

Original Article

TOTAL POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF *SILYBUM MARIANUM* CULTURES GROWN ON DIFFERENT GROWTH REGULATORS

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ABSTRACT

Objective: The present study was designed to study the effect of supplementing *Silybum marianum* cultures with different combinations and concentrations of growth regulators on the total phenolic content and the antioxidant activity of the produced cultures.

Methods: The total polyphenolic content of 23 extracts of calli was calculated as μg of gallic acid/mg of dry weight extract (DWE) using Folin-Ciocalteu reagent. The antioxidant activity was calculated as trolox equivalent antioxidant capacity using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay for extracts containing more than 10 μg gallic acid/mg DWE.

Results: Most of the combinations showed an increase in the total polyphenolic concentration compared to the wild plant. However, cultures grown on media supplemented with combinations of benzyl aminopurine (BAP) and gibberellic acid (Gb), adenine (Ad) and kinetin (Kin), BAP and indole acetic acid (IAA) possessed the highest total polyphenolic contents 20.5, 13.09 and 12.15 μg gallic acid/mg DWE, respectively. However, only (BAP+Gb), (BAP+1-Naphthaleneacetic acid (NAA), IAA, kin and (BAP+Ad) showed a significant increase in the antioxidant activity calculated as trolox equivalent with 2.65, 1.56, 1.56, 1.35 and 1.22 folds increase over the wild plant, respectively.

Conclusion: The results obtained clearly indicated that changing the growth regulator system of *Silybum marianum* cultures serves as an easy and reliable method for increasing the total phenolic content and the antioxidant activity of cultures.

Keywords: *Silybum marianum*, Growth regulators, Polyphenolic, Antioxidant, Trolox, DPPH, Folin-Ciocalteu

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INTRODUCTION

Liver is one of the largest organs in the human body that performs several critical functions to detoxify the body of harmful substances [1]. Worldwide, liver diseases are regarded a major health problem. Globally in 2010, liver cirrhosis mortalities increased to over 1 million [2, 3]. Additionally, World Health Organization (WHO) fact sheets indicate that 130-150 million people globally have chronic hepatitis C infection, of which a significant number would develop liver cirrhosis or liver cancer and 500,000 yly mortality due to complications of late-stage liver diseases. In Egypt, the problem is even worse [4, 5]. Approximately 20% of Egyptian blood donors are anti-hepatitis C virus (anti-HCV) positive [6, 7].

Other estimates that Egypt has the highest prevalence of HCV worldwide, ranging from 6% to more than 40% among regions and demographic groups [8]. In Egypt, almost one-fifth (18.1%) of male mortalities 45-to 54-years old are due to liver cirrhosis [3]. In spite of the tremendous advances in modern medicine, no effective medicines are available to treat some liver problems. This fact puts a challenge for scientists to explore the hepatoprotective activity of plants based on traditional use or try to increase the activity of available drugs [9-11]. *Silybum marianum* is a well-known active hepatoprotective agent [12-14]. Silymarin, obtained from *S. marianum* is a widely used plant-derived hepatoprotective agent [15-19].

The properties of being antioxidant, free radical scavenger and stabilizer of the cell membrane are considered the important mechanisms of silymarin hepatoprotective action [20-24]. Thus, the aim of this work was to study the effect of different growth regulators combinations and concentrations on *S. marianum* cultures in an attempt to increase the activity using the antioxidant activity and the total polyphenolic concentration as a measure of the hepatoprotective activity of the grown cultures.

MATERIALS AND METHODS

Plant material

S. marianum aerial parts and fruits were collected from Khuorsheed, Alexandria. The fruits were collected during late April 2014. The aerial parts were collected from the same area during January 2015 in the pre-flowering stage and identified by the faculty of Science, University of Alexandria. Voucher specimens of the plant materials were deposited in the faculty of Pharmacy, University of Alexandria, specimen numbers (S. mar.1.14 and S. mar.1.15).

Chemicals and reagents

Murashige and Skoog (MS; Caission Laboratory, USA); BAP, Gb, Ad, Kin, IAA, NAA, 2,4-D, DPPH, chitosan, high molecular weight, trolox, folin-ciocalteu reagent and methyl jasmonate (Sigma Aldrich, USA); agar (Roko, Spain); phenylalanine (Universal Fine Chemicals PVT. LTD., India); sodium hypochlorite (5.25%, Clorox®); tween 20 (Serva, Germany); gallic acid (Mallinckrodt Chemical Inc., USA); sucrose, sodium nitroprusside and all other solvents (El Nasr Pharmaceutical Chemicals Company, Egypt).

Instruments

Microplate reader, SPECTRO star Nano (BMG LABTECH, Germany) was used to get the results of both the total polyphenolics and the antioxidant capacity.

Cell culture preparation and extraction procedure

Fruits were surface sterilised in 20% "clorox" bleach (30 min), washed three times in sterile purified water. Hypocots were initiated on semi-solid MS media for 14 d (d). The hypocots were then scored on their abaxial sides with a sterile scalpel blade and cut into 1 cm² pieces. Explants were cultured on 25 ml aliquots of MS regeneration medium with 1.5 mg/l BAP, 1.5 mg/l 2,4D, 30 g/l sucrose, and semi-solidified with 0.8% (w/v) agar, pH 5.6, in 9 cm

diameter petri-dishes. The explants were transferred onto fresh medium until callus was produced.

Cell suspensions were established by transferring 1-2 g fresh weight (f. wt.) of rapidly growing, friable undifferentiated callus and maintained in 100 ml MS liquid medium with same growth regulators, but no agar was added, pH 5.6 in 100 ml Erlenmeyer flasks. Cultures were maintained on a rotary shaker at 100 rpm and incubated under a 16 h photoperiod, with day and night temperatures of $23\pm 1^\circ\text{C}$.

For each growth regulator combination, aliquots of 2.5 ml packed cell volume (PCV) with 2.5 ml spent medium of the mother-stock were transferred to six 100 ml erlenmeyer flasks, each containing 45 ml of fresh media supplemented with the different growth regulators combinations. The cultures were kept at $23\pm 1^\circ\text{C}$, with a 16 h photoperiod and maintained on a rotary shaker at 100 rpm. After 28 d, cultures were harvested, extracted in 70% ethanol, dried and dissolved in dimethyl sulfoxide (DMSO) with a concentration of 1 mg/ml. Similarly, the fresh leafy samples were extracted and treated. Each experiment was repeated at least twice. All statistical analyses were performed by one-way analysis of variance (ANOVA) test, (design 2 between subject factors, $p < 0.000001$).

Estimation of total phenolic contents in calli tissues using folin-ciocalteu reagent (F-C)

The assay that utilizes F-C describes a microplate-adapted colorimetric total phenolics assay. The assay depends on electrons transfer in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined at 765 nm spectroscopically. The total phenolic content was expressed as gallic acid equivalents in mg/g of DWE.

Twenty μl of each extract, gallic acid standard concentrations (200-6.25 $\mu\text{g/ml}$) or DMSO (solvent blank) was placed in a 96 well plate to which 40 μl 10% F-C were added, the plate was gently shaken, then 160 μl 700 mmol Na_2CO_3 were added into each well and the plate was incubated at room temperature for 2 h. The color intensity was assayed using a microplate reader spectrophotometer adjusted at 765 nm. Total phenolics were calculated as gallic acid equivalents using the regression equation between gallic acid standards and A_{765} [25]. Calibration curve of gallic acid (μg concentration vs absorbance at 765 nm) is given in fig. 1.

Determination of the total antioxidant capacity-DPPH assay

The antioxidant capacity of *Silybum* extracts was determined relying on the redox potential of DPPH. The DPPH molecule is a stable free radical with a delocalized spare electron showing an absorption band in methanol at 520 nm yielding a deep violet colour.

In the presence of an antioxidant, DPPH is reduced with the consequent loss of the violet colour. 1 mmol DPPH solution (0.394 mg/ml) in methanol was prepared and then diluted 1:10 to obtain a 100 μM solution ($\text{Abs}_{515\text{ nm}} = 0.5-0.6$). 100 μl of each extract, trolox standard concentrations (25-0.78 $\mu\text{g/ml}$) or DMSO (solvent blank) was placed in a 96 well plate to which 100 μl of 100 μM DPPH solutions were mixed. Negative control was prepared with 100 μl of methanol and 100 μl of DPPH solution. All solutions were incubated in the dark at room temperature for 15 min. Absorbance was read at $\lambda = 515\text{ nm}$. Results were expressed as the percentage of reduction of the radical absorbance [26].

$$[\text{Abs max (negative control)} - \text{Abs (sample+DPPH)}] * 100 / \text{Abs max}$$

Where Abs max (negative control) is the absorbance 100 μl of methanol and 100 μl of DPPH solution and Abs (sample+DPPH) is the absorbance of 1000 μl of the sample and 100 μl of 100 μM DPPH solutions at 515 nm. Calibration curve of trolox antioxidant activity (μg concentration vs %inhibition) is given in fig. 2.

RESULTS AND DISCUSSION

Results for total phenolic contents in calli tissues and total antioxidant capacity are presented in table 1.

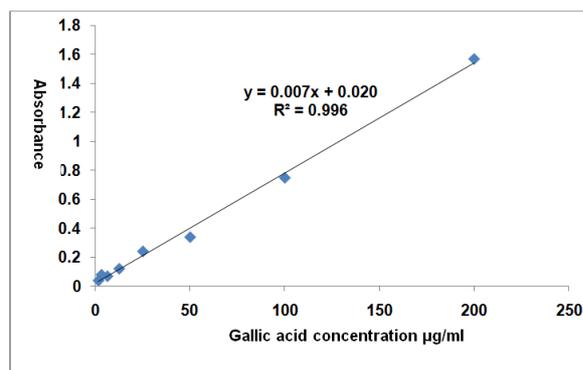


Fig. 1: Standard calibration curve of gallic acid (μg concentration vs absorbance at 765 nm)

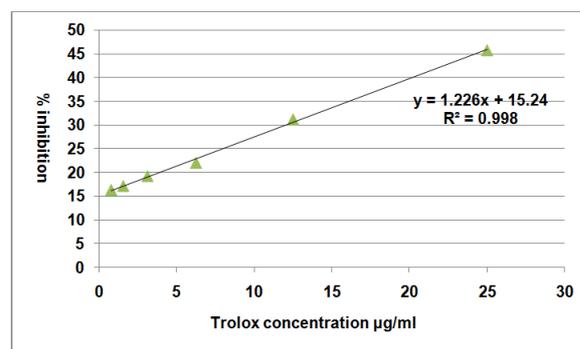


Fig. 2: Standard calibration curve of trolox antioxidant activity (μg concentration vs % inhibition)

Silymarin, the purified fruit and seed extract of *S. marianum* (commonly known as milk thistle), has long been considered as the most powerful hepatoprotective agent. It was first thought to be a single compound with the structure of 7-chromanol-3-methyl-taxifolin; recently it was found to be a mixture of several compounds namely silybin, isosilybin, silydianin and silychristin [22, 23]. Silymarin's hepatoprotective effects are accomplished via several mechanisms including antioxidation, free radical scavenging properties and stabilisation of cell membrane [20, 21, 24, 27], inhibition of lipid peroxidation [28, 29], protection of glutathione depletion [30]. Recently, studies have shown silymarin exhibits several anti-inflammatory effects [31, 32]. Silymarin has also promotes hepatic tissue regeneration through increasing hepatocyte protein synthesis [33]. Recent studies showed that silybin can also slow or even reverse fibrosis [34, 35] and has immunomodulatory effects on diseased liver [36, 37].

Antioxidant activity is considered the most important and is strongly linked to the polyphenolic content of the plant [38, 39]. In this study, supplementing the media with different growth regulators resulted in a general significant increase in the total phenolic content and consequently affected the antioxidant activity of *S. marianum* cell culture extract. Examining the results, it is clear that presence of IAA and/or BAP in the culture media induces a significant increase in phenolic synthesis pathway. Our results are in accordance with Tavieria *et al.* (2009) and Sayd *et al.* (2010) that showed that media supplemented with increased BAP produced higher phenolic compound content [40, 41]. Furthermore, Anastasia *et al.* (2010) showed that IAA significantly increases the production of phenolics in lentil plants [42]. Moreover, combining Gb and BAP resulted in the highest total phenolics production (20.5 μg) and this is in concurrence with the results of Jeong *et al.* (2007) who concluded that, hairy roots of *Panax ginseng* requires the presence of gibberellic acid and cytokinins in order to have an increase in the total phenolic compounds in culture [43]. However, only cultures supplemented with (BAP+Gb), 3 mg/l IAA, (BAP+NAA), 3 mg/l Kin or (BAP+Ad) showed a significant increase in the antioxidant efficiency.

Table 1: Mean µg of gallic acid/mg DWE and mean trolox equivalent antioxidant capacity of samples of *S. marianum* cultures grown on different concentrations and combinations of growth regulators

S. No.	Sample	Mean µgs of gallic acid/mg DWE	Mean µgs trolox equivalent antioxidant capacity/mg DWE
1	Fresh leaves (Khourshed)	4.77±0.09	5.42±0.24
2	Ad 1.5 mg/l+Kin 1.5 mg/l	13.09±0.13 (p<0.0001)	1.55±0.11 (p<0.0001)
3	IAA 1.5 mg/l+Ad 1.5 mg/l	6.75±0.97 (p<0.1116)	-
4	BAP 1.5 mg/l+NAA 1.5 mg/l	10.01±0.2 (p<0.0001)	8.48±0.09 (p<0.0003)
5	BAP 1.5 mg/l+IAA 1.5 mg/l	12.15±0.12 (p<0.0001)	3.75±0.12 (p<0.0002)
6	NAA 1.5 mg/l+IAA 1.5 mg/l	9.09±0.1 (p<0.0001)	-
7	NAA 1.5 mg/l+2Ph ind 1.5 mg/l	8.82±0.03 (p<0.0001)	-
8	IAA 3 mg/l	10.00±0.01 (p<0.0001)	8.49±0.12 (p<0.0003)
9	Kin 1.5 mg/l+NAA 1.5 mg/l	7.22±0.05 (p<0.0001)	-
10	Kin 1.5 mg/l+BAP 1.5 mg/l	9.27±0.15 (p<0.0001)	-
11	2,4D 1.5 mg/l+IAA 1.5 mg/l	10.66±0.16 (p<0.0001)	3.45±0.15 (p<0.0022)
12	IAA 1.5 mg/l+2ph ind 1.5 mg/l	12±0.27 (p<0.0001)	6.65±0.73 (p<0.1809)
13	Kin 1.5 mg/l+Gb 1.5 mg/l	9.75±0.06 (p<0.0001)	-
14	BAP 1.5 mg/l+Gb 1.5 mg/l	20.5±0.3 (p<0.0001)	14.35±1.32 (p<0.0026)
15	BAP 1.5 mg/l+Ad 1.5 mg/l	11.22±0.29 (p<0.0001)	6.63±0.15 (p<0.0124)
16	IAA 1.5 mg/l+Gb 1.5 mg/l	9.36±0.12 (p<0.0001)	-
17	2 ph Ind 1.5 mg/l+Kin 1.5 mg/l	8.46±0.03 (p<0.0001)	-
18	Kin 3 mg/l	10.19±0.19 (p<0.0001)	7.31±0.18 (p<0.0319)
19	2 ph ind 1.5 mg/l+Ad 1.5 mg/l	6.37±0.01 (p<0.0001)	-
20	2,4D 3 mg/l	9.01±0.15 (p<0.0001)	-
21	IAA 1.5 mg/l+Kin 1.5 mg/l	10.53±0.42 (p<0.0002)	4.07±0.34 (p<0.0319)
22	2 ph ind 1.5 mg/l+Gb 1.5 mg/l	9.15±0.14 (p<0.0001)	-
23	BAP 3 mg/l	8.49±0.14 (p<0.0001)	-
24	BAP 1.5 mg/l+2 ph ind 1.5 mg/l	8.63±0.37 (p<0.0005)	-

Values represent the mean of three replicates. Values are given as mean±SD, Ad= adenine; BAP= benzylaminopurine; 2,4D= 2,4-dichlorophenoxyacetic acid; Gb= gibberellic acid; IAA= indoleacetic acid; NAA= 1-naphthaleneacetic acid; 2Ph ind= 2-phenylindole.

CONCLUSION

The results obtained clearly indicated that changing the growth regulator system of *S. marianum* cultures serves as an easy and reliable method for increasing the total phenolic content and thus the antioxidant activity of cultures. Further studies are required may be with the addition of some elicitors or precursors.

CONFLICT OF INTERESTS

Declared none

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