Effect of different carbon sources on callus formation, pigment accumulation, and antiviral activity in cell cultures of *Euphorbia milii*

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Background and aim
Humans have long been relying on plants for food, shelter, and most importantly for medicines. *In vitro* culture techniques, which ensure rapid, consistent, and uniform mass production, are indispensable for manufacturing important plant-derived metabolites. However, insufficient information is available regarding the tissue culture of *Euphorbia milii* and its important bioactive compounds. This study mainly aims to explore the impact of different carbon sources on callus formation, pigment accumulation, and antiviral activity of *E. milii*.
Materials and methods
Using inflorescence tissues as explants, we investigated the effects of different types and concentrations of plant growth regulators (2,4-dichlorophenoxyacetic acid [2,4-D], alone or combined with benzyl aminopurine) on callus induction frequency. Moreover, we studied the effects of different concentrations of carbon sources (glucose, fructose, and mannose) on callus growth, pigment (chlorophylls, carotenoids, and anthocyanins) accumulation, and antiviral activities.
Results and conclusion
The results indicated that 2,4-D alone (1.0 mg/l or 1.5 mg/l) induced callus formation most effectively. Furthermore, glucose was detected to be the more effective carbon source for the accumulation of chlorophylls, carotenoids, and anthocyanins compared with fructose and mannose. The highest accumulation of pigments was induced by 20 or 40 mg/l glucose supplements, with no significant difference between the effects of different concentrations. Unlike glucose, fructose or mannose did not enhance pigment accumulation; moreover, high concentrations had negative effects on pigment accumulation. Similarly, glucose was more suitable for the antiviral activity of *E. milii* extracts than fructose and mannose. The results will support the effective production of secondary metabolites and offer insights into the antiviral potency of *E. milii*.

Keywords:
antiviral activity, callus induction, carbon source, *Euphorbia milii*, pigment accumulation

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Introduction

Euphorbia milii, also known as the crown of thorns, is an important medicinal plant species belonging to the family Euphorbiaceae. Euphorbia plants are used as traditional medicine for treating scorpion and snake bites, liver diseases, respiratory disorders, asthma, rheumatism, tumors, and warts for hundreds of years [1,2]. These plants are medicinally valuable due to their diverse secondary metabolites [3]. Previous reports have shown that plant pigments, showing antitumor, antioxidant, antiaging, and anti-inflammatory activities, are associated with several health benefits [4]; therefore, they are widely used in the modern food, cosmetic, and healthcare industries.

Currently, plant cell culture is a crucial strategy for the production of disease-free plants, rapid multiplication of valuable genotypes, nonseasonal crop production, and production of plant-derived metabolites with high commercial value [5,6]. This technology is advantageous for the production of active compounds, as it allows for a more reliable production as well as faster and more efficient isolation of phytochemicals compared with the isolation processes involving whole complex plant organs [7]. Calluses of numerous plant species are widely used in industrial applications. However, a few studies on the callogenesis of *E. milii* have been reported.

The effectiveness of a tissue culture protocol primarily depends on the media composition [8,9]. In particular, the carbon source in tissue culture medium is an

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absolute requirement for growth, development, and metabolite production in plant cell culture systems [10]. Different concentrations of carbon sources can remarkably impact the performance of the culture, which is attributable to their effects on the energy available for the cells and the osmotic potential of the medium. Dysregulation of these physiological factors related to cell survival potentially leads to significant alterations in cell expansion and division, and the biosynthesis of secondary metabolites [7].

To the best of our knowledge, no previous report has demonstrated the effects of different types and concentrations of carbon sources on *E. milii* cells. Therefore, the current study aims to explore the effects of different carbon sources on callus formation, pigment accumulation, and antiviral activity in cell cultures of *E. milii*.

Materials and methods

Plant material and surface sterilization of explants

E. milii grown in the greenhouse at the National Research Centre was used as the primary source of explants. Inflorescences in their first developmental stage were excised, washed under running tap water for 15 min, immersed in 70% ethanol for 1 min, washed twice with sterilized distilled water, and rinsed using 25% commercial Clorox (5% NaOCl) for 10 min. Aseptic explants were washed three times with sterilized distilled water, blotted, and dried on sterilized filter papers (90 mm).

Callus induction

Two sets of experiments were conducted to optimize the type and concentration of growth regulators and carbon sources suitable for high-frequency callus induction. In the first experimental set, shoot segments (0.5–1 cm) were cultured using the MS medium [11] supplemented with either different concentrations of 2,4-D (0.5, 1, 1.5 mg/l) or combinations of 2,4-D and benzyl aminopurine (BAP) (1 mg/l 2,4-D with either 0.5 or 1 mg/l BAP); all media were supplemented with 3% sucrose and 0.7% agar, and adjusted to pH 5.8. The media were sterilized by autoclaving at 121°C for 20 min.

In the second experimental set, shoot segments (0.5 cm) were cultured on MS medium supplemented with different concentrations of glucose (20, 40, 60, and 80 g/l), fructose (20, 40, 60, and 80 g/l), or mannose (20, 40, 60, and 80 g/l). All media were adjusted to pH 5.8 and supplemented with 1 mg/l 2,4-D and 0.7% agar. In both sets of experiments, the cultures were incubated at 25°C ±2°C for 4 weeks. Explants with successfully induced calli were scored.

Chlorophylls and carotenoid determination

The chlorophyll and carotenoid contents were investigated in 30-day-old calluses. For this assay, briefly, 100 mg of fresh calli was crushed with 5 ml of 80% acetone and centrifuged at 2500 rpm for 5 min. Subsequently, the absorbance of the supernatant was measured at 660, 645, and 470 nm. Chlorophyll A (Chla), chlorophyll B (Chlb), and carotenoid contents were calculated in mg/g FW biomass using the following equations according to Wellburn [12]:

$$Chla = \left\{ \frac{[12.21(A660) - 2.81(A645)]V}{(1000 * \mathrm{FW})} \right\}.$$

$$Chlb = \left\{ \frac{[20.13(A645) - 5.03(A660)]V}{(1000 * \mathrm{FW})} \right\}.$$

$$Carotenoid = \left\{ \frac{[1000(A470) - 3.27(Chla) - 104(Chlb)]V}{(1000 * \mathrm{FW})} \right\}.$$

A660, A645, and A470 represent the absorbance values in nm; *V* is the extract volume; and FW is the weight of the fresh callus.

Anthocyanin determination

Anthocyanin content was measured in 30-day-old calli following the method reported by Mori *et al.* [13] Anthocyanin compounds were extracted from 0.1 g of fresh callus ground in 10 ml of acidic methanolic solution [MeOH : HCl=99 : 1 (V/V)]). The solution was maintained in the dark for 24 h at 25°C, and subsequently, centrifuged for 10 min at 4000 rpm, and the supernatant was analyzed using a spectrophotometer at 550 nm. Anthocyanin content was calculated using the extinction coefficient (ϵ) of 33 000 cm/mol and according to the formula

A = €bc;

absorbance=A, cell width=1 cm=b, and anthocyanin concentration=c. Total anthocyanin content is presented as μmol/g FW.

Cell culture, transfections, and infections

Huh7.5 human hepatoma cells were gifted by C.M. Rice (Rockefeller University, New York, USA). These cells were incubated for 24 h, and subsequently, transfected with the 4a (ED43/CNS2/NS5A) plasmid, which provided by the Jens Bukh Department of Infectious Diseases and Clinical

Research Centre, University of Copenhagen, Denmark, using a mixture of Opti-MEM Reduced-Serum Medium (ThermoFisher Scientific, Waltham, Massachusetts, USA) and Lipofectamine 2000 reagent (Invitrogen). Subsequently, the cells were incubated for 24 h, and the medium was replaced with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% nonessential amino acids and 10% fetal bovine serum (FBS). After incubation for 48 h, culture filtrates were collected every 2 days for 7 days, as infectious hepatitis C virus (HCVs) were secreted into the supernatants of each infected cell, and the cells were preserved. Collected culture filtrates were concentrated using Amicon Ultra 15 centrifugal filter units at −4°C for 30 min. The concentered virus was preserved at −80°C [14,15].

Hepatitis C virus infection and immunostaining

Huh7.5 cells were seeded in Corning Biocoat collagen 8-well culture slide to reach confluency after incubation for 24 h in a humidified cell incubator maintaining 5% CO_2 and 95% air at 37°C with DMEM supplemented with 1% penicillin-streptomycin, 1% nonessential amino acids, and 10% FBS. After incubation, the concentrated virus mixed with seven different extracts (concentration 2 mg/ml) was incubated with the cells for 2 h. An experimental set without any viral infection was considered the negative control. The treated seven well-cultured slides were washed; DMEM supplemented with 1% penicillin-streptomycin, 1% nonessential amino acids, and 10% FBS were added to cover each well, and the slides were incubated at 37°C for 48 h. After incubation, the cells were washed with phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde in PBS for 1 h at room temperature, followed by permeabilization for 15 min in 0.1% Triton X-100 in PBS. A blocking buffer containing bovine serum albumin, FBS, and PBS was added and further incubated for 1 h at room temperature. The cells were incubated with primary antibodies (anti-NS5a) diluted in PBS for 2 h at room temperature. After washing the cells with PBS three times, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) was added to them at a 1 : 1000 dilution followed by incubation for 1 h at room temperature in the dark. After staining, slides were washed with PBS and mounted using ProLong Antifade. The slides were examined under a Zeiss Axiostar fluorescence microscope [16].

Statistical analysis

All the experiments were performed using a completely randomized design considering three replicates of each. The results are presented as mean±SE of triplicate measurements. IBM SPSS Statistics Subscription (IBM, Armonk, New York, USA) was used for statistical analyses of data; significant differences between means were determined using one-way analysis of variance and Duncan's test at P value less than 0.05.

Results

Callus induction

In general, explants cultured in media containing auxin (2,4-D) alone or in combination with cytokinin (BAP) induced callus formation at different frequencies (Table 1). In contrast, a medium free of any plant growth regulator (PGR) did not induce callus formation in investigated explants. The results showed more frequent callus formation induced by media containing 2,4-D alone compared with that induced by 2,4-D combined with BAP; the frequency of callus induction ranged from 20.00 ±5.78 to 36.67±6.67% on the media supplemented with 2,4-D alone. MS medium supplemented with 1 mg/l or 1.5 mg/l 2,4-D induced the highest frequencies of calli, with no significant differences between them.

Media with different carbon sources induced callus formation at varying degrees (Table 2). Media supplemented with glucose proved to have a higher ability to induce callus than those supplemented with either fructose or mannose. Moreover, 40 g/l glucose was the most effective for callus induction after 4 weeks of culture, with a callus fresh weight of 7.10±0.10 g. It was observed that high concentrations of all carbon sources affected callogenesis adversely.

Chlorophyll, carotenoid, and anthocyanin contents

The effect of three types of carbon sources (glucose, fructose, and mannose) on the accumulation of chlorophylls in callus culture has been studied and

Table 1 Effects of 2,4-D used alone or in combination with benzyl aminopurine on callus induction frequency

Concentrations of plant growth regulator (mg/l)	Callus induction frequency (%)
Control	$0.00±0.00^{d}$
0.5 2,4-D	$20.00±5.78^{b}$
1.0 2,4-D	$36.67±6.67^{a}$
1.5 2,4-D	$33.33±3.33^{a}$
1.0 2,4-D+0.5 BAP	$23.33±3.33^{b}$
1.0 2,4-D+1.0 BAP	$10.00±5.78^{c}$
1.0 2,4-D +1.5 BAP	$6.67±3.33^{c}$

Data are expressed as mean±SE of three replicates. 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine. Treatments denoted by different letters within a column are significantly different ($P<0.05$) according to Duncan's test.

compared with that of culture techniques involving sucrose as the carbon source (Table 3). Total chlorophyll content differed significantly between callus culture sets; a higher level of chlorophylls was observed in calli grown using either 20 or 40 g/l of glucose (4.93±0.09 mg/g FW and 5.03±0.18 mg/g FW, respectively, with no significant difference between these two groups) comparing with the chlorophyll content in callus supplemented with 30 g/l sucrose (control). In contrast, fructose or mannose supplements in media did not enhance the total chlorophyll content but rather had adverse effects in some cases.

Table 4 shows the effects of different carbon sources on the carotenoid content in cultured calli. Total carotenoid contents ranged from 1.33±0.09 to 0.27 ±0.07 mg/g FW. Maximum carotenoid contents were recorded to be 1.33±0.09 and 1.23±0.09 mg/g FW in calli cultured using 20 and 40 g/l of glucose, respectively, which indicated no significant difference between these two groups; whereas calli cultured using 80 g/l fructose or mannose showed the least carotenoid content (30.00±0.10 and 0.27±0.07 mg/l FW, respectively), indicating no significant differences between the effects of fructose and mannose.

Similarly, in terms of anthocyanin accumulation in callus cultures, glucose was detected to be a more effective carbon source than fructose and mannose (Table 5). Maximum anthocyanin accumulation was observed in calli cultured using 20 and 40 g/l glucose (156.67±6.67 and 160.00±5.77 μmol/g FW, respectively), which suggested no significant differences between these groups. The lowest anthocyanin contents were recorded in calli cultured using 80 g/l fructose or mannose (46.67±8.82 or 50.00 ±5.77 μmol/g FW, respectively), and no significant differences was detected between these two groups.

Table 2 Effects of different carbon sources on callus growth

Treatments	Concentration (g/l)	Callus fresh weight (g)
Control (sucrose)	30	6.03±0.12^{b}
Glucose	20	6.03±0.15^{b}
	40	7.10±0.10^{a}
	60	6.07±0.15^{b}
	80	5.13±0.15^{c}
Fructose	20	6.07±0.12^{b}
	40	6.13±0.09^{b}
	60	5.63±0.20^{bc}
	80	4.23±0.15^{d}
Mannose	20	6.00±0.12^{b}
	40	5.10±0.15^{c}
	60	5.17±0.17^{c}
	80	4.37±0.12^{d}

Data are expressed as mean±SE of three replicates. Treatments denoted by different letters within a column are significantly different ($P<0.05$) according to Duncan's test.

Antiviral activity

To explore the anti-HCV activity of *E. milii* through an immunostaining assay, HCV-infected Huh7.5 cells were treated with different *E. milii* extracts obtained from calli cultured using various carbon sources (Fig. 1 and Table 6). The results demonstrated different levels of fluorescence rate among the tested extracts; low fluorescence intensity indicated inhibition of HCV entry into cells reflecting the high degree of anti-HCV activity. Extracts obtained from calluses induced using either 20 or 40 g/l glucose showed the highest anti-HCV effect (indicated by the lowest intensity of fluorescence) than those obtained from calluses induced using either fructose (20 or 40 g/l) or sucrose (30 g/l), whereas, extracts obtained from

Table 3 Effects of different carbon sources on chlorophyll content

Treatments	Concentration (g/l)	Chlorophyll A (mg/g FW)	Chlorophyll B (mg/g FW)	Total chlorophyll (mg/g FW)
Control (sucrose)	30	1.10±0.12^{b}	2.03±0.09^{b}	3.13±0.12^{b}
Glucose	20	1.70±0.12^{a}	3.23±0.09^{a}	4.93±0.09^{a}
	40	1.77±0.17^{a}	3.27±0.17^{a}	5.03±0.18^{a}
	60	1.17±0.12^{b}	2.20±0.06^{b}	3.37±0.09^{b}
	80	1.03±0.09^{b}	2.07±0.09^{b}	3.10±0.12^{b}
Fructose	20	0.47±0.07^{c}	1.43±0.09^{c}	1.90±0.15^{c}
	40	1,03±0.03^{b}	2.00±0.06^{b}	3.03±0.15^{b}
	60	1.17±0.12^{b}	2.07±0.12^{b}	3.23±0.13^{b}
	80	0.50±0.06^{c}	2.13±0.15^{b}	2.63±0.19^{bc}
Mannose	20	0.43±0.09^{c}	1.40±0.06^{c}	1.83±0.15^{c}
	40	1.07±0.09^{b}	2.03±0.09^{b}	3.10±0.12^{b}
	60	1.13±0.15^{b}	2.03±0.13^{b}	3.17±0.12^{b}
	80	0.47±0.12^{c}	1.43±0.09^{c}	1.90±0.15^{c}

Data are expressed as mean±SE of three replicates. FW, fresh weight. Treatments denoted by different letters within a column are significantly different ($P<0.05$) according to Duncan's test.

Table 4 Effects of different carbon sources on carotenoid content

Treatments	Concentration (g/l)	Carotenoids (mg/g FW)
Control (sucrose)	30	0.70±0.06[b]
Glucose	20	1.33±0.09[a]
	40	1.23±0.09[a]
	60	0.73±0.03[b]
	80	0.77±0.07[b]
Fructose	20	0.67±0.03[b]
	40	0.80±0.06[b]
	60	0.77±0.09[b]
	80	0.30±0.10[c]
Mannose	20	0.63±0.03[b]
	40	0.73±0.09[b]
	60	0.70±0,06[b]
	80	0.27±0.07[c]

Data are expressed as mean±SE of three replicates. FW, fresh weight. Treatments denoted by different letters within a column are significantly different ($P<0.05$) according to Duncan's test.

Table 5 Effects of different carbon sources on anthocyanins content

Treatments	Concentration (g/l)	Anthocyanins (μmol/g FW)
Control (sucrose)	30	113.33±8.82[b]
Glucose	20	156.67±6.67[a]
	40	160.00±5.77[a]
	60	106.67±3.33[b]
	80	110.00±5.77[b]
Fructose	20	106.67±6.67[b]
	40	93.33±8.82[b]
	60	96.67±12.02[b]
	80	46.67±8.82[c]
Mannose	20	110.00±5.77[b]
	40	116.67±8.82[b]
	60	103.33±3.33[b]
	80	50.00±5.77[c]

Data are expressed as mean±SE of three replicates. FW, fresh weight. Treatments denoted by different letters within a column are significantly different ($P<0.05$) according to Duncan's test.

mannose-induced (20 or 40 g/l) calluses showed the lowest anti-HCV activity.

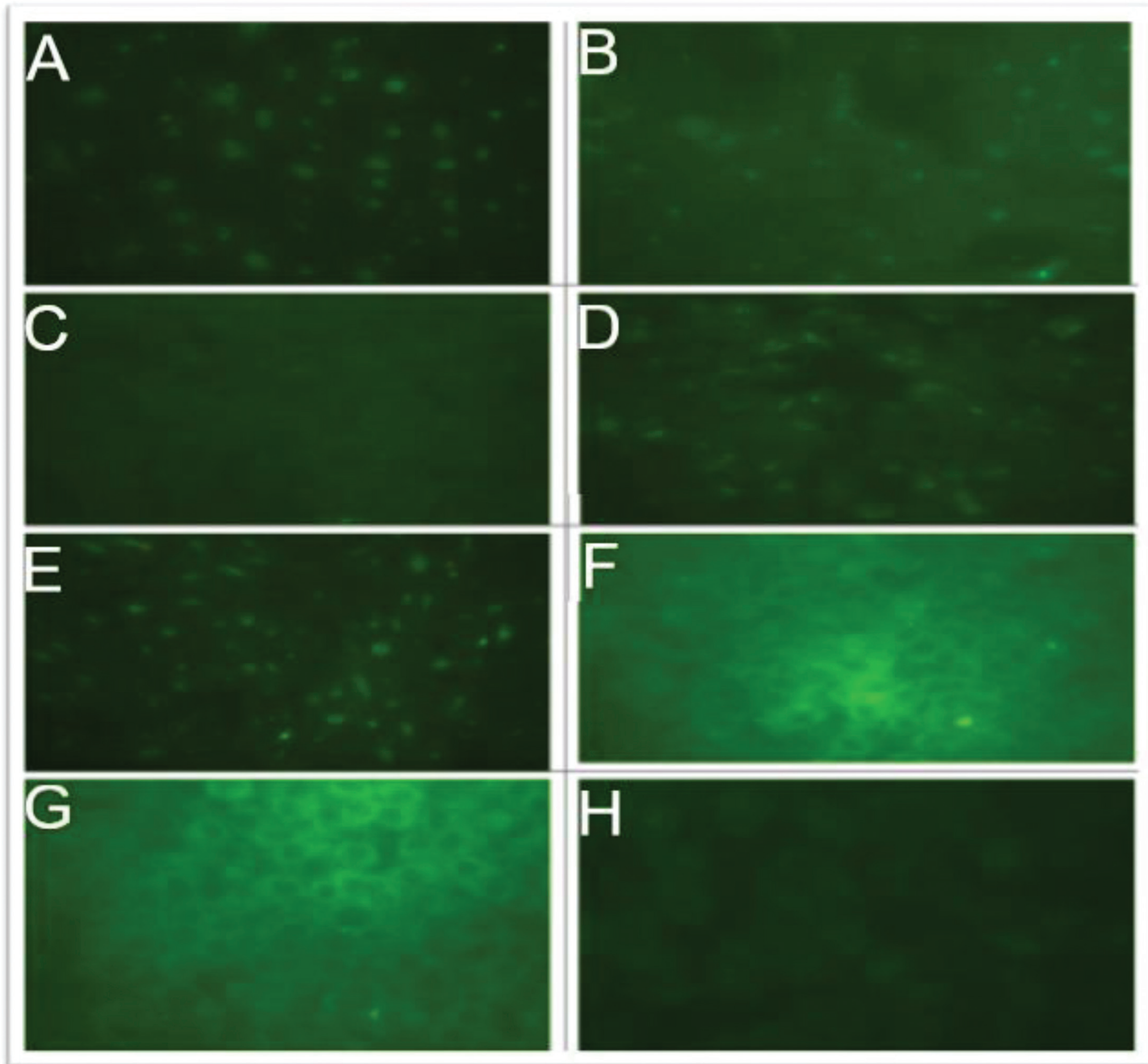
Discussion

Therapeutic phytochemicals have unique characteristics, and currently, they are considerably valued due to their natural origin, efficacy, safety, and minimal side effects [17,18]. In particular, medicinal plants have been used worldwide for centuries as the source of various biologically active compounds to treat multiple diseases, even those of unknown etiology [19,20]. *E. milii* was reported to exhibit numerous bioactivities including antioxidant, anticancer, antibacterial, and antifungal activities [21–23]; however, no report about its anti-HCV activities is available. In this study, all tested *E. milii* extracts showed various degrees of anti-HCV effects (Fig. 1 and Table 6), suggesting the potential of *E. milii* as an anti-HCV agent. Our results are comparable with previous reports on other medicinal plants such as *Valeriana wallichii* [24], *Iberis gibraltarica* [25], and *Artocarpus heterophyllus* [26]. The variable antiviral effects of *E. milii* extracts is possibly attributable to the differences in their phytochemical constituents induced by different carbon sources. Previous reports have confirmed that the carbon source affects the performance of the cultures, including the biosynthesis of secondary metabolites [7,10].

The ability of plant hormones promoting development in tissue cultures has been of interest to plant scientists for many years. Growth regulators play a major role in many factors associated with plant growth and development, including callus formation [27]. In our experiments, no callus was induced in explants cultured without growth regulators, even after 4 weeks of incubation (Table 1). Moreover, 2,4-D alone significantly stimulated callus formation. Conflicting reports are available on this topic; some of them demonstrated that 2,4-D alone acts as an effective growth regulator stimulating callus induction [28], while others suggested the enhanced effectiveness of 2,4-D combined with cytokinins (such as BAP) in increasing callus induction frequency [29]. These differences in opinions are possibly attributable to various endogenous hormone levels of the study materials. Callus formation is mainly controlled by the balance of endogenous and exogenous PGRs. As callogenesis is affected by multiple factors, which renders a comparison of the various outcomes of previous studies complicated. For example, genotype, explant type, and physiological status modulate the endogenous levels of growth regulators and, consequently, the effects of exogenous growth regulators [6,30]. It was hypothesized that equal amounts of auxin and cytokinin promote callus induction; however, in practice, the association differs greatly due to variable endogenous levels of phytohormones in plants [31]. The mode of interaction of these PGRs varies in different plant species and organs [32].

Cultured plant tissues need a continuous supply of carbohydrates from the medium for their *in vitro* growth and survival. The selection of specific carbohydrates as carbon and energy sources influences cell biomass accumulation owing to the low CO_2 levels in closed cultures during the

Figure 1



Immunostaining to investigate hepatitis C virus (HCV) in cultured Huh7.5 human hepatoma cells after treatment with different *Euphorbia milii* extracts obtained from calluses augmented with various carbon sources. (a) Callus induced using 30 g/l sucrose, (b) callus induced using 20 g/l glucose, (c) callus induced using 40 g/l glucose, (d) callus induced using 20 g/l fructose, (e) callus induced using 40 g/l fructose, (f) callus induced using 20 g/l mannose, (g) callus induced using 40 g/l mannose, and (h) negative control (no virus infection).

Table 6 Antiviral activities of different extracts of *Euphorbia milii* against hepatitis C virus

Extract code	Response
S30	++
G20	+++
G40	+++
F20	++
F40	++
M20	+
M40	+
Negative control	++++

+, low antiviral activity; ++, moderate antiviral activity; +++, high antiviral activity; ++++, very high antiviral activity (no virus).

photoperiod [30]. Although sucrose is the most commonly used in tissue culture as a carbon source, several reports have indicated the positive effects of other sugars [33]. Although the present study clearly showed that all four tested carbon sources supported callus growth, media supplemented with optimum concentrations (20 and 40 g/l) of glucose demonstrated significantly higher callus induction efficiency than those containing sucrose, fructose, or mannose (Table 3). This result demonstrates the significant influence of the type of carbon source on callus growth and indicates that glucose is the preferred carbon source during callus induction in *E milii* culture. Similar results were reported by Petersen and Krogstrup [34], which indicated best callus induction and embryogenic callus formation from leaf explants stimulated by the glucose supplement

compared with that achieved using either sucrose, fructose, maltose, or glucose combined with fructose.

The present study demonstrated that the accumulation of pigments, such as total chlorophyll, carotenoid, and anthocyanin, was significantly higher in *E. milii* calli induced by the glucose-supplemented media (Tables 3–5). Mathur *et al.* [35] reported that anthocyanin accumulation, associated with the cytodifferentiation process, is strongly influenced by the culture environment and medium variables. Previous cell culture investigations demonstrated that the elicitation of bioactive pigments in plants was affected by numerous factors, including elicitor specificity, concentration, and time of exposure [36]. It was also observed that callus cultures supplemented with 20 or 40 g/l glucose enhanced the biosynthesis of bioactive pigments compared with that achieved with 60 or 80 g/l glucose supplements. This supports a previous finding reported by Fauz *et al.* [36], which indicates exposure to higher concentrations of elicitors does not necessarily increase the accumulation of secondary metabolites. Treating plants with different concentrations of elicitor can facilitate optimization of the elicitor concentration for inducing bioactive compounds most effectively [37].

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Conflicts of interest
There are no conflicts of interest.

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