November 2006 Volume 2 Issue 3-4

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 2(3-4), 2006 [84-88]

# Production Of Silybin From Callus Culture Of *Silybum Marianum* (L.) Gaertn

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*Received: 22nd July, 2006*
*Accepted: 26th September, 2006*

*Web Publication Date : 14th November, 2006*

## ABSTRACT

*Silybum marianum* (L) Gaertn an important Indian medicinal herb has evolved wide interest for its silymarin (silybin) content, which is emerging as a very useful molecule. The callus culture was induced from leaf explant on MS medium supplemented with various growth regulators for the in vitro production of silybin. The best hormonal combination found was IAA + IBA + 2,4D and kinetin (1 ppm each). Growth kinetics of callus culture was studied using fresh weight parameter and results has shown that the cells grows very well, undergoing about 16-fold increase in total fresh weight at the end of the growth period. The content of silybin was estimated in six month old cultures by CAMAG HPTLC system using solvent system chloroform: acetone: formic acid (9:2:1). The chromatogram was developed and scanned at 254 nm. The $R_f$ value of standard silybin was found to be 0.63. The amount of silybin was found to 0.112 % w/w in natural leaf and 0.126 % w/w DW in leaf callus, which are 1.12 folds higher than that of natural leaf. The results obtained in the present investigation is the first tissue culture approach on production of silybin by leaf callus of *Silybum marianum*, which also indicate the significance of this method for large-scale production of silybin by plant tissue culture technique.


## KEYWORDS

Callus culture;
Silybin;
Silymarin;
HPTLC.

## INTRODUCTION

*Silybum marianum* (Compositae) commonly known as Milk Thistle, found in Western Himalayas Kashmir and Punjab. The plant is an important ingredient of various herbal formulations. *Silybum marianum* is mainly used for hepatoprotective[1], antioxidant[1], anti-inflammatory[2], and anti-cancer activity[3]. The plant contains mainly flavonolignans[4], alkaloids, saponin[1] and flavonoids[5]. Important flavonolignans are silibin, silidianin and silychristine. Silibin has been reported to possess antioxidant activity[6], and effective against human prostate cancer[7], lung tumour[8] and hepato cellular carcinoma[9]. Several authors have reported the tissue culture work on *Silybum marianum* for callus and suspension culture[10-12], regeneration[11] and micropropagation[13]. Cacho et al., have reported the effect of media composition[14], Alikaridis et al., have reported the effect of transformed or untransformed root cultures[15], Tumova et al., studied about the effect of cultivation time and hormonal combination[16] whereas Sanchez-Sampedro et al., studied the effect of $Ca^{++}$ and addition of elicitors for the production of flavonolignans[17-18]. Whereas, till now there is no report on the tissue culture work on the leaf explant as well as production of silybin by leaf callus. Hence, it was thought worthwhile to develop leaf callus of *Silybum marianum* and to estimate quantitatively the content of silybin in leaf calus as well in natural leaf by HPTLC.

## MATERIALS AND METHODS

Plant leaves were collected from herbal garden of Hamdard University New Delhi during the period of December-January. The plant was identified by the Taxonomist in the Department Botany of the University and voucher specimens have been maintained in the Department of Pharmacognosy and Phytochemistry of University.

### Initiation and maintenance of callus

The leaf explants were sterilized using mercuric chloride (0.5 %) for 5 min followed by washing with double distilled sterile water. The surface sterilized explants were inoculated on Murashige and Skoog's (MS) medium[19] supplemented with various growth hormones. The leaf callus was initiated, developed and maintained for 6 months on MS medium supplemented with IAA + IBA + 2, 4-D and kinetin (one ppm each) at 25±2° under 16 h diffused light (1600 lux) / 8 h darkness cycle.

### Growth kinetics study

The growth kinetics study of leaf callus culture was carried out for 15 weeks using fresh weight and dry weight parameters by regular sub culturing and recording its fresh and dry weights at every two weeks (0, 2, 4, 6, 8, 10, 12, 14 and 15$^{th}$ week).

### Preparation of sample

The powdered leaf (10 gm) was extracted with methanol (50ml) in Soxhlet for 4 hrs and the extract obtained was evaporated to dryness, which was redissolved in 10 ml of methanol. The leaf callus was dried at room temperature below 45°C for ten days and then 1 gm of dried callus was extracted with methanol (20ml) by shaking it for 24 hrs, filtered and filtrate was evaporated to dryness and redissolved in 3 ml of methanol. These samples were used for analysis of silybin by HPTLC.

### Standard silybin

Accurately weighed amount of reference standard silybin (Ranbaxy Laboratories Limited, Indore, India) was dissolved in HPLC grade methanol to get 500 µg /ml solution, which was then applied on TLC plate in different concentration for the preparation of calibration curve.

### Qualitative phytochemical Analysis

Preliminary phytochemical screening of methanol extracts of leaf and leaf callus were carried out for the detection of phytoconstituents using standard chemical tests.

### HPTLC instrumentation and conditions for quantification of Silybin

The samples were spotted in the form of bands of width 3 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (10 cm x 10 cm with 0.2 mm thickness, E.Merck, Germany) using a Camag Linomat V (Switzerland). A constant

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application rate of 150 nl/s was employed and space between two bands was 5.0 mm. Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase consists of Chloroform: acetone: formic acid (9:2:1). The optimized chamber saturation time for mobile phase was 10 min at room temperature. The length of chromatogram run was 60 mm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner IV in the absorbance mode at 254 nm. The source of radiation utilized was deuterium and tungsten lamp. The slit dimension was kept at 4 mm × 0.1 mm, and 20 mm/s scanning speed was employed.

**Preparation of calibration curves and validation of proposed method for Silybin**

Different volumes of standard solution of Silybine (500 µg/ml): 1, 2, 3, 4 and 5 µl were spotted in duplicate on TLC plate to obtain concentrations of 500, 1000, 1500, 2000, and 2500 ng per spot of Silybin respectively. The data of peak area obtained vs. drug concentration were treated by linear least-square regression and the regression equation thus obtained from standard curve was used for calculation of drug concentration in natural leaf and leaf callus.

The analyzed samples were spiked with extra 50, 100 and 150% of the standard Silybin and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug in proposed method.

The precision of method was obtained by determining the intra- and inter-day variation, which was carried out at two different concentration levels of 1000 and 1500 ng per spot.

**Quantification of Silybin in samples**

The 2µl of natural leaf extract and 3µl of leaf callus extracts were applied in duplicate on HPTLC plate for quantification. The silybin was quantified by using regression equation obtained from calibration curve and the mean of duplicate samples was calculated with respect to area. The average of amount of silybin obtained with respect to peak area was calculated.

## RESULTS AND DISCUSSION

The leaf callus of *Silybum marianum* was successfully initiated, developed and maintained on MS medium supplemented with IAA + IBA + 2, 4-D and kinetin (one ppm each).

The growth kinetics of callus culture showed that

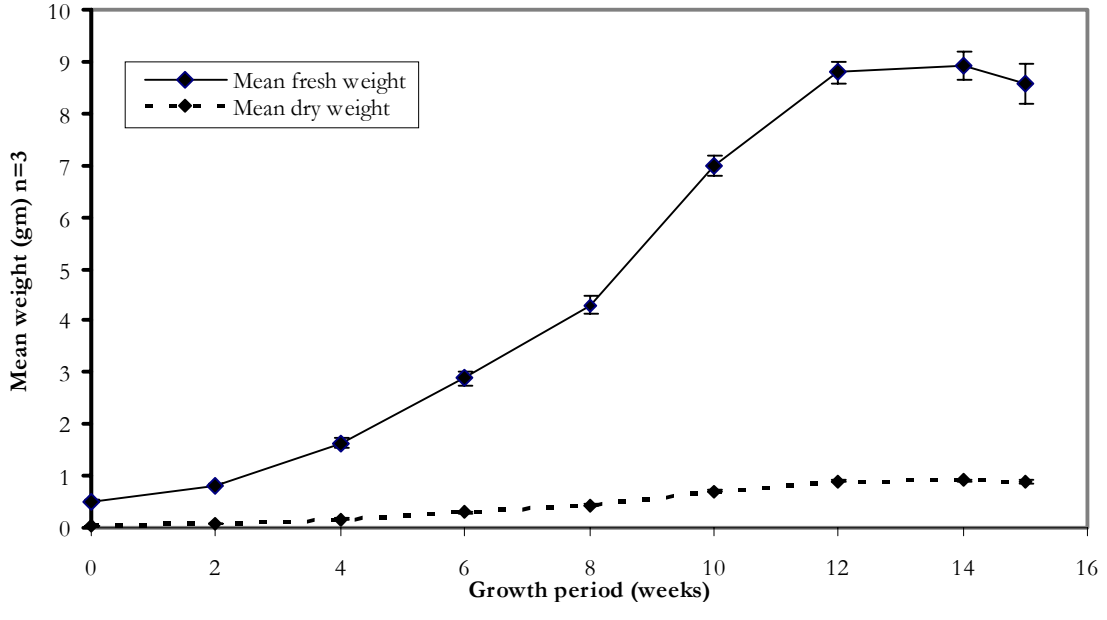


**Figure 1: Growth Kinetics of Callus Culture**

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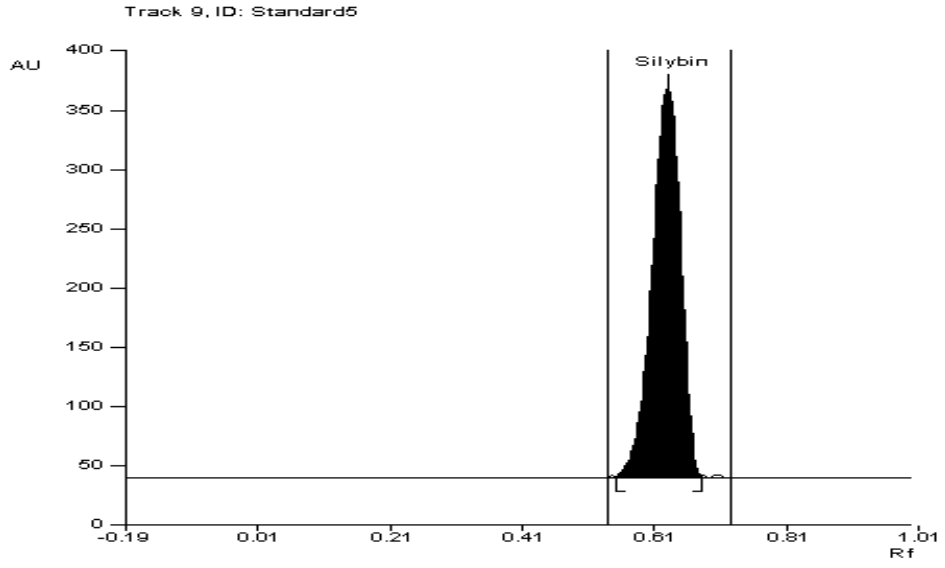


**Figure 2: A Simple HPTLC Chromatogram of Silybin**

the cells grow very well undergoing about 16-fold increase in total fresh weight at the end of the growth period. The growth circle is characterized by a relatively short lag-phase (8-10 days) followed by a long period of exponential growth until day 70, when the cell enter the stationary phase and senescent phase starts from 80-84 days (Figure 1).

Preliminary phytochemical screening of the aqueous alcoholic extracts of leaf and its in vitro culture revealed the presence alkaloids, amino acids, flavonoids, carbohydrates, phenolics, steroids and tannins in both the extracts.

HPTLC fingerprints of methanolic extracts were established using CAMAG HPTLC and chloroform: acetone: formic acid (9:2:1) as solvent system, which showed presence of 10 spots (Rf-value: 0.03, 0.07, 0.10, 0.19, 0.42, 0.51, 0.59, 0.63, 0.74, 0.79) and 8 spots (Rf-value: 0.03, 0.07, 0.19, 0.42, 0.52, 0.59, 0.63, 0.75) respectively, at 254 nm wavelength.

The solvent system used in the investigation was found to give compact spots for Silybine ($R_f$ value of 0.63 ± 0.05) figure 2. Densitometric analysis of Silybine was carried out in the absorbance mode at 254 nm. The linear regression analysis data for the calibration plots showed good linear relationship (r = 0.987) with respect to peak area in the concentration range 500-2500 ng per spot. The regression equation was found to be Y = 1309.907 + 2.381*X with respect to peak area.

The proposed method when used for extraction and subsequent estimation of silybin from natural plant after spiking with 50, 100 and 150% of additional drug afforded recovery of 98-101% as listed in TABLE 1.

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and results are depicted in TABLE 2, which revealed intra- and inter-day variation of silybin at two different concentration levels of 1000 and 1500 ng per spot.

The content of Silybin in different samples were calculated from the regression equations using values of area obtained from wincats software for different samples. The content of silybin was found to 0.112 % w/w in natural leaf and 0.126 % w/w D.W in leaf callus, which is 1.12 folds higher than that of

**TABLE 1: Recovery studies[a]**

| Excess drug added to the analyte (%) | Theoretical content (ng) | Content obtained (ng) | Recovery (%) |
|---|---|---|---|
| 0 | 1000 | 1007.38 | 100.74 |
| 50 | 1500 | 1527.28 | 101.82 |
| 100 | 2000 | 1965.53 | 98.28 |
| 150 | 2500 | 2485.87 | 99.43 |

[a] n=6

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**TABLE 2: Intra- and inter-day precision of HPTLC method[a]**

| Amount (ng per spot) | Intra-day precision | | | | Inter-day precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean Area | S.D. | %RSD | SE | Mean Area | S.D. | %RSD | SE |
| 1000 | 3993.31 | 63.07 | 1.58 | 25.75 | 3980.30 | 75.02 | 1.88 | 30.63 |
| 1500 | 5164.55 | 63.42 | 1.23 | 25.90 | 5144.29 | 77.09 | 1.49 | 31.48 |

[a] n=6

natural leaf. From the present investigations it can be stated that the higher amount of Silybin present in the in vitro cultures as compared to the natural leaf is responsible for the better carageenan and formaline induced anti-inflammatory activity of leaf callus as reported by Balian et al[20].

## CONCLUSION

The results obtained in the present investigations are significant from the point of view that the in vitro cultures generated from the leaf of Silybum marianum have all the enzyme and co-enzyme systems for the production of silybin. The method developed for quantification of silybin using CAMAG HPTLC system can be used for the quantification of silybin in natural products, pharmaceutical dosage forms and in bulk drugs. The results of the present methodology signify the potential of this method for large-scale production of silybin by plant tissue culture technique. However, advanced plant tissue culture techniques like suspension culture using precursor feeding, immobilized cell techniques, use of elicitors and genetically transformed tissue cultures using Agrobacterium can be tried for the better growth of mass as well as for the production of silybin in higher amount.

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