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Original Article

Influence of Plant Growth Regulators on Callus Induction, Silymarin Production and Antioxidant Activity in Milk Thistle (*Silybum marianum* L. Gaertn.) under Tissue Culture Medium

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Abstract

The *Silybum marianum* (L.) Gaertn. is the dicotyledonous herbs of the Asteraceae family that is important in medical industry. The biological active compound of *S. marianum* is a mixture of several flavonolignals generally known as silymarin. The purpose of this study was to optimize *S. marianum* tissue culture for callus induction, silymarin production and comparison of some biochemical traits between explants and its derived callus. In this experimental study, the seeds were surface sterilized and transferred to MS medium to achieve sterile seedlings. Then, the effects of different concentrations of 2,4-D either alone or in combination with Kin were investigated on callus induction and silymarin production of root and leaf explants from sterile seedlings. The experiment was performed in complete randomized design with three replicates. The results revealed that the highest percentage of callus induction and silymarin accumulation (14.4%) were observed with 0.5 and 1 mg/L 2,4-D and Kin in root explant after one month. The quantitative and qualitative data from HPLC method revealed that the major flavonolignans in the root and its derived callus was isosilybin B (ISBNB). But the main component in leaf explant and its derived callus was silydianin (SDN). The results showed that silymarin level in root and leaf explants was lower than their derived callus. Meanwhile lignin amount in the root and leaf explants was much higher than corresponding callus. In addition, peroxidase activity was significantly higher in callus derived from root explant, compared to leaf explant and its derived callus. The current data demonstrated that callus derived from root explants can be an efficient source for silymarin production.

Keywords: Antioxidant, Callus, Flavonolignan, *S*ilymarin.

Introduction

Plants are known to produce a large number of natural products which refers to as secondary metabolites. The secondary metabolites are economically important in medicinal and food industry. But the concentration of various plant secondary metabolites is very low and depends on the physiological and developmental stages [1]. *Silybum marianum* (L.) Garerth is herbaceous annual or biannual plant, native to the Mediterranean area, which is now widespread in other warm and dry regions [2]. The active component of *S. marianum* is known as silymarin. Silymarin is consisting of isomeric flavonolignans including, silybinin (SBN A and SBN B), silyadianin (SDN) silychristin (SCN) and Taxifolin [3].

Silymarin is a phytomedicine particularly used in the treatment of liver disease [4,5]. So far, several reports indicated different medicinal properties of silymarin, such as: its role in increasing protein synthesis and cell regeneration [6,7]. Meanwhile, silymarin has anticancer and antioxidant activity [8].

Silymarin is usually extracted from the dried fruits of field-grown plants. Dried fruits of *S. marianum* contain the highest level of silymarin (4%) [2]. In

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previous study, we measured silymarin amount in different parts of *S. marianum* (such as young and old leaves, root, stem, inflorescence and seeds) during different months. The data obtained from HPLC analysis showed that the highest amount of silymarin was observed in the seeds (32.77 mg/g dry weight). Also stems collected during May month showed high amount of silymarin (17.83 mg/g dry weight) [9].

The *in vitro* production of plant secondary metabolites is an excellent alternative. Hairy root, callus and cell suspension can be used as a source for silymarin production (10, 11). Callus induction of *S. marianum* has been previously reported [12]. They showed that flavonolignan content in 2 months old callus (1.5%) was lower than the values reported for seeds. Hassnloo *et al*. (2008) reported callus induction with cotyledons, shoot and root explants of *S. marianum* seedlings [13]. They have also showed the positive effect of picloram on callus growth and flavonolignan production. Rady *et al*. (2014) reported MS medium supplemented with 0.25 mg/L 2,4-D and Kin are a suitable medium for growth and maintenance of friable callus [14]. Other researchers reported callus induction from cotyledon by different concentrations of mineral element, plant growth regulators, amino acids and vitamins on MS medium culture (11). Meanwhile, Cimino *et al*., (2008) found B5 medium supplemented with 0.05 mg/L of BA and 0.5 mg/L of 2,4-D is the best medium for callus production and biomass production of *S. marianum* [15].

Cell suspension culture of *S. marianum* has been also reported previously [2,12]. So far, the effect of different concentrations of Iron, Calcium, KNo3, KHPO4, Methyl Jasmonate, Salisilic Acid, picloram and Ag^{+} on silymarin production was tested in cell suspension culture [7,16]. Silymarin production by hairy root culture of *S. marianum* has been also reported. But flavonolignan level in hairy root was lower than that is generally found in dried fruits of *S. marianum* [10,17].

Because of the interesting biological effects of silymarin and its increasing worldwide demand, in current research, we investigated the effect of different concentrations of plant growth regulators (2,4-D and Kin) on callus induction and silymarin production on different explants of *S. marianum* in tissue culture medium.

Materials and Methods

Plant Materials and Seed Collection

The seeds of *S. marianum* were harvested from north of IRAN area. The seeds were washed in running tap water for about 30 min. Surface sterilization was carried out by immersing in 20% (V/V) commercial bleach solution (containing two drops of tween 20) for 20 min. The seeds were then washed 5 times with sterile distilled water under laminar air-flow hood. The sterilized seeds were cultured into 100 ml capacity jars containing 20 ml Murashige and Skoog (MS) (1962) medium supplemented with 30 g/L sucrose and 8 g/L agar at pH 5.7 prior to autoclaving at 121 ˚C for 20 min. Five seeds were grown in each culture jar [18]. The cultures were incubated at 25±2 ˚C and 16h light/8h dark photoperiod in a growth chamber for 4 weeks. Germinated seedlings were used as a source of root and leaf explants.

Callus Induction

Segments of root and leaf were cultured on MS basal medium containing 30 g/L sucrose, 8 g/L agar, B_5 vitamins and different concentrations of 2,4-D (0, 0.1, 0.5, 1, 1.5 and 2 mg/L) either alone or in combination with Kin (0, 0.1, 0.5, 1, 1.5 and 2 mg/L). The pH of different media was adjusted to 5.7 prior to autoclaving at 121 ˚C for 20 min. The cultures were placed in a growth chamber at 16h light/ 8h dark photoperiod and 25±2 ˚C. Twenty replicates per each treatment were used and arranged in a completely randomized design. The cultures were grown for 30 days before recording percentage of callus production, fresh and dry weight measurement, determination of total phenol, total flavonoid, lignin, silymarin level, HPLC analysis and enzyme activity.

Flavonolignan Extraction

Flavonolignan extraction has been done according to Cacho *et al*., (1999) method [2]. To remove fats, the dried and powdered callus was treated with ethyl acetate for 5 h. Then Flavonolignan was extracted from the dried residue with 10 mL of methanol at 40 ˚C for 8 h. Methanol solution was evaporated under vacuum on a rotator evaporator instrument. The dried residue was re-dissolved in 2 mL of methanol and kept at 4 ˚C in the dark.

Silymarin Level Measurement by Spectrophotometric Method

Silymarin level was measured spectrophotometrically as described by Hassanloo

et al., 2005 [16]. In brief, The methanolic extract (1 mL) was added to 2 mL aliquot of 2,4-dinitriphenilhydrasine- sulfuric acid and then placed in a water bath for 50 min at 50 °C. The solution was made up to 10 mL with KOH-methanol 10%. 0ne milliliter of this solution was added to 20 mL aliquot of methanol and the mixture was centrifuged. Then aqueous phase was diluted to 50 mL volume with methanol. Absorbance was determined at 490 nm by spectrophotometer (Shimatzu-Japan). To measure silymarin, equation 1 was used, using the specific absorbance (A1%1cm= 537).

% Silymarin= A×50×50×10×100/A1%1cm×100×M

HPLC Analysis

The amount of flanonolignan components were determined by High performance liquid chromatography (HPLC) (Hitachi) equipped with a Nucleosil $C_{18}\mu$ (250 X 5 mm) column and aUV/VIS detector, a Diod Array pump and a Merck Hitachi7100–L software for peak integration. Mobile phase was a mixture of methanol and water (10:1 v/v). The mobile phase was filtered using 0.45µm membrane filter (Milipore) and degassed by vacuum prior to use. The flow rate was 1mL/min and flavonolignans were detected at 288 nm. All solvents were HPLC grade (Merk).

Silymarin Standard Solution

Stock standard solutions were prepared by dissolving of 0.7 mg of standard silymarin and silibinin in 1mL of methanol (Sigma, USA). The stock solutions were diluted with methanol to reach a series of solution at different concentrations. The obtained data from the analysis of each standard solution were plotted against corresponding concentrations to draw the standard curve. The quantity of flavonolignans in each sample was determined from standard curve (R^2=0.93). The average retention times for taxofillin (TXF), silychristin (SCN), silydianin (SDN), silybin A (SBNA), silybin B (SBNB), isosilybin A (ISBNA) and isosilybin B (ISBNB) were 3.56, 3.96, 4.86, 12.72, 14.14, 16.69 and 18.58 min, respectively.

Determination of Total Phenolic Content

Total phenolic content were measured using the Folin- Ciocalteau method [19]. For this purpose, 0.1 mL of extracted solution, 0.1 mL of aqueous Folin-Ciocalteaur reagent (50% v/v) and 2.8 mL distilled water were mixed in a test tube, thoroughly. Then2 ml 2% sodium carbonate was added. The mixture was kept 30 min at room temperature in the dark before measuring absorbance at 720 nm. All measurements were repeated tree times and results were expressed as Gallic acid equivalents.

Determination of total Flavonoid Content

Total flavonoid content was measured by aluminum chloride method as described by Chang *et al.* (2002) [20]. In brief, 0.5 mL of plant extract was mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate, 1.5 mL of 80% methanol and 2.8 mL of distilled water. The mixture was kept 40 min at room temperature in the dark. The absorbance of the samples was measured at 415 nm. Quercetin was used as the standard flavonoid. All measurements were performed in triplicate and results were expressed as quercetin equivalents.

Determination of Lignin Content

The lignin content was measured by acetyl bromide digestion [21]. All samples were powdered and passed through a sieve with 100 µm apertures. The samples were digested with solutions of 25 % (w/w) acetyl bromide in acetic acid. The reaction mixture were boiled at 70 ºC for 30 min and then cooled with ice and transferred to a 50 ml volumetric flask containing *2* M sodium hydroxide and acetic acid. The lignin content was determined by measuring the absorbance at 280 nm using the Specific Absorption Coefficient value for lignin, 20 g^{-1} litre cm^{-1}.

PAL Activity Measurement

Fifty mg of fresh explants tissues or callus were homogenized in 0.05M Tris-HCl buffer, pH=8, containing 0.8 mM Mercaptoethanol and 1% (w/v) PVP. The homogenate was filtered and centrifuged at 14000 g for 15 min at 4 °C. The PAL activity was determined by detection of produced cinnamic acid and the assay performed according to Syklowska-Baranek *et al.* method (2012) [22]. The reaction mixture was consisted of 1 ml tris-HCL buffer (pH8), 0.1 ml enzymatic extract, 0.5 mL l-Phe (10 mM). After incubation at 37°C for 1 hour, the reaction was stopped by addition of0.1 mL HCL (0.1 N). The absorbance of the samples was measured at 290 nm.

Antioxidant Enzymes Activity Measurement

To prepare crude enzyme extracts, fresh leaves (0.05 g) were ground with 2 ml of 0.1 M cool phosphate buffer (pH 6.8) as described by Kar and Mishra (1976) [24]. The obtained homogenate was then centrifuged at 15000 g for 15 min at 4 °C. The clear supernatant was used for assaying the activities of catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7).

Catalase activity was determined by monitoring the destruction of H_2O_2 at 240 nm [23]. The reaction mixture in a final volume of 3 mL contained 50 mM phosphate buffer (pH 6.8), 100 µL enzyme extract and 15 mM H_2O_2. The decrease in absorbance at 240 nm was recorded with a spectrophotometer (Shimadzu UV-160).

The peroxidase reaction mixture in a final volume of 3ml contained 20 mM guaiacol, 25 mM phosphate buffer (pH 6.8), 40 mM H_2O_2 and 10 µL from the crude enzymes extract. The increase in absorbance at 470 nm due to tetra-guaiacol formation was recorded spectrophotometrically.

Statistical Analysis

Analysis of variance (ANOVA) and Duncan's multiple range test at ρ=0.05 significant level were used to analyze data via SAS software (version 9).

Results

Callus Induction

The effect of different concentrations of 2,4-D and Kin on the callus induction of root and leaf explants of *S. marianum* is represented in table 1. No callus was induced in the MS medium without 2,4-D and Kin plant growth regulators. Addition of 2,4-D without Kin to MS medium showed stimulating effects on callus induction, after one month. Among 36 different combinations of 2,4-D and Kin treatment, callus induction was only observed in 11 treatments. Data from analysis of variance showed that different concentrations of 2,4-D and Kin had significant effect ($P \leq 0.05$) on callus induction of root and leaf explant (Table 2).

It seemed that by increasing 2,4-D concentration along with Kin, the callus induction has been increased significantly in root explants. The highest percent of callus induction were observed in root explants grown on MS medium supplemented with 1, 1.5 and 2, 1.5 mg/L 2,4-D and Kin, respectively. In addition, the morphology of the produced callus was friable and white in color (Fig. 1). Whereas, maximum callus induction in leaf explants was observed on MS medium supplemented with 0.5 and 1 mg/L 2,4-D and Kin, respectively. Most of the produced callus were whitish yellow and friable (Fig. 1E). Means comparison of the effect of explant type revealed that leaf explant is the most responsive to induce callus formation (Fig. 2A).

Callus Biomass Yield and Silymarin Production

The effect of 2,4-D and Kin treatments on callus biomass and silymarin amount was shown in Table 1.While the highest callus induction frequency (98%) in root explants was observed in MS medium supplemented with 1 and 1.5 mg/L 2,4-D and Kin, the combination of 0.5, 1 mg/L 2,4-D and Kin gave the highest amount of silymarin amount (14.4%) in obtained callus. Meanwhile maximum silymarin percent in derived callus from leaf explants was also observed on the MS medium supplemented with 0.5, 1 mg/L 2,4-D and Kin, respectively.

The highest fresh and dry weight were recorded in derived callus from leaf explants which grown on the MS medium contained 0.5, 1 mg/L 2,4-D and Kin, respectively. Data from analysis of variance showed that different concentrations of 2,4-D and Kin had significant effect ($P \leq 0.05$) on fresh weight and silymarin content of root and leaf explant. Meanwhile, type of explant and interaction between type of explant and different concentrations of 2,4-D and Kin had significant differences ($P \leq 0.05$) on fresh weight and silymarin content of root and leaf explant (Table 2).

Means comparison of the effect of explant type revealed that leaf explant is the most responsive to produce higher fresh weight, but root explant produced the highest percentage of silymarin content (Fig. 2B, D). Meanwhile, the current data showed that there was no significant difference in dry weight of callus derived from root and leaf explants (Fig. 2C).

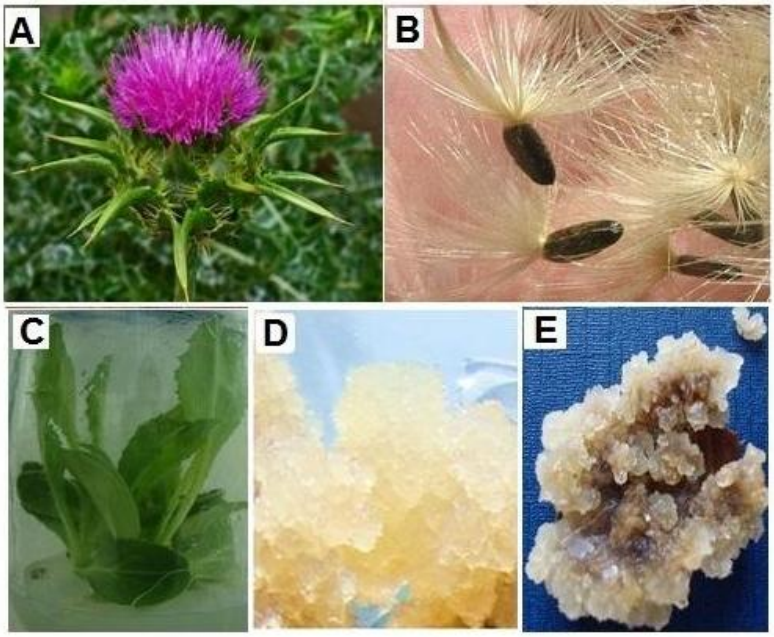

Fig. 1 A) Inflorescence, B) seeds, C) Sterile seedling of *Silybum marianum* (L.) Gaertn., D) Root -derived callus on MS medium supplemented with 1 and 1.5 mg/L 2,4-D and Kin, E) leaf -derived callus on MS medium supplemented with 0.5 and 1 mg/L 2,4-D and Kin.

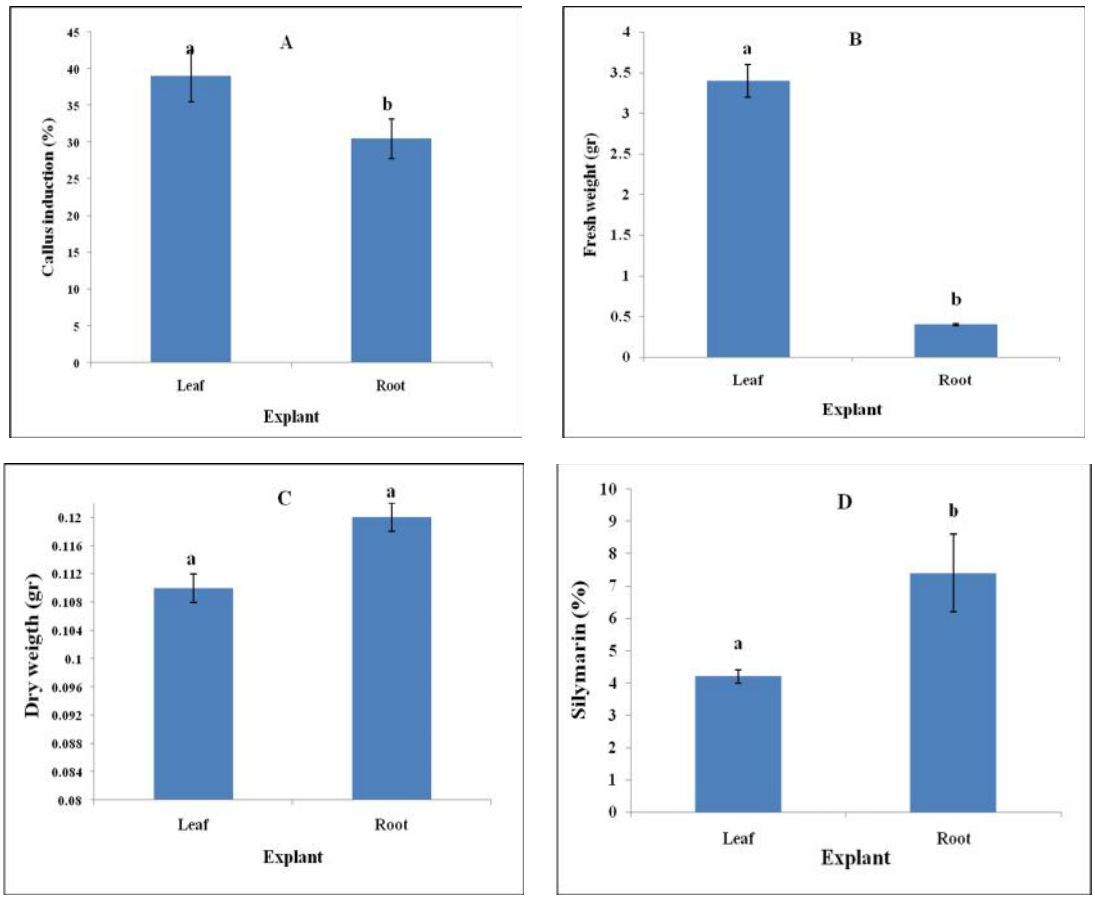

Fig. 2 Means comparison of the effect of explant type on A) callus induction, B) fresh weigth, C) dry weigth and D) silymarin amount of *Silybum marianum* (L.) Gaertn.. Different letters within each column indicate significance at $P \leq 0.05$ by Duncan's multiple range tests.

Table 1 Effect of different concentrations of 2,4-D and Kin on Callus induction, Callus quality, fresh weight, dry weight and silymarin content of root and leaf explants of *Silybum marianum* (L.) Gaertn.. Different letters within each column indicate significance at $P \leq 0.05$ by Duncan's multiple range tests.

Explant	PGR(mg/L)		Callus induction (%)	Callus quality	Fresh weight(g)	Dry weight (g)	Silymarin (% DW)
	2,4-D	Kin					
Root	1	0	19 g	whitish yellow, friable	0.24±0.01 h	0.02±0.0 f	absent
Root	1.5	0	21 f	whitish yellow, friable	0.3±0.05 h	0.02±0.001 f	absent
Root	2	0	21 f	whitish yellow, friable	0.25±0.03 h	0.02±0.002 f	1.45±0.50 e
Root	0.1	0.1	72 b	whitish yellow, friable	0.42±0.07 g	0.03±0.0 ef	2.34±0.56 d
Root	0.5	0.1	19 f	whitish yellow, friable	0.28±0.07 g	0.025±0.002 f	2.30±0.34 d
Root	0.5	1	52 d	whitish yellow, friable	0.87±0.05 e	0.024±0.001 f	14.44±1.2 a
Root	1	1	61 c	whitish yellow, friable	0.9±0.04 e	0.022±0.003 f	2.80±0.27 cd
Root	1	1.5	98 a	whitish yellow, friable	1.8±0.08 d	0.035±0.001 e	8.24±1.5 b
Root	1.5	1.5	20 gf	whitish yellow, friable	0.45±0.02 g	0.022±0.003 f	1.8±0.34 e
Root	1.5	2	79 b	whitish yellow, friable	0.32±0.04 h	0.023±0.003 f	1.24±0.45 e
Root	2	1.5	95 a	whitish yellow, friable	0.73±0.04 e	0.300±0.01 b	2.40±0.65 d
Leaf	1	0	18 g	whitish yellow, friable	0.45±0.06 g	0.032±0.01 e	absent
Leaf	1.5	0	31 f	Light green, friable	0.54±0.04 g	0.038±0.06 e	2.45±0.30 d
Leaf	2	0	50 d	whitish yellow, friable	0.88±0.1 e	0.034±0.04 e	3.67±1.1 c
Leaf	0.1	0.1	11 i	whitish yellow, friable	0.98±0.1 de	0.053±0.0 d	3.04±0.78 cd
Leaf	0.5	0.1	20 g	Light green, friable	0.86±0.03 e	0.045±0.02 d	1.65±0.20 e
Leaf	0.5	1	77 a	whitish yellow, friable	12.33±0.05 a	0.870±0.3 a	8.11±1.56 b
Leaf	1	1	70 c	whitish yellow, friable	8.3±0.12 b	0.400±0.08 b	absent
Leaf	1	1.5	60 c	whitish yellow, friable	4.5±0.28 c	0.200±0.05 c	4.22±0.46 c
Leaf	1.5	1.5	65 c	whitish yellow, friable	0.78±0.03 e	0.032±0.01 e	1.70±0.50 e
Leaf	1.5	2	38 e	whitish yellow, friable	0.88±0.06 e	0.023±0.01 f	3.45±0.44 c
Leaf	2	1.5	65 c	whitish yellow, friable	6.2±0.67 b	0.240±0.07 c	2.43±0.95 d

Table 2 Analysis of variance of effect of 2,4-D and Kin concentrations (PGR), explant and interaction between PGR and explant on callus induction, fresh weight, dry weight, and silymarin content of callus derived from leaf and root explants of *Silybum marianum* (L.) Gaertn.

Source of variance	df	F Value			
		Callus induction (%)	Fresh weight (g)	Dry weight (g)	Silymarin (%)
PGR	14	721.98^{**}	81.14^{**}	0.37^{ns}	4147.93^{**}
Explant	2	5.25^{**}	1760.48^{**}	0.16^{ns}	621.85^{**}
PGR × Explant	28	178.76^{**}	141.18^{**}	0.59^{ns}	341.08^{**}
Error	90				

^{ns} and ** means non-significant and significant on 5% probability levels.

Comparison of some secondary metabolites level between leaf and root explants and corresponding callus

To determine some secondary metabolites level such as: total phenol, total flavonoid and total lignin, we selected derived callus from root and leaf explants which showed the highest level of total silymarin in the medium culture. The total flavonoid of both root and leaf explants was similar to derived callus. But total flavonoid amount was higher in root and its derived callus compared to leaf explants and corresponding callus (Fig. 3A).

The measurement of total phenol showed an obvious difference between root explants and derived callus. But there is not any significant difference between leaf explants and corresponding callus (Fig 3B). Meanwhile, the obtained data indicated that lignin level in root and leaf explants was much higher than corresponding callus (Fig. 3C).

The current results indicated that total silymarin amount in callus was higher than corresponding explants. The highest level of total silymarin was observed in callus derived from root explant. While total silymarin in root explants was 4.35% dry weight, it was 14.4% dry weight in corresponding callus. Interestingly leaf and root explants showed similar total silymarin amount (Fig. 3D).

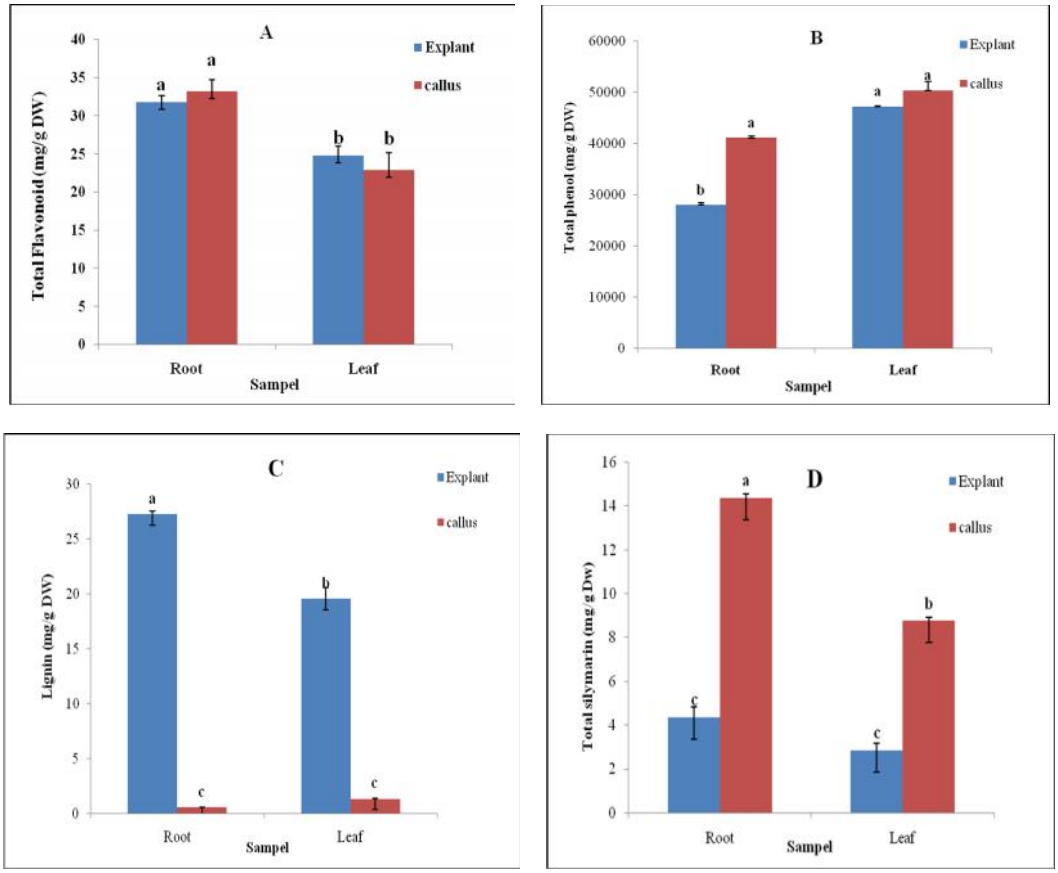

Fig. 3 **A**)Total flavonoid, **B**) Total phenol, **C**) lignin and **D**) Total silymarin level of root and leaf explants, callus derived from root and leaf explants of *Silybum marianum* (L.) Gaertn..The data were obtained from three independent experiments. Data are means ±SE. Columns with different letters indicate significant differences at $p \leq 0.05$, according to the Duncan's test.

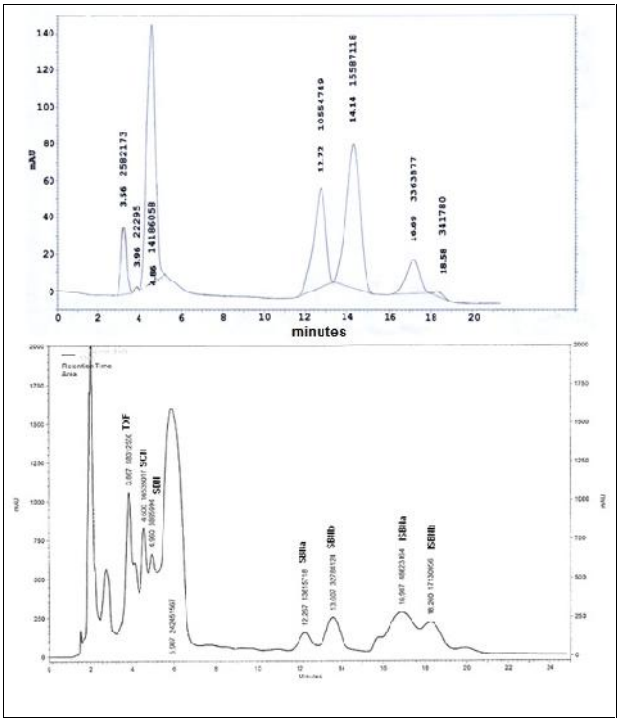

Fig. 4 HPLC Chromatograms of A) standard silymarin and B) metanolic extract of callus derived from root explants of *Silybum marianum* (L.) Gaertn.on MS medium supplemented with 0.5 and 1 mg/L2,4-D and Kin.

Table 3 HPLC analysis results of silymarin components (mg/g Dry weight) in rootandleaf explants, callus derived from root and leaf explants of Silybum *marianum*. Taxofillin (TXF), Silychristin (SCN), Silydianin (SDN), Silybin A (SBNA), Silybin B (SBNB), Isosilybin A (ISBNA) and Isosilybin B (ISBNB). Different letters within each column indicate significance at ρ=0.05 by Duncan's multiple range tests.

Sample	TXF	SCN	SDN	SBNA	SBNB	ISBNA	ISBNB	Total Silymarin
Root	0.55±0.05 b	0.037±0.05 c	0.43±0.02 c	0.03±0.01 d	0.34±0.005 c	0.51±0.05 b	0.97±0.05 c	2.86±0.2 c
Callus	0.15±000 c	0.63±0000 b	1.24±0.05 b	1.68±000 a	1.4±0000 a	3.58±0.05 a	25.2±0000 a	33.88±0.05 a
Leaf	0.506±0.03 b	0.855±0.04 a	1.35±0.15 b	0.274±0.024 c	0.419±0.011 c	0.211±0.038 c	0.335±0.015 cd	3.949± 0.21 c
Callus	0.87± 0.02 a	0.98±0.06 a	3.86±0.21 a	0.66±0.043 b	0.76±0.030 b	0.39±0.026 c	1.24±0.022 b	8.76±0.15 b

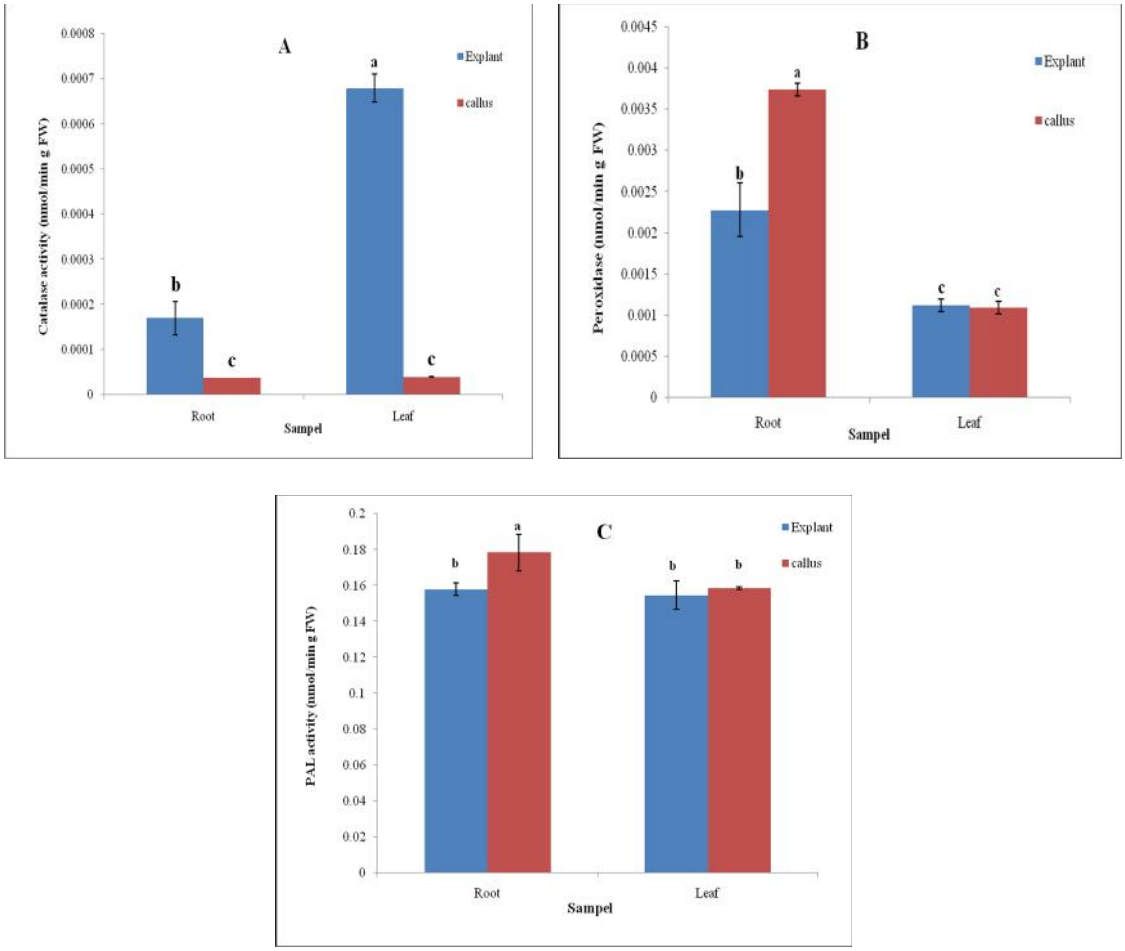

Fig. 5 A) Catalase, B) Peroxidase, C) PAL activity of root and leaf explants, callus derived from root and leaf explants of *Silybum marianum*. The data were obtained from three independent experiments. Data are means ±SE. Columns with different letters indicate significant differences at $p \leq 0.05$, according to the Duncan's test.

HPLC Analysis Results for Silymarin Constituents

To quantify the main constituents of silymarin, the HPLC method was selected. HPLC analysis identified seven isolated components: taxofillin (TXF), silychristin (SCN), silydianin (SDN), silybin A (SBNA), silybin B (SBNB), isosilybin A (ISBNA) and isosilybin B (ISBNB) from the explants and their derived callus (Fig. 4). The amount of silymarin in root and leaf explants was 2.86 and 3.94 mg/g dry weight, respectively (Table 3). Data from analysis variance showed that there was significant difference between silymarin means in root explant and its derived callus (Table 4). It was interesting that the amount of silymarin in callus derived from root was much higher than root explant. The highest amount of silymarin was observed in callus derived from root explant (33.88 mg/g DW). The major flavonolignan in the root and its derived callus was isosilybin B (ISBNB). But the main component in leaf explants and derived callus was silydianin (SDN) (Table 3).

Enzyme Activity Measurement in Root and Leaf Explants Versus Derived Callus

We further analyzed catalase, peroxidase and phenyl alanine ammonia lyase (PAL) activities in the explants and their corresponding callus. The obtained data showed that catalase activity in leaf explant was significantly higher than root explants. However catalase activity did not show any significant difference between derived callus from root and leaf (Fig. 5A).

Table 4 Analysis of variance of effect of explant on silymarin amount of callus derived from leaf and root explants of *Silybum marianum* (L.) Gaertn.

Source of variance	df	F Value
Explant	2	38.866**

In addition, peroxidase activity was significantly higher in derived callus with respect to root explants. There was not any significant difference in peroxidase activity between leaf explants and derived callus (Fig. 5B).

Significant differences were observed in PAL activity between root explants and its corresponding callus. But there was not any significant difference in PAL activity between leaf explant and its derived callus (Fig. 5C).

Discussion

So far several efforts have been concentrated on silymarin *in vitro* production under controlled conditions. But the amount of produced silymarin *in invitro* condition was lower than that obtained from field grown plants. In current study, the effect of plant growth regulators on callus induction and flavonolignans production of *S. marianum* has been studied. The fully expanded young leaves and roots segments were used for callus induction. Interestingly, addition of 2,4-D together with Kin to MS medium showed significant induction rate in callus induction. This is because auxin facilitates cell elongation; cell division and callus formation in the medium culture and cytokinin promote cell division and differentiation [25,26].

Exogenous application of an intermediate ratio of auxin and cytokinin induces callus formation (27). Meanwhile, 2,4-D is a synthetic auxin which induce callus formation much higher than other types of auxin. The optimum concentrations of auxin and cytokinin for callus induction vary among different species. In current research we found that 1and 1.5 mg/L 2,4-D and Kin were the most effective concentrations for callus induction in root explants.

Means comparison of the effect of explant type on callus induction showed that leaf explants are more effective, compared to root explants. This finding was contradicted with other researchers who reported that the cotyledon explants was the best for callus induction [13,15].

According to obtained data supplementing MS media with different concentrations of Plant growth regulators influenced callus induction and silymarin accumulation. The highest amount of fresh weight and total silymain has` been seen in callus derived from root explants which induced on MS medium supplemented with 1,1.5 mg/L and 0.5, 1 mg/L 2,4-D and Kin, respectively. This findings shows that callus biomass did not correlate with silymarin production in investigated concentrations of 2,4-D and Kin.

Our preliminary experiment revealed that 0.5 and 1 mg/L 2,4-D and Kin concentration had an induction effect on total silymarin production on callus derived from root explants to reach 14.4 % dry weight. Silymarin is usually isolated from the dried fruits of *S. marianum*. The dried fruits contain the highest amount of silymarin (1-4%). Cacho *et al*. (1999) reported that the flavonolignan content in 2 months old callus was lower than the values obtained for fruits [2]. A likely explanation for this discrepancy is that they tried with cotyledons explants of *S. marianum* to induce callus formation. On the other hand, Rahnema *et al*. (2008) used hairy root culture method to produce silymarin. But the yield percentage was lower than that is generally extracted from dried seeds of *S. marianum* [10]. El sheriff *et al*. (2013) found the highest total silymarin concentration (up to 6.98% dry weight) in the *in vitro* regenerated shoot tip culture explants when exposed to gamma irradiation [28].

Data from HPLC analysis revealed while the main component in the leaf explants and derived callus was silydianin (SDN), the major flavonolignans in the root and its derived was isosilybin B (ISBNB). This is in agreement with some previously reported results in *S. marianum* tissue culture. El Sherif *et al*. (2013) reported that the major component of silymarin in the *in vitro* regenerated shoot tip explants under gamma irradiation treatment was ISBNB [28]. So far, several researches have shown that the main component (both quantitatively and

therapeutically) of silymarin is SBN (2, 16). Our previous study also showed that the major flavonolignan in dried fruits collected from IRAN-Gorgan is SBNB (9). Saba *et al*., (2000) revealed that the amount of total silymarin and its component depends on developmental stage and composition of medium in the *S. marianum* tissue culture [29].

The comparison of some antioxidant enzyme activity in the explants and their derived callus showed that peroxidase enzyme activity in root explants and its derived callus was higher than leaf explants and its corresponding callus. Flavonolignans are synthesized from the phenylalanine produced by the shikimic acid pathway. The phenylalanine ammonia-lyase (PAL) is one of the most important enzymes which catalyze deamination of phenylalanine to produce coumaric acid. In the next steps, chalcone synthase (CHS) catalyzes the condensation of three acetyl units from malonyl-CoA with p-coumaryl CoA to produce naringinin chalcon [30, 31].

Toxifolin, a precursor of silymarin biosynthesis, is derived from naringinin chalcone. It has been demonstrated that silymarin is formed biosynthetically from toxifolin and coniferyl alcohol (alcoholic monomeric precursor of lignin biosynthesis), in an oxidative process which catalyzed by peroxidase enzyme [32]. The current results suggest that induction of peroxidase activity plays a significant role in flavonolignan metabolism in root explants and its corresponding callus.

The current data revealed that there is not any significant difference between root explants and its corresponding callus in total flavonoid level. But there is an inverse relation between root explants and its derived callus with lignin and total silymarin content. These results explain the importance of lignin biosynthesis in growth and development of root organ.

Conclusion

As a summary, callus induction from root explants can be a suitable source for silymarin production. In our experiment, 0.5 and 1 mg/l 2,4-D and Kin strongly induced callus induction and silymarin accumulation in root explants.

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Conflict of Interest Statement

We declare that we have no conflict of interest.

References

1. Nikolova MT, Ivancheva SV. Quantitative flavonoid variations of *Artemisia vulgaris* L. and *Veronica chamaedrys* L. in relation toaltitude and polluted environment. Acta Biol Szeged. 2005;49:29-32.
2. Cacho M, Moran M, Corchete P, Frarandez-Tarrago J. Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L) Gaertn. Plant Sci. 1999;144:63-68.
3. Kurkin VA, Zapeschnaya GG, Volotsueva AV, Avdeeva EV, Pimenovm KS. Flavolignans of *Silybum marianum* fruit. Chem Nat Prod. 2001;37:315-317.
4. Flora K, Hahn M, Banner K, Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am J Gastroenterol. 1998;93:139-143.
5. Kren V, Walterova D. Silybin and silymarin-New effects and application. Biomed Pap. 2005;149:29-41.
6. Kropácová K, Misúrová E, Haková H. Protective and therapeutic effect of silymarin on the development of latent liver damage. Radiats Biol Radioecol. 1998;38:411-415.
7. Santosh k. Silymarin and skin cancer prevention: Anti-inflammatory, antioxidant and immunomodulatory effects. Int J Encol 2004;18:101-112.
8. Sanchez-Sampedro MA, Fernandez-Tarrago J. Enhanced silymarin accumulation is related to calcium deprivation in cell suspension cultures of *Silybum marianum*. Plant Physiol. 2005;162:1-6.
9. Kordi H, Aghdasi M, Khalafi M. An investigation on flavonolignans in different organs of *Silybum marianum* L. in Gorgan region. Iranian J Med Aroma Plants. 2013;3:651-665.
10. Rahnama H, Hasanloo T, Shams MR, Sepehrifar R. Silymarin production by hairy root culture of *Silybum marianum* (L.) Gaertn. Iranian J Biotech. 2008;6:113-118.
11. Elhaak M, Zayed M, Mattar M, Gad G, Dietz K. Optimization of *Silybum marianum* L. callus production and magnifying callus silymarin accumulation by elicitors or precursors. Int J Adv Pharm Biol Chem. 2016;5:148-161
12. Asghari G, Saidfar GA, Mahmudi S. Biotransformation of Aromatic Aldehydes by Cell Cultures of Peganum harmala L. and *Silybum marianum* (L.) Gaertn. Iranian J Pharm Res. 2004;3:127-130.
13. HasanlooT, Khavari-Nejad RA, Majidi E, Shams Ardakani MR. Flavonolignan Production in Cell

Suspension Culture of *Silybum marianum*. Pharmacol Biol. 2008;12:876-882.
14. Rady MR, Matter MA, Ghareeb HA, Hanafy MS, Saker MM, Eid SA, Hammoda FM, Imbaby SI, Nazief NH. In vitro cultures of *Silybum marianum* and *silymarin accumulation*. J Genet Engend Biotechnol. 2014;12:75-79.
15. Cimino C, Cavalli SV, Spina F, Natalucci C, Priolo N. Callus culture for biomass production of milk thistle as a potential source of milk clotting peptidases. Elect J Biotechnol. 2006;9:1-4.
16. Khalili M, Hasanloo T, KazemiTabar SK, Rahnama H. Influence of exogenous salicylic acid on flavonolignans and lipoxygenase activity in the hairy root cultures of *Silybum marianum*. Cell Biol Int. 2009;33:988-94.
17. Alikaridis F, Papadakis D, Pantelia K, Kephalas T. Flavonolignan production from *Silybum marianum* transformed and untransformed root cultures. Fitotrapia. 2000;71:379-384.
18. Murashing T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 1962;15:473-497.
19. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and pralin contetents in Burkina Fasan honey, as well as their scavenging activity. Food Chem. 2005;91:571-577.
20. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002;10:178-182.
21. Iiyama K, Wallis AFA. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. J Sci Food Agric. 1990;51:145-161.
22. Sykłowska-Baranek k, Pietrosiuk A, Naliwajski MR, Kawiak A, Jeziorek M, Wyderska S, Lojkowska E, Chinou I. Effect of L-phenylalanine on PAL activity and production of naphthoquinone pigments in suspension cultures of Arnebiaeuchroma (Royle) Johnst. In Vitro Cell Develop Biol. 2012;48:555-564.
23. Kar M, Mishra D. Catalase, peroxidase, and Polyphenoloxidase activities during Rice leave senescence. J Plant Physiol. 1976;57:315-319.
24. Chance B, Maehly AC. Assay of catalase and peroxidases. Methods Enzymol. 1995;11:755-764.
25. Hobbie LJ. Auxins, Molecular genetic approach in Arabidopsis. Plant Physiol Biochem. 1998;36:91-102.
26. Mok MC. Cytokinins and plant development–An overview. In Cytokinins: Chemistry, Activity, and Function, ed. DWS Mok, MC Mok. Boca Raton, Florida: CRC Press. 1994;155-66.
27. Skoog F, Miller CO. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp Soc Exp Biol. 1957;11:118-130.
28. El sheriff F, KhattabS, Ibrahim A. Improved silymarin content in elicited multiple shoot cultures of *Silybum marianum* L. Physiol Mol Biol Plants. 2013;19:127-136.
29. Saba M, Iqbal PS, Srivastav A. In Vitro Micropropagation of *Silybum marianum* L. from Various Explants and Silybin Content. J Plant Biochem Biotechnol. 2000;9:81-87.
30. Heller W, Forkmann G. Biosynthesis. In: Harbone JB (ed) the flavonoids. London, UK: Chapman and Hall. 1988.
31. Schmid JPW, Doemer SD, Clouse RA, Dixon CJ, Lam B. Developmental and environmental regulation of a bean chalkone synthase promoter in transgenic tobacco. Plant Cell. 1990;2:619-631.
32. Freudenberg K, Neish AC. Constitution and Biosynthesis of Lignins. New York, USA: Springer Verlag. 1968.