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ORIGINAL ARTICLE

Phytochemical characterization of callus cultures from the endangered plant *Crocus scepusiensis* (Rehm. & Woł.) Borbás ex Kulcz.

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Abstract

Crocus scepusiensis (Rehm. & Woł.) Borbás ex Kulcz., a critically endangered herbaceous plant which serves as a valuable source of bioactive compounds found across Europe and Asia. The aim of this study was to produce a calli from two different plant parts (leaf and shoot tip) for the critically endangered *C. scepusiensis* through tissue culture techniques, characterize the resulting calli through chemical profiling, with a focus on identifying key phytoconstituents, and lay the groundwork for future research on the biological activities of these calli extracts. Leaf disc and micro shoot tip explants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA) to induce organogenic calli. The resulting calli exhibited distinct biochemical profiles. Moreover, a phytochemical analysis was conducted to compare the metabolite composition of callus 1 (derived from leaf discs) and callus 2 (derived from shoot tips). Callus 1 displayed a higher total phenolic content (30.3558 ± 1.3564 mg (GAE)/g) compared to callus 2 (29.1543 ± 0.9754 mg (GAE)/g). Similarly, callus 1 exhibited a greater total flavonoid content (26.0089 ± 1.8029 mg (RE)/g) than callus 2 (18.4464 ± 1.4797 mg (RE)/g). Liquid chromatography-photodiode array-electrospray ionization-tandem mass spectrometry (LC-PDA-ESI-MS/MS) analysis revealed the presence of 26 and 25 constituents in callus 1 and 2, respectively. Fourteen and thirteen of these identified compounds have been previously reported in other *Crocus* species, with 22 constituents common to both calli. Twelve constituents were reported here in *Crocus* for the first time as far as we know.

Key message

Tissue culture successfully produced *Crocus scepusiensis* calli with distinct phytoconstituents profiles, potentially offering novel sources of bioactive compounds.

Keywords *Crocus scepusiensis* · Calli · LC–MS/MS · Tissue culture · Total flavonoid · Total phenolics

Introduction

The genus *Crocus* L., belonging to the Iridaceae family, encompasses over 80 known species distributed primarily across Europe and Asia (Escribano et al. 2000). These flowering plants are prized for their vibrant autumnal or early spring blooms, with *Crocus sativus* L. (saffron) being the most commercially valuable member due to its saffron spice derived from its stigmas. Saffron possesses a long history of use in culinary applications and traditional medicine; however, its high production cost and labor-intensive harvesting limit its widespread availability (Souret and Weathers 2000; Verma et al. 2016).

In contrast to *C. sativus*, *Crocus scepusiensis* (Rehm. & Woł.) Borbás ex Kulcz. is a lesser-known yet ecologically

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important species native to Central and Eastern Europe. This endangered herbaceous perennial is threatened by habitat loss and overcollection. Human activities such as urbanization, industrialization, and other land disturbances, as well as overcollection, contribute to habitat loss (Freytag et al. 2017). Notably, *Crocus* exhibits triploid sterility, meaning it relies solely on vegetative propagation for reproduction (Fernández 2004). This mode of reproduction restricts genetic diversity within the population, further jeopardizing its long-term survival (Fernández et al. 2011).

Plant tissue culture is one of the advanced biotechnological techniques which exploit the totipotency of plant cells and is widely applied to achieve different purposes. This technique offers an alternative way to traditional agriculture, plant breeding, and is a sustainable conservation strategy for endangered species like *Crocus* (Elshahawy et al. 2022). Applying tissue culture systems, also hold biotechnological value for *Crocus* as it's an efficient alternative to produce biological active compounds from important germplasms (Demeter et al. 2014; Gantait and Vahedi 2015). Plant tissue culture provides a transformational application by influencing secondary metabolite synthesis in plants. This can be accomplished through controlled growing conditions and genetic modification (Anushi et al. 2023). Since *Crocus* is a triploid hybrid (does not reproduce sexually), its genetic diversity is declining in part (Fernández et al. 2011). Thus, tissue culture can be an excellent way to conserve valuable genotypes of *Crocus* wild growing species. On the other hand, the main issue with micropropagation is contamination, which makes it difficult to produce many high-quality seedlings in a short amount of time (Altan et al. 2010). It's also important to take into consideration that tissue culture protocols can vary depending on the specific plant species, as each species may have unique requirements for optimal growth and multiplication. It's worth noting that the application of tissue culture techniques for *Crocus* species are not as thoroughly documented as other commercially relevant plants (Gantait and Vahedi 2015).

The scarcity of literature on *Crocus* scepusiensis tissue culture methods compared to other *Crocus* species can be attributed to several factors. Limited access to the plant itself might be a contributing factor, as some *Crocus* species are difficult to obtain due to rarity or conservation restrictions. Existing research on *Crocus* tissue culture demonstrates a variety of successful approaches. Demeter et al. (2010) achieved embryogenic calli in *C. heuffelianus*, while Sivanesan et al. (2012, 2014) established somatic embryos from *C. vernus* shoot regeneration using corm explants. Additionally, research documented callus culture establishment in numerous *Crocus* species, including *C. scepusiensis*, C. *tommasinianus*, *C. vittatus*, and *C. banaticus* (Freytag et al. 2017). However, the application of these diverse techniques to *C. scepusiensis* seems less explored. While studies on other *Crocus* species investigate methods like plant regeneration from floral explants, vegetative tissue callus cultures, direct micropropagation from corms skipping the callus stage, and even protoplast culture (Freytag et al. 2017), similar research for *C. scepusiensis* remains scarce.

This study addresses this gap in knowledge by establishing a callus culture system for *C. scepusiensis* using leaf disc and shoot tip explants. Callus cultures are undifferentiated masses of cells that can be a source of valuable secondary metabolites and serve as a platform for plant regeneration (Efferth 2019). We further compared the total phenolics, flavonoids content and the phytoconstituent profiles of the established calli, as these phytoconstituents are known to possess various biological activities.

Materials and methods

Source of *C. scepusiensis* explant

Leaves and shoot tips samples (30 days old seedling) of *C. scepusiensis* (Rehm. & Woł.) Borbás ex Kulcz plant were obtained from the Tatra Mountains and Podtatrze region in Poland (Latitude: Approximately 49°10' N to 49°30' N, Longitude: Approximately 19°30' E to 20°30' E). These old seedlings were used as explant sources for two parts of leaves and shoot tips in a 128 small size 0.5–1 cm, respectively. For decontamination, explants were sterilized in 70% ethanol for 10 min., followed by 10 min immersion in 40% commercial Clorox mixed with five drops of Tween 20. Thereafter, explants were rinsed multiples times in sterile distilled water (dist. H_2O).

Tissue culture conditions

Sterilized ex-plants were placed and maintained on the culture medium in a sterilized glass.

Petri dish (100 mm), contained 20 mL of sterilized full-strength MS medium 135 (Murashige and Skoog 1962), 3% (w/v) sucrose and solidified with 7% (w/v) Difcoagar (DifcoAgar Type, Sigma Co., USA) to stimulate growth. This MS media comprising a blend of macronutrients, micronutrients, vitamins (Table 1).

Also, a different concentration of plant growth regulators (PGR) was used in range from: 0.5

to 5 mg/L NAA, combined with 0.1 to 5 mg/L BA (Table 2). The pH value of MS medium was

adjusted to 5.8 before adding gelling agents. The media were autoclaved at 1.1 kg/cm^2 (121 °C) for 20 min. All cultures were incubated at a temperature-controlled growth

Table 1 Composition of the MS medium

Component	Elements	mg/L
Macro elements	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
Micro elements	KI	0.83
	H_3BO_3	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	Na_2.EDTA	37.3
	$FeSO_4.7H_2O$	27.8
Vitamins and organics	myo-Inositol	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
	Glycine	2.0
Others Agar	Agaropectin	7 g
	Sucrose	30 g

Table 2 The composition of MS medium used for callus induction

Code	Media composition
1	MS + 0.5 mg/L NAA + 0.5 mg/ L BA
2	MS + 0.5 mg/LNAA + 2.5 mg/L BA
3	MS + 1.5 mg/Ll NAA + 0.1 mg/Ll BA
4	MS + 1.5 mg/L NAA + 0.5 mg/Ll BA

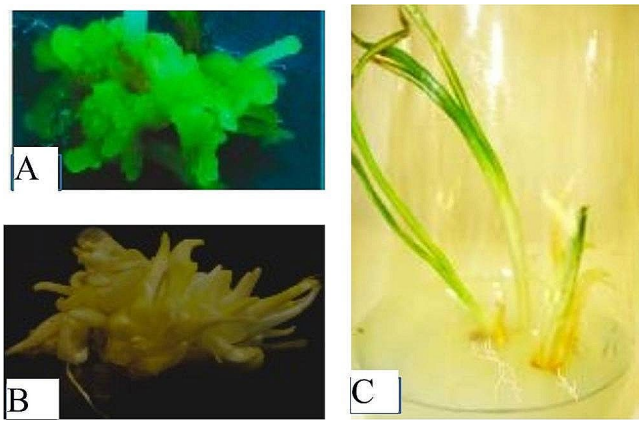

Fig. 1 Illustration of the different stages of regeneration to achieving intact plant. (**A**) Initiation of regenerated shoot form callus of shoot tip. (**B**) Initiation of regenerated shoot form callus of leaf. (**c**) Whole intact plant

room at 25 ± 2°C with 16 / 8 h. light / dark cycles (Intensity of used light is 50–100 µmol/ m^2/sec provided by cool white fluorescent lamps). Humidity was approximately 60–70%. After 30–45 days, jars were checked, photographed and the calli frequencies were scored. This experiment was consisted of 5 petri dishes per treatment as replicates.

To promote quick multiplication, the actively growing tissue was frequently (each 5 weeks) transferred to the appropriate fresh modified MS medium. Once sufficient shoots were produced, they were transferred to a rooting basal full and half strength MS medium for root development. Once plantlets had formed roots, they were gradually exposed to external environmental conditions such as increased light and humidity for acclimatization to their natural environment. Rooting and shooting will be completed and further studied in the future. Figure 1 will clarify the outline the steps of initiation process of future studies from regeneration to achieving a complete intact plant.

According to a statistical study, each treatment used five replicate Petri dishes with five explants per treatment (modified media). One way analysis of variance was used to compare means in the data analysis using ANOVA, and the significant was determined by Duncan's (1955) multiple range test using SAS (V.9.2).

Extract preparation

Following incubation, calli cultures from the most effective treatment were collected, dried, and ground into a fine powder. The powder was then macerated in 70% methanol for three days. Subsequently, the extracts were concentrated to dryness using a rotary evaporator under reduced pressure at 40 °C. The resulting dried extracts were immediately collected and stored at 4 °C in screw-cap containers for further phytochemical analysis.

Phytochemical study

Total phenolics content of both calli were determined using Folin-Ciocalteu colorimetric method (Attard 2013). The total flavonoid contents of both calli were determined using aluminum chloride colorimetric method (Kiranmai et al. 2011). Additionally, the phytochemical profiles of both calli were characterized using advanced Liquid chromatography-photodiode array-electrospray ionization-tandem mass spectrometry (LC-PDA-ESI-MS/MS) analysis to identify and differentiate the presence of bioactive compounds.

Total phenolics

Gallic acid standard stock solution in methanol at a concentration of 1 mg/mL was made, and eight serial dilutions at concentrations of (25, 50, 100, 200, 400, 600, 800, and 1000 µg/mL) were prepared, then callus samples were then made at concentrations of 5 mg/mL in absolute methanol. By using the Folin-Ciocalteu technique, the total phenolic

content was determined (Attard 2013). 100 µL of diluted Folin-Ciocalteu reagent was combined with 10 µL of sample or standard in a 96-well microplate. The mixture was then mixed with 80 µL of 4 N Na_2CO_3, and it was left for 20 min. in the dark at room temperature (25 °C). The blue complex color that resulted after the incubation period was measured at 630 nm. The data are shown as means ± standard deviation (SD). Microplate reader FluoStar Omega was used to capture the data. The total phenolic content of the extract was expressed as mg gallic acid equivalents (GAE)/ g dry weight.

Total flavonoids

Ten serial dilutions of the 1 mg/mL rutin standard stock solution in absolute methanol were created at concentrations of 1000, 800, 500, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL. The samples were then made at a 20 mg/mL concentration in MeOH. With a few minor adjustments to be carried out in microplates, the aluminum chloride method as described by (Kiranmai et al. 2011) was used to determine the total flavonoids content. In a 96-well microplate, 15 µL of sample/ standard was first added. Next, 175 µL of methanol and 30 µL of 1.25% $AlCl_3$. After 5 min. of incubation, 30 µL of 0.125 M $C_2H_3NaO_2$ was added. Then the resulting yellow color was detected at 420 nm. Data is displayed as means SD. FluoStar Omegaas, a microplate reader that measures at 420 nm, was used to record the results. Data was displayed as means ± SD. FluoStar Omega, a microplate reader, was used to record the outcomes. Total flavonoid content of the extract was expressed as mg rutin equivalents (RE)/ g dry weight.

Thin layer chromatography (TLC)

As a preliminary qualitative evaluation of phytoconstituents of the produced calli. A part of each extract (5 mg) was dissolved in absolute methanol (3 mL), spotted on precoated silica gel thin layer chromatography (TLC) plates (Merck), and developed in four different solvent systems EtOAc-Hexane (8:2), EtOAc-MeOH (6:4), and MeOH–$CHCl_3$ (2:8). The plates were firstly visualized under UV light. at 254 and 365 nm first and then visualized by spraying with anisaldehyde reagent and heating at 100 °C (Wagner and Bladt 1996).

Liquid chromatography-photodiode array-electrospray ionization–tandem mass spectrometry (LC-PDA-ESI-MS/MS) analysis of calli' phytoconstituents

LC-PDA-ESI-MS/MS analysis was performed on a Shimadzu LC-20AD solvent delivery system equipped with binary pump, refrigerated autosampler, mobile phase degazer, column oven and interfaced to photodiode array detector (PDA) and a Bruker amaZon Speed mass spectrometer. The mobile phase was freshly prepared by filtering through membrane disc filter (0.45 µM). High-performance liquid chromatography separation was conducted via reversed phase C_{18} HPLC (Inertsil ODS-3, 4.6 × 150 mm, GL Sciences Inc.) at flow rate 0.45 mL/min. and oven temperature 40 °C, eluted with a gradient of two solvents; 0.1% acetic acid in H_2O (solvent A) and 0.1% acetic acid in CH_3CN (solvent B). Gradient program was performed as follows: 10–100% B from 0 to 12 min., 1000% B from 12 to 15 min. MS interface parameters (source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 50 L/ hr., cone voltage 50 eV, capillary voltage 3 kV, and desolvation gas flow 600 L/hr.). Spectra were recorded between 50 and 2000 *m/z*. The mass spectra were detected in negative and positive modes, a Compass Data Analysis viewer software was used to compare retention times, UV spectra at the range between 200 and 500 nm obtained from PDA and *m/z* values obtained by MS/MS2.

Results

Calli initiation

For morphogenetic responses of culture conditions are summarized as shown in Fig. 2. Surface sterilized 0.5–1 cm of leaf parts and shoot tip explants derived from young seedlings were transferred to a culture MS medium containing different concentration of NAA and BA for the initiation of first step of tissue cultures system as summarized in Table 3.

Generally, initiation of calli were appeared during 3 weeks. After 45-day-old culture *C. scepusiensis*, shoot tips on MS medium supplemented with the highest level of auxin (5 mg/L NAA and 0.5 mg/L BA) appeared the most suitable for calli induction 3.4 ± 0.24 (Fig. 2B left plate). The number of calli induction/shoot tip explant on the same medium was 3.0 ± 0.00, these calli were ready for subculture/maintenance. When NAA concentration was reduced to 2.5 mg/L and the same BA concentration 0.5 mg/L, yellowish, friable and smooth calli were appeared on explant of leaf discs (Fig. 2A). A healthy green, compact and smooth calli per explant was produced from shoot tips than leaf explants. Media supplemented with 2.5 mg/L NAA and 2.5 mg/L BA was used in maintaining calli culture of shoot tips (Fig. 2B right plate). The calli culture of both explants was maintained on the previous specific MS medium with 2.5 mg/L NAA and 2.5 mg/L BA for an extended period due to their slow and healthy growth, allowing for the long-term maintenance of the calli. All calli cultures were maintained at a

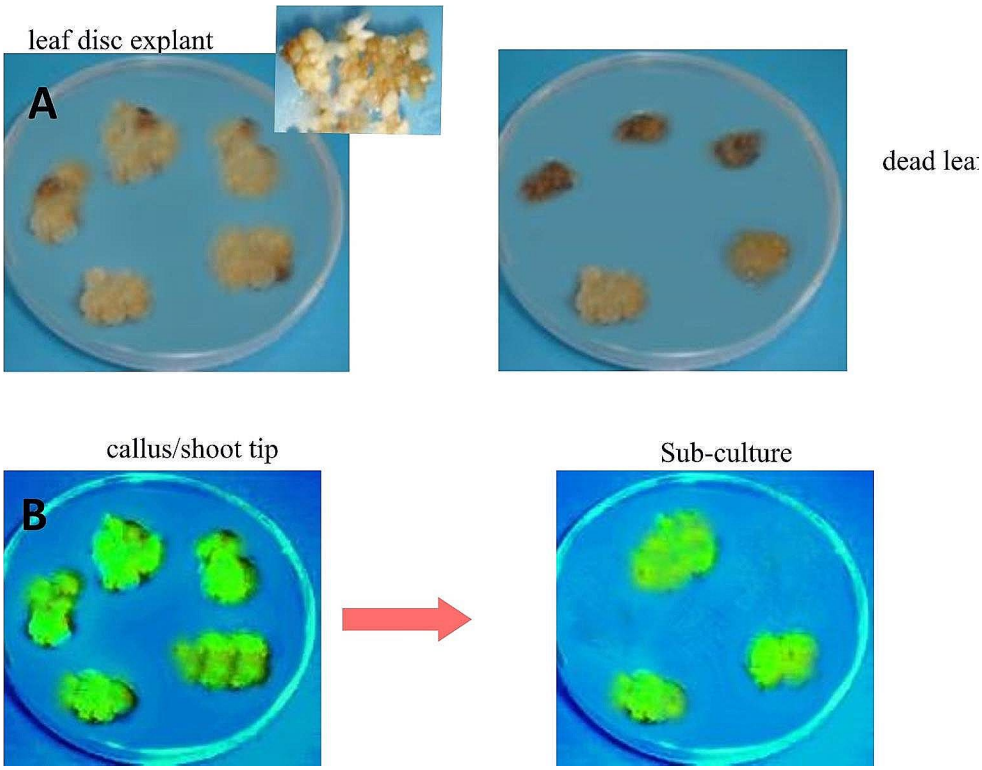

Fig. 2 (**A**) Yellowish, friable and smooth calli of *C. scepusiensis* leaf disc on MS medium supplemented with 2.5 mg/L NAA and 0.5 mg/L BA (left plate) compare to calli per leaf disc on 5 mg/L NAA + 0.5 mg/L BA (right plate). (**B**) Healthy green callus formation/ shoot tip explants cultured on MS medium with 5 mg/L NAA and 0.5 mg/L BA (left plate) and maintenance callus/shoot tip on modified MS medium with 2.5 mg/L NAA and 2.5 mg/L BA (right plate)

Table 3 Effect of different concentrations of NAA and BA on induction of calli from two different explants of *C. scepusiensis*

MS media supplemented with NAA + BA	Incubation condition at temperature-controlled growth room at 25 ± 2˚ C with 16 / 8 h./ dark cycles			
	Callus induction		No. of callus per explant	
	Leaf disc	Shoot tip	Leaf disc	Shoot tip
2.5 mg/L NAA + 0.1 mg/L BA	0.76 ± 0.199B	0.8 ± 0.20D	0.00 ± 0.00D	0.6 ± 0.24 C
2.5 mg/L NAA + 0.5 mg/L BA	1.17 ± 0.10 A	2.6 ± 0.24B	1 ± 0.00 A	1.2 ± 0.37BC
2.5 mg/L NAA + 2.5 mg/L BA	1.02 ± 0.23AB	2.4 ± 0.24BC	0.7 ± 0.15AB	1.6 ± 0.24B
5 mg/L NAA + 2.5 mg/L BA	1.02 ± 0.23AB	1.8 ± 0.37 C	0.3 ± 0.00CD	1.8 ± 0.20B
5 mg/L NAA + 0.5 mg/L BA	1.00 ± 0.01AB	3.4 ± 0.24 A	0.5 ± 0.16BC	3.0 ± 0.00 A

Values are the mean of 5 replicates/treatment ± SE.

Means followed by same letters within a column are not significantly different.

Table 4 Total phenolics and total flavonoids content of induced calli from the two explants of *C. scepusiensis*

Callus sample	Total phenolics mg gallic acid equivalents (GAE)/ g D.W.	Total flavonoids mg rutin equivalents (RE)/ g D.W.
Leaf explant 1	30.3558 ± 1.3564	26.0089 ± 1.8029
Shoot explant 2	29.1543 ± 0.9754	18.4464 ± 1.4797

temperature-controlled growth room at 25 ± 2°C fitted with lighting system that delivers an illumination with intensity of (50–100 µmol/m^2/sec.) for 16/8 hr. day and night periods.

Phytochemical study

Total phenolics and total flavonoids

Total phenolics and flavonoid contents of the calli induced by the two explants were determined and the results are presented in Table 4. The results showed that the mean phenolic content of Leaf callus is 30.3558 mg/g Dry Weight which is slightly higher than that of shoot callus which is 29.1543 mg/g D.W. Leaf callus also exhibits higher flavonoid content (26.0089 mg/g D.W.) compared to shoot callus which is 18.4464 mg/g D.W.

Thin layer chromatography (TLC)

By comparing the TLC of both extracts, both extracts showed identical images which indicates no notable difference in their chemical constituents.

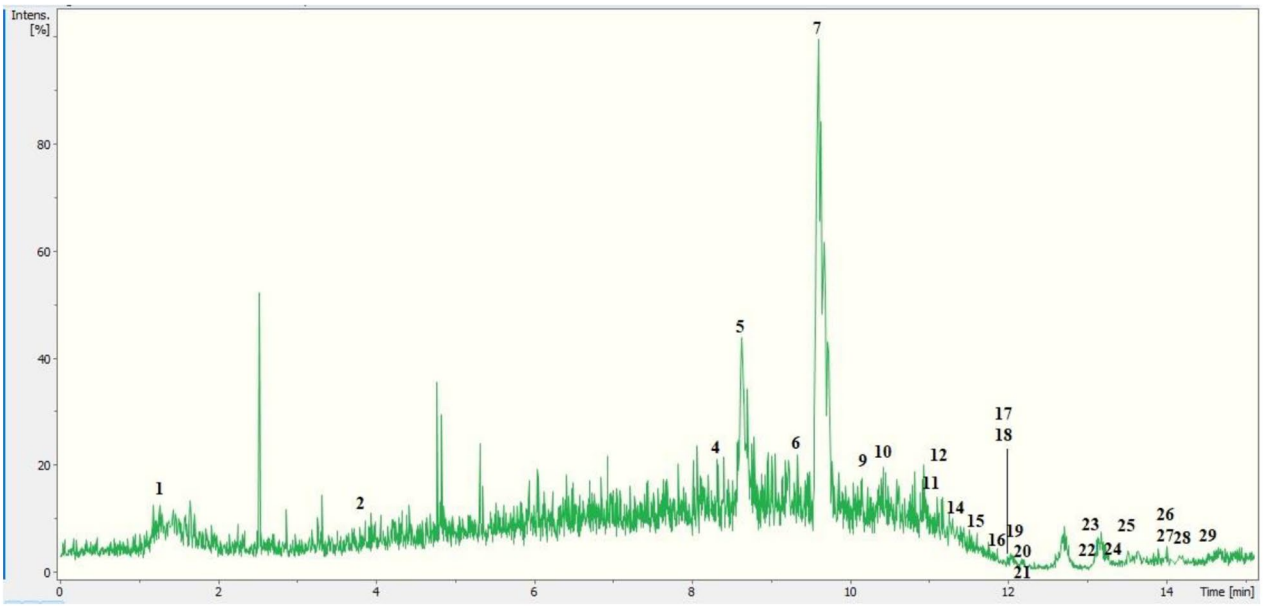

Fig. 3 Base peak chromatogram of callus 1 by LC-PDA-ESI-MS/MS in a negative ion mode

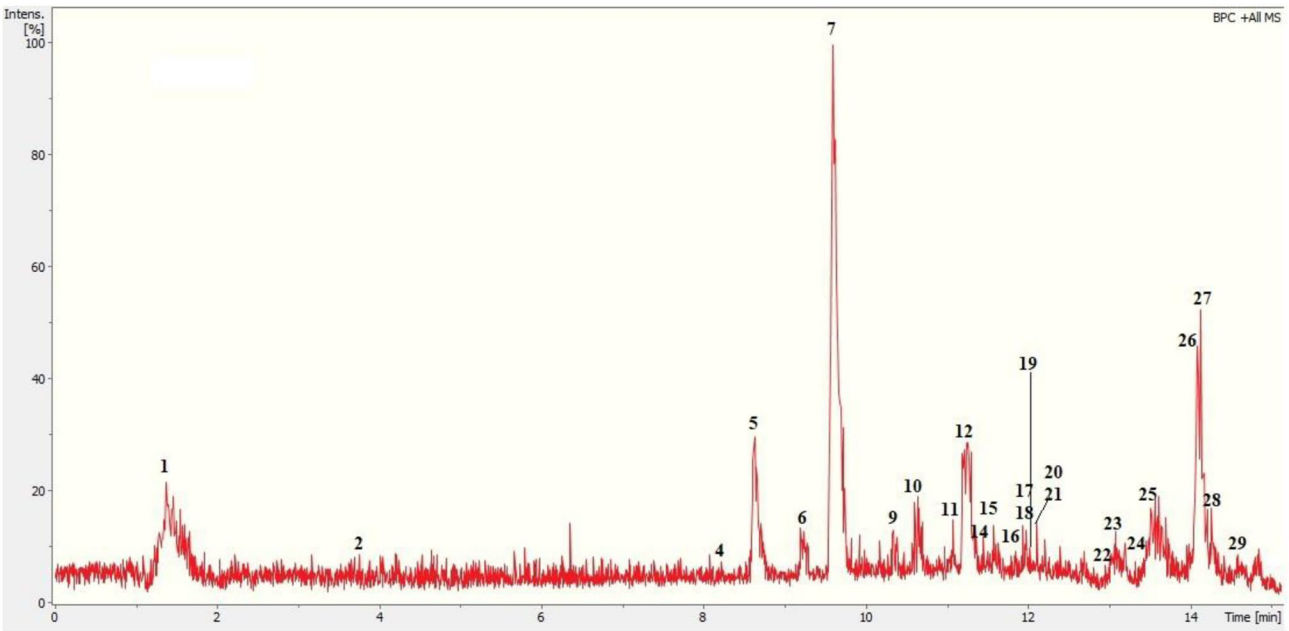

Fig. 4 Base peak chromatogram of callus 1 by LC-PDA-ESI-MS/MS in a positive ion mode

Liquid chromatography-photodiode array-electrospray ionization–tandem mass spectrometry (LC-PDA-ESI-MS/MS) analysis of calli' phytoconstituents

To determine the phytoconstituents of callus 1 and callus 2 extracts, each were analyzed using LC-PDA-ESI-MS/MS in negative and positive ion modes. Their base peak chromatograms are illustrated in Figs. 3, 4, 5 and 6. The phytoconstituents were identified according to how closely their retention times R_t and mass data matched those in the literature and online mass databases. Table 5 summarizes the tentatively identified phytoconstituents along with their retention time, mass data and relative intensity.

Twenty-six constituents were characterized in callus 1, 14 of them were reported previously in other *Crocus* species.

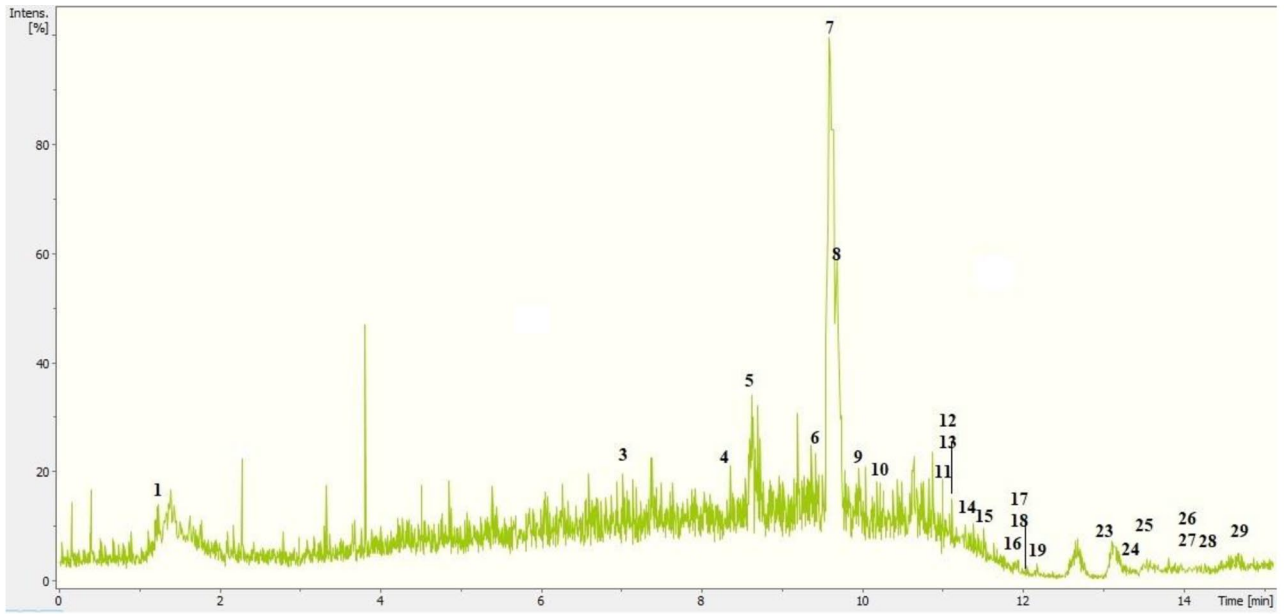

Fig. 5 Base peak chromatogram of callus 2 by LC-PDA-ESI-MS/MS in a negative ion mode

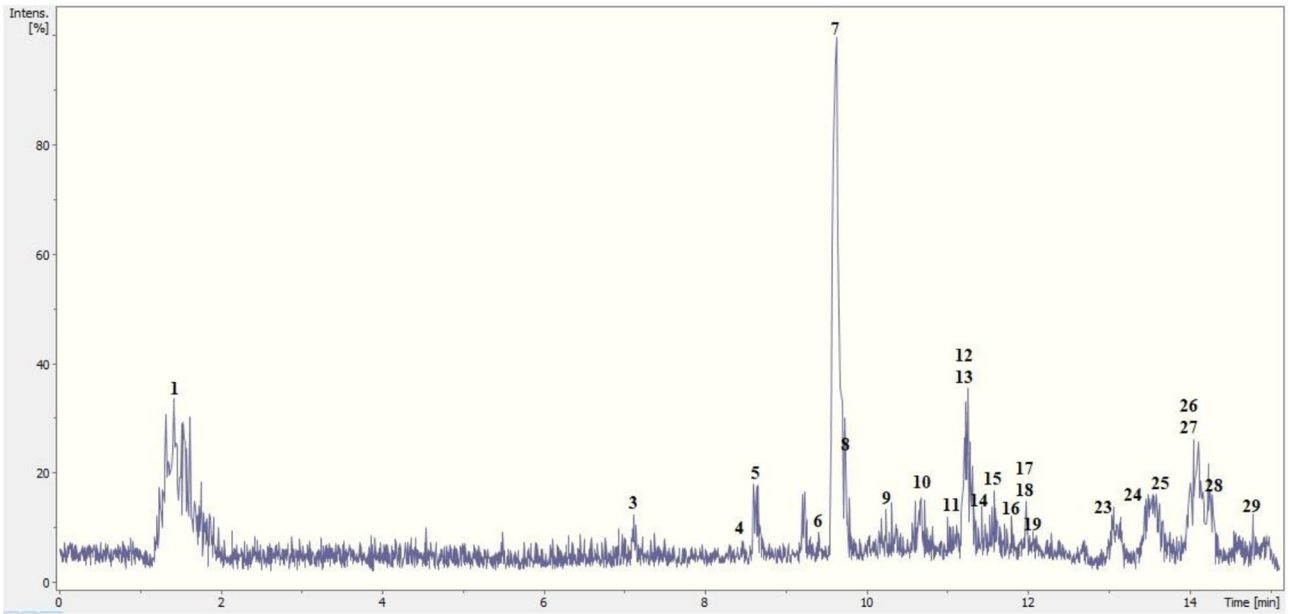

Fig. 6 Base peak chromatogram of callus 2 by LC-PDA-ESI-MS/MS in a positive ion mode

Twenty-five constituents were characterized in callus 2, 13 of them were reported previously in other *Crocus* species. Twenty-two constituents were common in both calli 1 and 2.

Flavonoids are the major constituents of *Crocus* species. According to Mykhailenko et al. 2019; 61 flavonoids and their glycosides were reported previously in different *Crocus* species. Eleven flavonoids were detected in both extracts in this study. They were taxifolin (1), epicatechin (4), kaempferide (10), scoparin (11), diplacone (12), catechin gallate (17), apigeninidin (19), scaposin (24), kaempferol pentoside (25), vitexin (26), and naringenin (29). Taxifolin (1), epicatechin (4), kaempferide (10), vitexin (26), and naringenin (29) are well known constituents of *C. sativus* L. (Li and Wu 2002; Termentzi and Kokkalou 2008; Goupy et al. 2013; Baba et al. 2015; Sánchez-Vioque et al. 2016; Stelluti et al. 2021). Moreover, peaks 11, 12, 17, 19, 24 and 25 were detected in both extracts and showed a characteristic fragmentation pattern of flavonoids. As far as we know,

Table 5 Tentatively identified phytoconstituents of calli

Peak no.	R_t (Min.)	Callus 1 A%	Callus 2 A%	Constituent	M. Wt.	Uv	Molecular formula	$[M-H]^-$	MS/MS (-ve)	$[M+H]^+$	MS/MS (+ve)	References
1	1.3	10.42	1.97	Taxifolin*	304	273	$C_{15}H_{12}O_7$	303	339 $[M+Cl]^-$, 289, 272, 257, 242, 183	305	287, 269, 258, 232, 195	(Horai et al. 2010; Baba et al. 2015)
2	3.7	0.21	-	Neotussilagolactone*	344	234	$C_{21}H_{28}O_4$	343	247, 231	345	362 $[M+NH_4]^+$, 337, 205, 173	(Xu et al. 2019)
3	7.1	-	1.24	Resveratrol*	228	228	$C_{14}H_{12}O_3$	227	141	229	211, 187, 175, 169, 122	(Horai et al. 2010; Ege et al. 2019)
4	8.3	0.13	0.75	Epicatechin*	290	227	$C_{15}H_{14}O_6$	289	245, 203, 151	291	329 $[M+K]^+$, 273, 261, 231, 199, 175, 147	(Horai et al. 2010; Stelluti et al. 2021)
5	8.7	6.17	2.77	Crocetin*	328	226	$C_{20}H_{24}O_4$	327	298, 232, 201	329	311, 287, 273, 251, 175	(Xu et al. 2022; Fiehn Lab 2023)
6	9.3	1.92	1.21	Chavicol glucoside*	296	226	$C_{15}H_{20}O_6$	295	141	297	341 $[M+2Na-H]^+$, 279, 225, 200, 125, 107	(Xu et al. 2019)
7	9.6	24.18	23.87	Coniferin*	342	226	$C_{16}H_{22}O_8$	341	326, 311, 249	343	325, 249, 153	(Xu et al. 2019)
8	9.6	-	2.7	Isorhamnetin rutinoside*	624	225	$C_{28}H_{32}O_{16}$	623	-	625	663 $[M+K]^+$, 335, 317	(Horai et al. 2010; Goupy et al. 2013)
9	10.4	1.09	0.36	Blumenol C glucoside*	372	226	$C_{19}H_{32}O_7$	371	233, 193	373	405 $[M+CH_3OH+H]^+$, 449 $[M+2\ K+H\ ]^+$, 333, 315, 301, 282, 211, 171	(Goupy et al. 2013)
10	10.6	2.43	1.47	Kaempferide*	300	225	$C_{16}H_{12}O_6$	299	283, 253	301	285, 272, 255, 211, 147	(Xu et al. 2019)
11	11.1	0.56	0.72	Scoparin	462	226	$C_{22}H_{22}O_{11}$	461	413, 374, 327	463	377, 337, 243, 215, 199	(Li and Wu 2002; Horai et al. 2010)
12	11.3	7.74	4.44	Diplacone	424	226	$C_{25}H_{28}O_6$	423	445 $[M+Na-2\ H]^-$	425	365, 327, 309, 281	(Fiehn Lab 2023)
13	11.3	-	2.39	Ginsenoside F2	784	n.d	$C_{42}H_{72}O_{13}$	783	819 $[M+Cl]^-$, 532, 437	785	827 $[M+ACN+H\ ]^+$, 425	(Du et al. 2021)
14	11.4	0.26	0.23	Trihydroxy octadecenoate*	344	n.d	$C_{19}H_{36}O_5$	343	326, 267, 155	345	362 $[M+NH_4]^+$, 327, 295, 229, 185, 129	(Xu et al. 2019)
15	11.6	1.14	1.86	Sinapoyl malate	340	226	$C_{15}H_{16}O_9$	339	385 $[M+FA-H]^-$, 223, 135, 113	341	321, 296, 227, 209, 175	(Horai et al. 2010)
16	11.9	1.02	1.02	Thymidylic acid	322	n.d	$C_{10}H_{15}N_2O_8P$	321	-	323	309, 283, 128	(Horai et al. 2010)
17	12	0.4	0.29	Catechin gallate	442	226	$C_{22}H_{18}O_{10}$	441	397, 331, 271, 248	443	427, 385, 315, 228, 166, 148	(Horai et al. 2010)
18	12	0.32	0.69	Gummosin	382	n.d	$C_{24}H_{30}O_4$	381	-	383	399 $[M+NH_4\ ]^+$, 339, 311, 296, 275, 149	(Fiehn Lab 2023)
19	12.1	0.38	0.45	Apigeninidin	254	226	$C_{15}H_{10}O_4$	253	196, 154	255	239, 219, 200, 193, 177, 165, 157, 123, 116, 109	(Fiehn Lab 2023)
20	12.2	0.26	-	Tetrahydro etheno dimetheno benzodioxacyclodocosin triol	440	n.d	$C_{28}H_{24}O_5$	439	-	441	422, 384, 315, 291, 209	(Horai et al. 2010)
21	12.2	0.27	-	Crocusatin K*	184	226	$C_{10}H_{16}O_3$	183	155, 125	185	217 $[M+CH_3OH+H]^-$, 167, 149, 125, 113	(Li et al. 2004)

Table 5 (continued)

Peak no.	R_t (Min.)	Callus 1 A%	Callus 2 A%	Constituent	M. Wt.	Uv	Molecular formula	[M-H]$^-$	MS/MS (-ve)	[M+H]$^+$	MS/MS (+ve)	References
22	13	1.04	-	Tomentogenin*	368	226	$C_{21}H_{36}O_5$	367	331, 307	369	391 [M+Na]$^+$, 333, 283, 229	(Xu et al. 2019)
23	13.1	0.38	0.34	Perrottetin E	426	225	$C_{28}H_{26}O_4$	425	-	427	409, 387, 367, 324, 315, 303, 271, 209	(Horai et al. 2010)
24	13.5	0.11	0.15	Scaposin	390	225	$C_{19}H_{18}O_9$	389	370, 359, 328	391	349, 319, 279, 167	(Horai et al. 2010)
25	13.6	5.01	4.36	Kaempferol pentoside	418	225	$C_{20}H_{18}O_{10}$	417	285, 257, 227	419	343, 300, 285, 223	(Horai et al. 2010)
26	14.1	4.07	9.95	Vitexin*	432	225	$C_{21}H_{20}O_{10}$	431	341, 311, 283, 117	433	375, 357, 313, 299, 131	(Horai et al. 2010; Sánchez-Vioque et al. 2016)
27	14.1	6.08	1.11	Voacristine	384	225	$C_{22}H_{28}N_2O_4$	383	367, 291, 281, 251	385	363, 327, 290, 273, 161	(Oliveros-Diaz et al. 2021)
28	14.3	0.25	0.12	Tetramethyl oxotetrahydro naphthalenyl methylpentanoic acid	320	n.d	$C_{20}H_{32}O_3$	319	-	321	338 [M+NH$_4$]$^+$, 303,268, 247, 233, 219, 191, 163, 149, 135, 111	(Fiehn Lab 2023)
29	14.6	1.23	1.6	Naringenin*	272	226	$C_{15}H_{12}O_5$	271	225, 152	273	189, 173, 155, 123	(Termentzi and Kokkalou 2008; Horai et al. 2010)

* Reported in *Crocus* species.
- not detected.

this is the first report of these flavonoids in *Crocus* species. Peak 11 detected at R_t 11.1 min. with the molecular formula $C_{22}H_{22}O_{11}$ produced the molecular ions [M-H]$^-$ at m/z 461 and [M+H] $^+$ at 463. According to its characteristic mass fragments at m/z 377, 337, 243, 215, 199 at the positive ion mode and its fragments at m/z 413, 374, 327 at the negative ion mode, peak 11 was assigned as scoparin (Horai et al. 2010). Scoparin *O*-rhamnosyl glucoside was previously isolated from *C. reticulatus* leaves (Harborne and Williams 1984). Peak 12 (R_t 11.3 min.) showed the molecular ions in positive and negative modes at m/z 423 [M-H]$^-$ and 425 [M+H]$^+$, respectively. The MS_2 spectrum of peak 12 at the negative ion mode showed the mass fragments at m/z 365, 327, 309, 281. Peak 12 was tentatively assigned as diplacone (MassBank of North America MoNA) (Fiehn Lab 2023). Peak 17 with the molecular formula $C_{22}H_{18}O_{10}$ and the molecular ions at m/z 441 [M-H]$^-$ and 443 [M+H]$^+$ was characterized as catechin gallate according to its characteristic mass fragments at 427, 385, 315, 228, 166, 148 in the positive ion mode (Govindaraghavan 2019). Catechin and catechin hydrate are well known constitute of *C. sativus* L. and *C. cancellatus* subsp. Damascenus (Mykhailenko et al. 2019; Ege et al. 2019; Stelluti et al. 2021). Peak 19 with the molecular ions at m/z 253 [M-H]$^-$ and 255 [M+H]$^+$ was assigned as the anthocyanin, apigeninidin (Horai et al. 2010). Its identification was based on its mass fragments (+ve mode) at m/z 239, 219, 200, 193, 177, 165, 157, 123, 116, and 109. Peaks 24 and 25 were characterized as scaposin and kaempferol pentoside, respectively. The identification of peaks 19, 24 and 25 was supported by the reported anthocyanin, flavone contents and kaempferol derivatives of other *Crocus* species (Mykhailenko et al. 2019).

One more flavone derivative was detected only in the callus 2 at Rt. 9.6 min. (Peak 8). It produced the molecular ion and the characteristic mass fragments at m/z 625 [M+H]$^+$, 663 [M+K]$^+$, 335, and 317. It was tentatively characterized as isorhamnetin rutinoside, the well-known constituent of *C. sativus* (Goupy et al. 2013).

Beside the common detected flavonoids in both calli, peaks 5, 6, 7, 9, 14, 15, 16, 18, 23, 27, and 28 were also detected in both calli. Reviewing the available literature, peaks 5, 6, 7, 9, and 14 were characterized as crocetin, chavicol glucoside, coniferin, blumenol C glucoside, and trihydroxy octadecenoate, which are *C. sativus*'s constituents (Xu et al. 2019). According to the European mass bank database, peaks 15, 16, and 23 were characterized as sinapoyl malate, thymidylic acid, and the lignan, perrottetin E, respectively (Horai et al. 2010). Presence of sinapic acid, and nucleosides were reported in other *Crocus* species previously (Mykhailenko et al. 2019). According the MassBank of North America (MoNA), peaks 18, 27, and 28 were assigned as the coumarin derivative, gummosin, the

alkaloid, voacristine, and the diterpenoid, tetramethyl oxotetrahydro naphthalenyl methylpentanoic acid, respectively (Oliveros-Díaz et al. 2021; Fiehn Lab 2023). The presence of coumaric acid, alkaloids and diterpenoids were previously reported from other *Crocus* species (Mykhailenko et al. 2019).

Peaks 2, 20, 21, and 22 were only detected in callus 1 extract. Peaks 2, 21, and 22 were characterized as neotussilagolactone (Terpene lactone), crocusatin K (Monoterpenoid), and tomentogenin (Steroid), respectively (Xu et al. 2019). They are well known constituents of *Crocus sativus*. Peak 20 with the molecular ion [M + H] + at m/z 441 and mass fragments at m/z 422, 384, 315, 291, 209 was characterized as the lignan derivative, tetrahydro etheno dimetheno benzo dioxacyclo docosin triol, according to the European mass bank database (Horai et al. 2010).

On the other hand, peaks 3, 8 and 13 were only detected in callus 2. Peaks 3 and 8 were characterized as the stilbene derivative, resveratrol and the flavone derivative, isorhamnetin-3-O-rutinoside, respectively. Both are reported constituents of *Crocus sativus* (Horai et al. 2010; Xu et al. 2019; Ege et al. 2019). Peak 13 was assigned as the triterpenoid derivative, ginsenoside F2 (Horai et al. 2010; Goupy et al. 2013; Du et al. 2021). Triterpenoids such as ginsenoside Rh4 and Azafrin were reported previously in *C. sativus* (Mykhailenko et al. 2019; Xu et al. 2019).

As far as we know, this is the first report of Scoparin (11), Diplacone (12), Ginsenoside F2 (13), Sinapoyl malate (15), Thymidylic acid (16), Catechin gallate (17), Gummosin (18), Apigeninidin (19), Tetrahydro etheno dimetheno benzodioxacyclodocosin triol (20), Perrottetin E (23), Scaposin (24), Kaempferol pentoside (25), Voacristine (27), Tetramethyl oxotetrahydro naphthalenyl methylpentanoic acid (28) in *Crocus* species.

Discussion

While research on plant callus and regeneration specific to *C. scepusiensis* is scarce, this study successfully established callus cultures from leaf disc and shoot tip explants. Our findings are aligning with previous studies demonstrating callus induction in *Crocus* species like *C. sativus* and the influence of plant growth regulators on callus formation. Our results are in agreement with Blazquez et al. (2009) who produced Saffron embryogenic nodular callus in 6 weeks from corm tissue cultures. Also, induction of embryogenic callus from bulblet explants happened in the presence of BA and NAA (Ahuja et al. 1994). In addition, Freytag et al. (2017) produced tissue cultures and plant regeneration from different explants of endangered *Crocus* species such as *C. scepusiensis*, C. *tommasinianus*, *C. vittatus* ("Verni" series of the genus) and *C. banaticus* by using a plant growth regulator (PGR) combination of 10 mg/LNAA and 1 mg/L BA.

Both calli showed similar elution pattern on TLC plates. Both calli showed nearly similar total phenolics content. But callus 1 showed higher flavonoids content than callus 2. The higher flavonoids content of callus 2 was confirmed by the LC-PDA-ESI-MS/MS analysis results.

Comparing the peaks' relative intensities (A%) of both explants' extracts LC-PDA-ESI-MS/MS chromatograms, 77.07 and 66.06% of the detected peaks in callus 1 and callus 2 were identified, respectively. The major class of the identified phytoconstituents were flavonoids by 32.48% in callus 1 and 28.85% in callus 2. The major peak was the alcohol derivative, coniferin (Peak 7) 24.18% in callus 1 and 23.87% in callus 2. The callus 1 had significant higher intensities of peaks 1, 5, 9, 10, 12 and 27. While peaks 11, 15, 18 and 26 were detected in higher intensities in the callus 2.

Our analysis revealed variations in the accumulation of phenolic compounds and flavonoids between the two *Crocus* calli. This variation could be attributed to several factors, including the type of explant used. Leaves, being directly exposed to environmental factors and potentially harmful agents, are known to accumulate secondary metabolites like phenolics as a defense mechanism. This suggests that the origin of the explant (leaf vs. shoot tip) might influence the metabolic profile of the resulting callus. Additionally, different plant tissues typically express distinct sets of genes related to phenolic and flavonoid production. Furthermore, growth conditions, such as the composition of the culture medium, light exposure, and temperature, can significantly influence the production of secondary metabolites. Variations in these conditions between leaf and shoot tip callus cultures could contribute to the observed differences in their metabolite profiles (Feduraev et al. 2019). For instance, the composition of phytoconstituents metabolites in callus cultures can be different from that of intact plants. Kothari et al. (2021) reported that, saffron, a valuable secondary product obtained from *Crocus sativus*, are harvested from the stigma of the flower. The chemical composition and concentration of the saffron compounds may not be the same if extracted from leaves or shoot tips. Also, some literature mention that, different parts of the *Crocus* plant, such as the flowers, corms, or stigmas, can contain varying concentrations of secondary metabolites, which are compounds not directly involved in the plant's growth but often have important roles, such as in defense or attraction (Mykhailenko et al. 2019).

Conclusion

This research lays the groundwork for efficient, large-scale callus induction in the critically endangered *C. scepusiensis*. The study successfully established callus cultures using two explant types: leaf discs and shoot tips. The type of explant significantly impacted callus production. Shoot tip explants yielded the highest callus biomass within the shortest time (3.4 ± 0.24 weeks) on a medium containing 5 mg/l NAA and 0.5 mg/l BA. Conversely, leaf disc explants formed yellowish, friable, and smooth calli on a medium with 2.5 mg/l NAA and the same BA concentration. The established callus cultures from both explants are a first step for micropropagation protocol which serves as a valuable resource for maintaining *C. scepusiensis* germplasm under controlled conditions. This research paves the way for further biochemical and cytological studies to elucidate the developmental processes in *Crocus* species. These callus cultures are a valuable asset for a variety of reasons. Callus cultures provide a consistent and controlled environment for the production of bioactive compounds, regardless of seasonal fluctuations. This assures a steady supply for pharmaceutical, biological, and research purposes. The study revealed variations in the content of total phenolics and flavonoids between the two calli. LC-PDA-ESI-MS/MS analysis confirmed the presence of diverse bioactive compounds in *C. scepusiensis* calli. Further investigation is warranted to understand the factors contributing to the observed differences in phenolic and flavonoid accumulation between leaf and shoot tip callus cultures. This knowledge can be crucial for optimizing callus cultures for targeted metabolite production.

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Data availability All the data of this study are available within the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

Author contribution statement Heba D. Khlifa and Heba-tollah M. Sweelam performed the tissue culture study, analyzed, explained the data and wrote the manuscript. Ahmed H. El-Desoky, M. Raslan and Heba-tollah M. Sweelam performed the phytochemical study, analyzed, characterized the data and wrote the manuscript. The manuscript was revised by all authors.

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