) and fresh weight analysis is the most common and simple method with no need of sophisticated equipment (Mohamad and Abdullah, 2018).

Based on the analysis of growth curve carried out in the present study, the cells of *S. marianum* should be subcultured every 16-20 days for the maintenance and cells viability. The duration for maintaining cells in suspension cultures recorded differences based on the initial volume of cells, growth conditions and plant species (Ramulifho *et al.,* 2019). For example, the medicinal plant; *Abrus precatorius*, suspended cells require subculturing every 14 days (Rahman *et al.,* 2012), while *Jatropha curcas* and *Jatropha gossypifolia* cell suspensions require subculturing every 20 days (Ramos *et al.,* 2013).

S. marianum cell suspension cultures offer an alternative source for liver protective drugs and would have a significant impact either pharmacologically and ecologically. Although *S. marianum* cell cultures can produce silymarin and phenolic compounds, the produced amounts are lower than that in the field grown plants (Abbasi *et al.,* 2010). Moreover, the increasing demand for *S. marianum* worldwide for the pharmaceutical purposes is endangering its populations in the Mediterranean region (Ahmad *et al.,* 2008). Therefore, the present study was directed towards using the precursor of the active constituents for better silymarin and certain phenolic compounds accumulation in cell cultures of *S. marianum*.

The precursor; phenylalanine has been reported to enhance secondary metabolite production in plant cell suspension cultures (Masoumian *et al.,* 2011). In this study, the effect of phenylalanine feeding on biomass and secondary metabolites production in *S. marianum* cell suspension cultures was investigated. From the above results it was obvious that biomass and the concentration of silymarin and phenolic compounds content in cell suspension culture of *S. marianum* was strongly affected by phenylalanine concentration. The biomass expressed as fresh and dry weight of cells significantly increased by phenylalanine supplementation at 302.68 µM, compared to the control. Cellular proliferation in suspension cultures, which is expressed as biomass, is the first step for establishing a large-scale production of active compounds (Indu *et al.,* 2013). Also, phenylalanine supplementation elevated the level of silymarin and some phenolic compounds. This may be caused by increasing metabolic flux through the phenylpropanoid biosynthetic pathway; however, appropriate concentration of the precursor should be applied for successful feeding (Rahimi *et al.,* 2011). Phenylalanine caused a marked silymarin accumulation; it was significantly increased

by increasing phenylalanine concentration. Phenylalanine elevated accumulation of silymarin by about 3-fold at the highest concentration of 908.05 µM, compared to the callus on the control medium without phenylalanine. The application of phenylalanine as the biosynthetic precursor of phenylpropanoid pathway enhanced *in vitro* silymarin accumulation in *S. marianum* with 31.4% increase in callus at 25 µM phenylalanine as reported by Elhaak *et al. (*2016) and with 1.89-fold increase in hairy root culture at 10 µM phenylalanine, compared to the control as found by Rahimi *et al. (*2011). Also, phenylalanine was frequently used *in vitro* as a precursor, such as enhancing *in vitro* production of isoflavones from *Psoralea corylifolia* cell cultures (Shinde *et al.,* 2009a), flavonoid from *Hydrocotyle bonariensis* (Masoumian *et al.,* 2011) and phenolic acids from *Exacum affine* (Skrzypczak-Pietraszek *et al.,* 2014).

In this study, supplementing the media with different concentrations of phenylalanine resulted in a broad variation in the phenolic content and consequently will affecting the antioxidant activity of *S. marianum* cell culture extract. Antioxidant activity is considered the most important link associated with the phenolic content of the plant cell and tissue (Fidranny *et al.,* 2015; Hano and Tungmunnithum, 2020). The results are in harmony with that of Tůmová *et al.* (2006), who concluded that feeding *S. marianum* cell cultures *in vitro* with coniferyl alcohol; the precursor of biosynthetic pathway of flavonolignans, enhances the accumulation of some components of silymarin complex and flavonoid-taxifolin and does not influenced the other components. Gad *et al.* (2021) reported that coniferyl alcohol and taxifolin are may be synthesized *in vitro* from dicaffeoylquinic acid by caffeoyl-CoA pathway.

The suggested pathway for silybin biosynthesis, from dicaffeoylquinic acid, starts from coniferyl alcohol and taxifolin as substrates for flavonolignane biosynthesis. Coniferyl alcohol and taxifolin are synthesized through the p-coumaroyl-CoA and caffeoyl-CoA pathways. This pathway involves a series of enzymes. In the same way, Shinde et al. (2009b) reported that phenylalanine at 2 mM concentration enhanced isoflavones levels in Psoralea corylifolia hairy root cultures, compared to the control, however isoflavones production was inhibited when 10 mM phenylalanine was applied. The reason for the enhanced accumulation of silymarin after phenylalanine feeding (as a biosynthesis precursor), while some phenolic compounds were not affected could be that some precursors are not completely soluble in aqueous media and therefore the reaction rate could fairly be detected (Tůmová *et al.,* 2006). Also, Gad *et al*. (2021) reported a well-documented phenomenon of the increase of peroxidase activity in elicited *in vitro* cell cultures that cause the degradation of secondary metabolites and may contribute to their low amounts.

The first step in the phenylpropanoid pathway is catalysed by phenylalanine ammonia lyase, which converts phenylalanine to ammonia and trans-cinnamic acid (PAL).Therefore, phenylalanine is involved in the biosynthesis of flavonoids, phenyl-propanoids and lignins in plants (Solekha *et al.,* 2020). On this base, the results obtained in the present study indicated that all the three concentrations of added phenylalanine may stimulated PAL activity and consequently supported the use of this precursor as the substrate for the first step of phenylpropanoid pathway (Rahimi *et al.,* 2011).

CONCLUSION

The production of silymarin and other phenolic compounds in *S. marianum* cell suspension cultures was considerably boosted when the precursor amino acid L-phenylalanine was used. In the *S. marianum* cell suspension culture system, this approach provides an alternative method for large-scale synthesis of bioactive secondary metabolites. This can also help in preventing the plant from becoming endangered due to the overcollection of plant material for extracting bioactive compounds. Interestingly, *S. marianum* cell suspension culture has the potential to scale up to the level of the bioreactor for enhancing the production of silymarin-rich extracts for possible commercial use.

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تراكم السيليمارين في المزارع المعلقة لنبات شوك الجمل عن طريق التغذية ببادئ

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السيليمارين والمركبات الفينولية لها العديد من الاستخدامات الصيدلانية، لا سيما كعوامل واقية من مرض الكبد. ويعتبر محتوى السيليمارين في ثمار عشائر النباتات البرية لنبات شوك الجمل منخفض مع وجود اختلافات معنوية بين الأفراد النباتية كما أن المناطق التي تتواجد بها هذه العشائر قلت بسبب الاهتمام المتزايد بالنبات. لذلك فإن تقنية مزارع الخلايا المعلقة هي إجراء بديل ومستدام للإنتاج الحيوي من النبات وللحفاظ عليه. الهدف من هذه الدراسة هو إنتاج السيليمارين وبعض المركبات الفينولية من مزارع الخلايا المعلقة لنبات شوك الجمل عن طريق إضافة بادئ للمركبات الفعالة وهو الحمض الأميني فينيل ألانين. تم جمع البذور من النباتات البرية على طريق الإسكندرية الصحراوي وتم انباتها معمليًا. تمت زراعة أجزاء من جذور النبتات على بيئة موراشيجي وسكوج مع إضافة ٤,٤٤ ميكرو مول ٤،٢-ثنائي كلورو فينوكسي حمض الخليك و٤,٥٥ ميكرو مول ٦-بنزيل أدينين لإنتاج وتضاعف الكالس. تم إنشاء الخلايا المعلقة في نفس البيئة المغذية مضافًا إليها الفينيل ألانين بتركيزات مختلفة (٣٠٢,٦٨ و٦٠٥,٣٦ و٩٠٨,٠٥ ميكرو مول). تم إنتاج السيليمارين وبعض المركبات الفينولية معمليًا من مزارع الخلايا المعلقة وأدى إضافة الفينيل ألانين إلى زيادة الكتلة الحيوية للخلايا وتراكم السيليمارين وبعض المركبات الفينولية. بلغ أعلى تراكم للسيليمارين بزيادة معنوية بلغت ٣٣.٦٦٪ مقارنة بالكنترول. هذه الدراسة تسلط الضوء على قدرة الحمض الأميني فينيل ألانين كبادئ للسيليمارين في تعزيز إنتاج المستخلصات الغنية بالسيليمارين من مزارع الخلايا المعلقة لنبات شوك الجمل وحماية الفلورا والتنوع الحيوي من خلال منع التجميع الجائر للنبات وكذلك التحكم في كمية الإنتاج ونوعيته ووقته.

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