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Qualitative and Quantitative Analysis of Sinigrin in Different Parts *In Vitro* and *In Vivo* of *Brassica nigra* Plants

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Abstract Plants produce a wide range of organic compounds that are not directly involved in their reproduction and normal growth and development. A Laboratory and field study were conducted to optimize the concentration of Sinigrin which produced by *Brassica nigra* plants. Sinigrin concentrations in plant tissues at various plant parts were monitored using High Performance Liquid Chromatography (HPLC) technique. Concerning, the results of (HPLC) analysis of callus extracts showed that average amount of sinigrin were between 0.1-71.4 (µg g^{-1}). Furthermore, the results of HPLC analysis inducted that sinigrin concentration in seeds, Cotyledon leaves, true leaves and stems of field grown plant were ranged between 6.8-12.75(µg g^{-1}). In conclusion, this study demonstrated that sinigrin is located in all *B. nigra* plant parts of different concentrations with the highest amount in the mature seeds.

Keywords: *Brassica nigra, callus, sinigrin, High Performance Liquid Chromatography HPLC*

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1. Introduction

B. nigra cultivation is very limited in the north region of Iraq. Although, it's an annual plant which grows in many types of soils and adapted to acidic, neutral and alkaline conditions. *B. nigra* is an important plant which has many medicinal applications [1]. *B. nigra* contains isothiocynate glycoside named sinigrin and myrosin, which yield 0.7-1.3% mustard volatile oil. Over 90% of this oil is allyl isothiocyanate. The seeds contain about 4% of sinigrin which is a natural aliphatic glucosinolate, also known as (allyl-glucosinolate or 2-propenyl-glucosinolate) [2]. Sinigrin is an aliphatic glucosinolate compound which occurred naturally in some plants as thiosugar [3]. Sinigrin is very important pharmaceutical compound which has a wide range of medicinal uses such as anticancer, antibacterial, antifungal, antioxidant, anti-inflammatory, wound healing and biofumigation [4].

Tissue culture could be used to grow this important plant throughout the year. Tissue culture has been used successfully in the propagation of many medicinal plants and in the production of secondary metabolites from callus and cell cultures [5]. This technology provides the medical industry with pharmaceutical compounds throughout the year with high amount per space unit. Besides, tissue culture induces somaclonal variation in the cultured tissue which provides the opportunity to select the cells and the plants with high secondary metabolic production [6]. Improvement of the secondary metabolites production *in vitro* can also be achieved by the manipulation of medium components as well as the addition of precursors and growth regulators in the callus induction medium *in vivo* [6].

Lately the importance of sinigrin, draw the attention of many researchers. Thus the objective of the current study was to analyze the sinigrin in deferment parts of different *B. nigra* via parts *in vitro* and *in vivo* using High Performance Liquid Chromatography HPLC method.

2. Materials and Methods

2.1. Sterilization of *B. nigra* Seeds and SEed's Viability *in vitro* Test

The mature seeds of B. nigra plant were surface sterilized by submerging into a solution of 70% ethanol with continuous gentile stirring for 1 min. Then seeds were washed with sterile distilled water one time for 5 minutes. Seeds were then sterilized with different concentration of local disinfectant solution (bleach) which contains 6% sodium hypochlorite using 10% for 5 minutes. Then they were washed with sterile distilled water three times for 5 minutes each. Sterilized seeds were cultured under aseptic conditions in a Laminar Air Flow Hood on MS medium without plant growth regulators in plastic Petri dishes [7]. Ten seeds were cultured in each Petri dish. These Petri dishes were wrapped with parafilm and all the cultures were placed in light in the growth room at 21 ± 2°C with 16/8 hours light/dark photoperiod of a light

intensity 1000 Lux provided by cool white fluorescence lamps. After 14 days of incubation germination percentage (%) was calculated using the following formula:

$$\text{Germination percentage} = \frac{\text{number of germinated seeds}}{\text{number of total cultured seeds}} \times 100.$$

Callus induction In Vitro

Full strength hormone free (MS) medium was used for mature seeds germination [7]. Sucrose 30g L-1 was added to the medium. The pH was adjusted to 5.7 using 1N NaOH or 1N HCl. The final volume was complete to 1000 ml with distilled water and 7g L-1 agar type (Agar-Agar) was added as shown in (Table 1). In vitro grown seedlings were used as explants source. Fully expanded true leaves, cotyledon leaves, stems and roots (Figure 1) were excised and cultured on basal (MS) medium containing different combinations of growth regulators as shown in (Table 1) [8]. The explants were cultured by placing 5 explants in plastic Petri dishes with 10 replications. Petri dishes were wrapped with parafilm and incubated in the growth room at 21 ± 2°C and 16 /8 day and night with light intensity of 1000 Lux. The explants were observed for development of callus. After 4 weeks callus induction percentage (Cip) was calculated using the following formula [9]:

$$C_{ip} = \frac{\text{number of explants forming callus}}{\text{total number of cultured explants}} \text{x}100.$$

2.2. Field Experiments Planting Parts *in vivo*

Seeds of *B. nigra* were sown in pots of 30 cm in diameter which contain peat moos and sand in a 1:1 ratio. The pots were kept in the field until maturity. The plants were watered and fertilized as needed. Seeds of *B. nigra* germinated within one week. As the young seedlings emerged the cotyledon leaves were collected for sinigrin extraction. Two months later the plants reached the flowering stage (Figure 2).

At maturity, the stems and fully expanded leaves as well as the dry seeds were harvested and used to analyze sinigrin.

Table 1. Composition of callus induction medium used for *B. nigra* [11]

Medium	Modified MS-Medium Composition
MS	MS Salts (4.91 g L^{-1}) + Sucrose (30 g L^{-1}) + Agar (7 g L^{-1}) + 2 mg L^{-1} (2,4-D) + 2 mg L^{-1} (Kin)

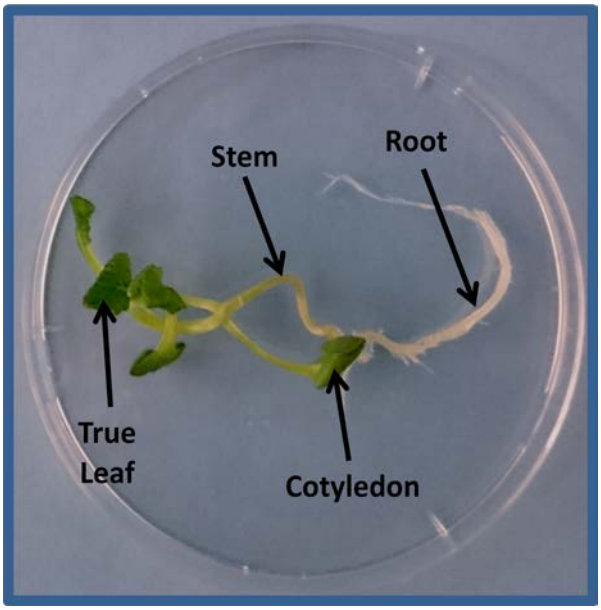


Figure 1. Seedling of *B. nigra*

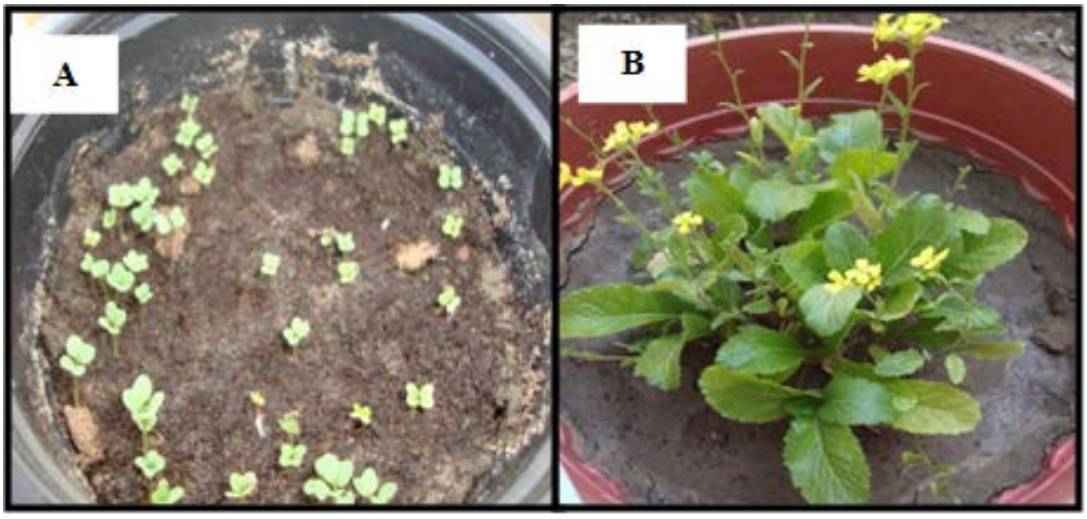


Figure 2. *Brassica nigra* plants grown in pots in the field: (A) within one week, (B) after two month

2.3. Extraction of Sinigrin from *in vitro and in vivo* Tissues

Sinigrin was extracted from mature mustard seeds, fully expanded leaves, cotyledonal leaves and stems (*in vivo*) Figure 3. All samples were dried in the oven at 60°C for 48hrs and ground to fine powder with mortar and pistil. The samples were prepared by dissolving 1g powder in 10 ml methanol in a test tube and kept for 24 hrs at room temperature for solvent extraction [10]. Each extract was centrifuged at 1780 rpm, 4°C for 20 min and then the supernatant was filtered through a 0.45µm PVDF membrane filter. The filtrate was collected and stored for further studies.

The stock solution of 30 ppm of stander sinigrin was prepared by dissolving 0.03 mg of pure sinigrin in 1ml deionized water using volumetric flask.

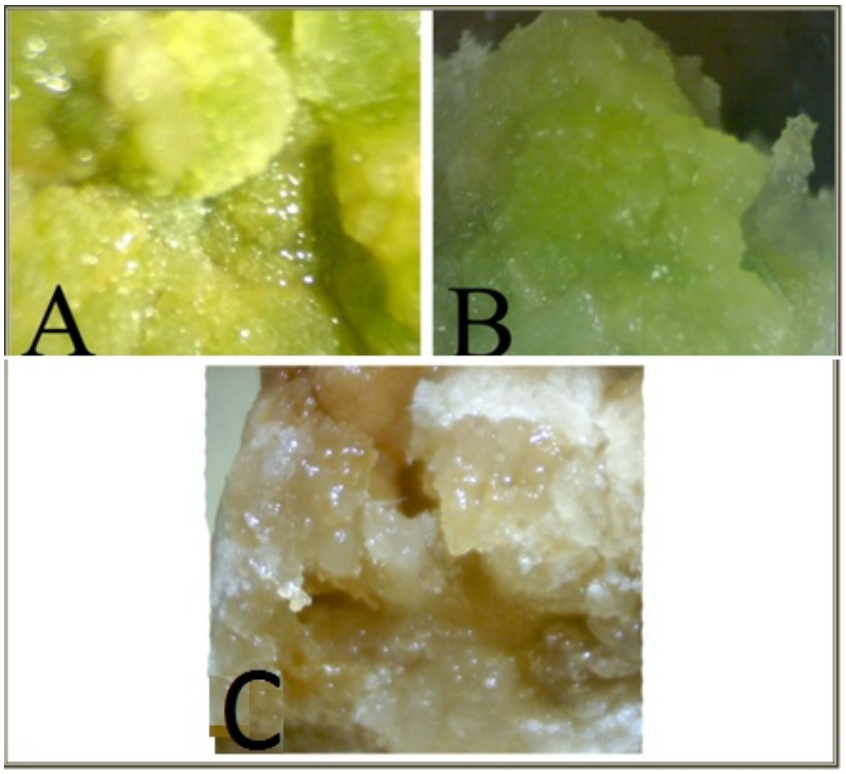


Figure 3. Callus varied in color induced from: (A) Cotyledon on MS medium, (B) stem on MS medium, (C) True leaf on MS medium

2.4. Qualitative and Quantitative Analysis of Sinigrin by HPLC

The chromatographic analyses were carried out on an HPLC system (Shimadzu Corporation, LC-2010 AHT, Japan). Sinigrin was separated on a plantium ODS (C18), 25cm Column (250×4.6mm, particle size 5mm), thermo stated at room temperature. The flow rate was 0.5 ml/min and the injection volume was 20µl. The mobile phase consisted of Acetonitrile: water (70:30) with U.V. detection at 280 nm. The samples were run in the mobile phase for all the treatments with three replications for each [11]. The concentration of active substances was quantified using the following equation [12].

$$\text{Concentration}_{\text{sample}}\,(\mu g/ml) = \frac{\text{Area of sample}}{\text{Area of standard}} \text{X conc. of standard X conc. of standard.}$$

3. Results and Discussions

3.1. Detection and Determination of Sinigrin in Different Parts of *B. nigra* in the Field Grown Plants Using HPLC

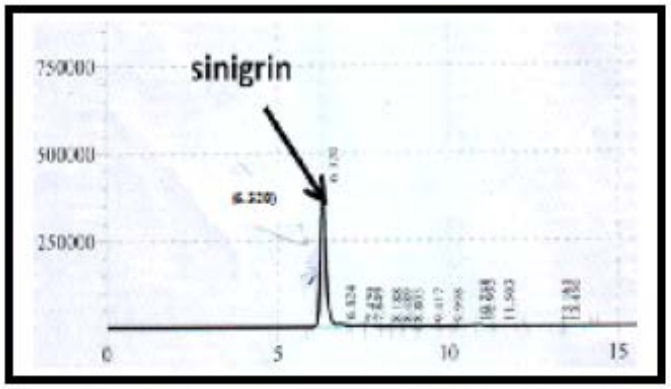
The results obtained from HPLC analysis showed that the retention time for the pure sinigrin was 6.32 min (Figure 4), while the retention time for the extracted sinigrin from the field grown plant parts was between 6.3 – 6.5 min (Figure 4 A, B, C and D). Quantitative analysis showed that seeds extract contained the highest amount of sinigrin with an average of 12.75 µg g^{-1} which was significantly different from the other plant parts (Table 2). In addition no significant differences between cotyledon leaves, true leaves and stems extracts in the amount of sinigrin which gave values of 7.0, 7.1 and 6.8 µg g^{-1} respectively (Table 2, Figure 4 A, B, C and D).

The result of the current study is in agreement with [13] who concluded that the seeds of *B. juncea* contain the highest level of in the stem of *B. juncea*. In addition, these results were in disagreement with those of [14] who reported that sinigrin content in the seeds was similar to that in the cotyledon and hypocotyl tissues in most B. species tested.

The amount of sinigrin is effected by myrosinase enzyme activity which hydrolysis sinigrin to allylisothiocyanate. However it has been found that the heat and low moisture reduce the activity of myrosinase [15]. Therefore; in the current experiment sinigrin was extracted from oven dry tissue by 100% methanol at 60ºC which prevent the sinigrin from the hydrolysis process. Also methanol extraction is more effective than aqueous method due to its polarity which degrades the cell walls of the seeds and releases the metabolites from the cells [16]. Variable extraction methods for the quantification of sinigrin by HPLC have been studied by several researchers. [17] reported that the 70% methanol at 70°C and the boiling 50% acetonitrile methods are more effective than water for the extraction of sinigrin from mustard seed.

Table 2. Sinigrin Content (µg g^{-1}) in Different Parts of *Brassica nigra* Field Grown Plants Using HPLC Technique

Field grown plant parts	Sinigrin concentration (µg g-1)
Seeds	12.75
Cotyledon	7.0
True leaves	7.1
Stems	6.8
LSD (p≤0.05)	0.64



HPLC chromatogram for the standard pure sinigrin

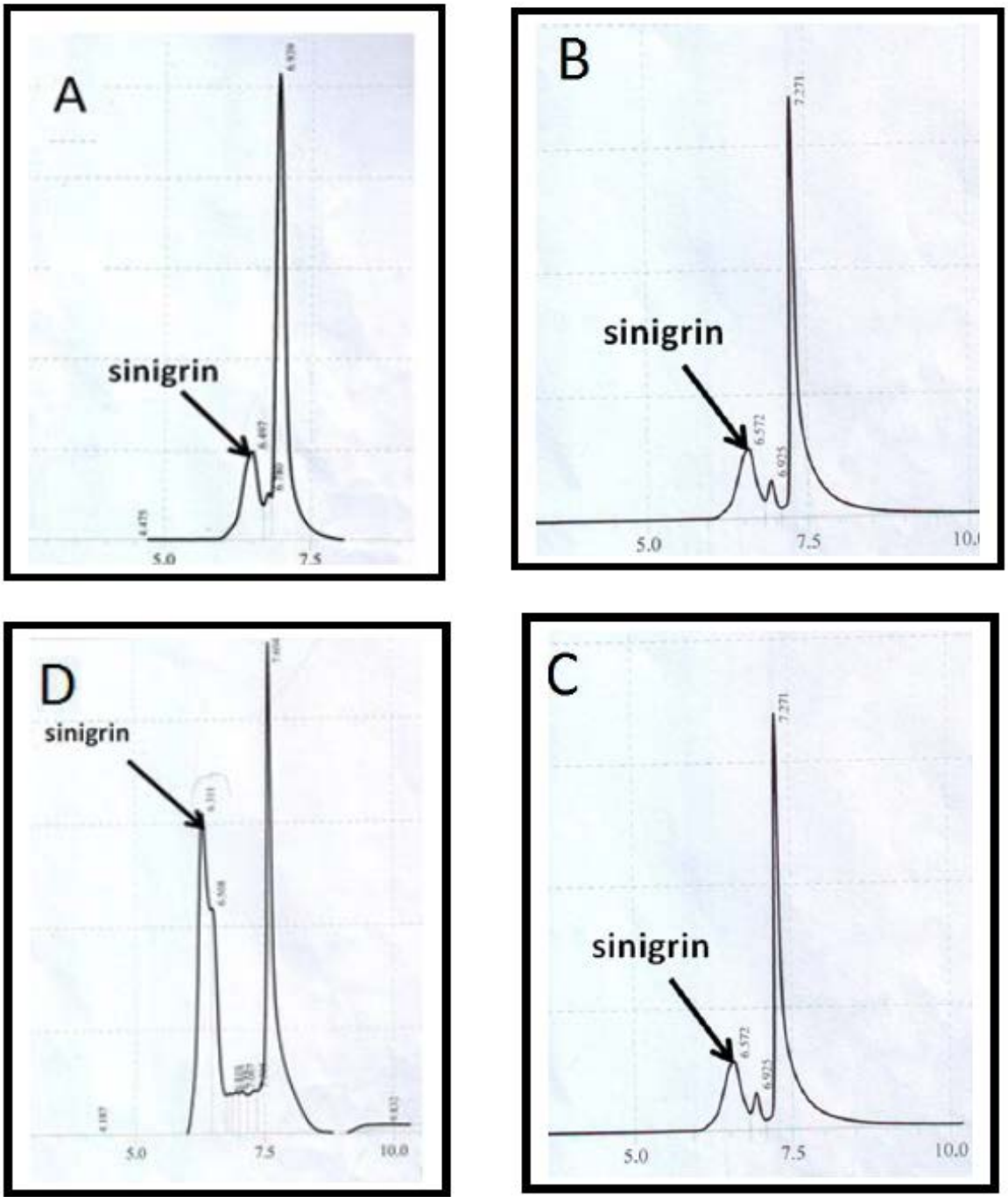


Figure 4. HPLC chromatograms of sinigrin extracted from plant parts of *B. nigra* grown *In vivo*: (A): Seeds (B): cotyledonal leaves (C): True leaves (D): Stems

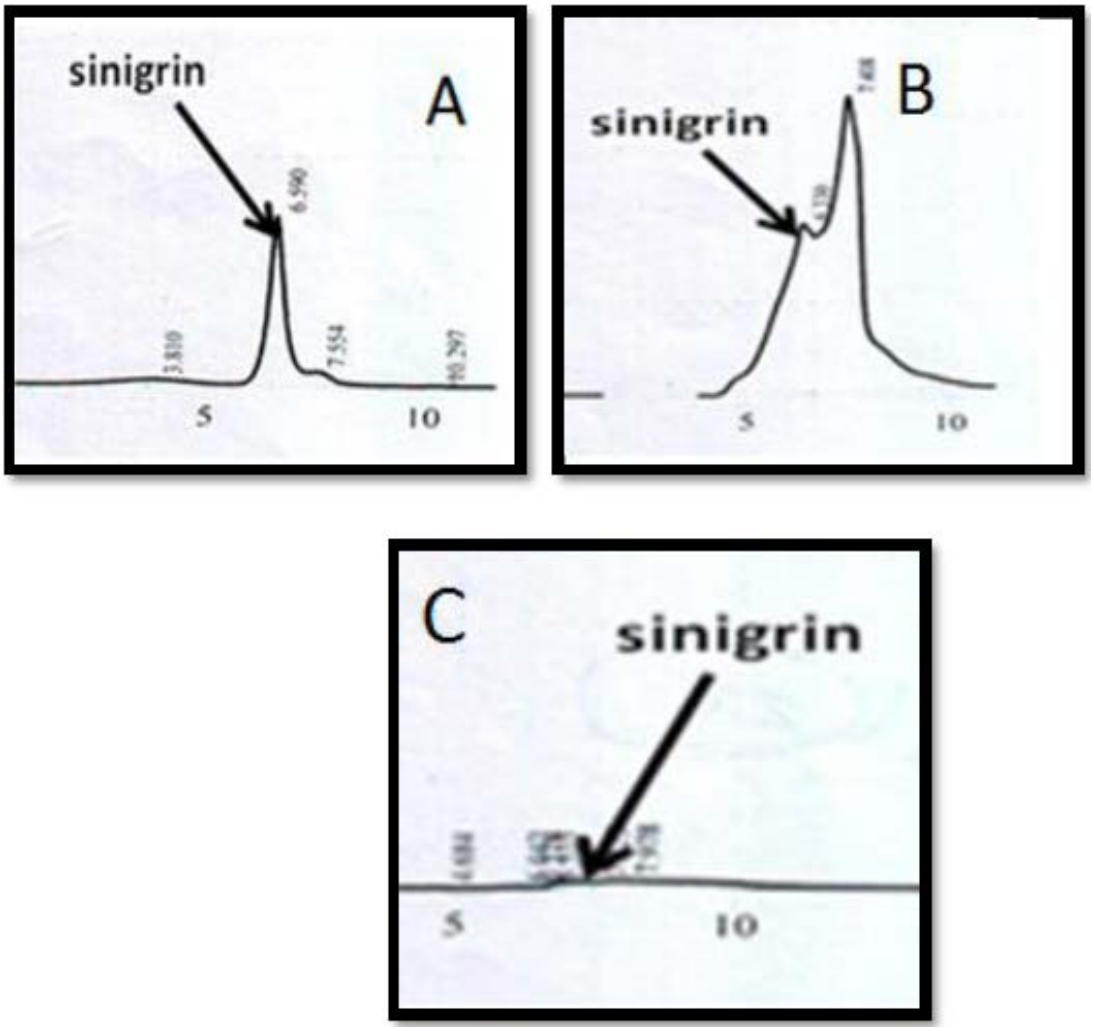


Figure 5. HPLC chromatograms of sinigrin extracted from callus induced from cotyledonal leaves of *Brassica nigra*: (A): Standard pure sinigrin. (B): Extract of callus induced from cotyledonal leaves of seeds treated with control (phosphate buffer) and grown on medium supplemented with L-methionine. (C): Extract of callus induced from cotyledonal leaves of seeds treated with control

This result indicated that there were highly significant differences between the samples in the amount of sinigrin compared with the seeds (Figure 5). Additionally, HPLC analysis showed that the highest amount of sinigrin was accumulated in the extract of callus induced from cotyledonal leaves excised from seeds treated with control (phosphate buffer) and grown on medium with an average of 71.4 µg g^{-1} about 560 % of the amount in the mature seeds (Table 2, Figure 4).

The results of the current study showed that the production of sinigrin *in vitro* is increased compared with the *in vivo* grown plants. Another study showed that the addition of sucrose to MS medium is the most effective factor for the induction of anthocyanin biosynthesis in *Arabidopsis thaliana* seedlings [18]. The addition of some amino acids as precursors to the culture media was used to increase the production of secondary metabolites in many plants. These results are in agreements with the results of the current study in the improvement of secondary metabolites production by the addition of precursors.

4. Conclusions

According to this study demonstrated that sinigrin is located in all *B. nigra* plant parts of different concentrations with the highest amount in the mature seeds. HPLC analysis showed that the mature seeds extract gave the highest amount of sinigrin compared with the other plant parts. Besides, the current study showed that the production of sinigrin *in vitro* is increased compared with the *in vivo* grown plants.

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