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Research Article
Phytoconstituents Profile and UPLC-ESI-MS/MS Analysis of *Centaurea pumilio* L. Callus Culture Following Elicitation

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Abstract

Background and Objective: *Centaurea pumilio* L. (Asteraceae) is a wild plant rich in valuable secondary metabolites. The aim of this study was the induction and maintenance of callus culture in addition to, the analysis of secondary metabolites in the *in vitro* induced tissues. Biotic and abiotic elicitors were used to enhance the production of flavonoids and phenolics in the callus. **Materials and Methods:** Calli were induced from seedling explants cultured on Murashige and Skoog solid media (M&S) supplied with different combinations of phytohormones. Callus growth parameters besides its productivity of flavonoids and phenolics were monitored for each treatment. M&S media supplied with 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} benzyl amino purine showed the maximum growth parameters while media with 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} kinetin exhibited the maximum flavonoids and phenolics productivity compared with other media. **Results:** The best-applied elicitor was the yeast elicitor and the highest productivity of both flavonoids and phenolics were observed on the 6th day after elicitor addition. Moreover, aqueous extract of *Spirulina platensis* added to M&S media as a substitute for phytohormones showed an increase in callus growth parameters, flavonoids and phenolic contents. Thirty-two secondary metabolites were tentatively identified in callus grown on M&S with 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} kinetin using UPLC-ESI-MS/MS technique in positive and negative ionization mode. **Conclusion:** The M&S media supplemented with 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} benzyl amino purine was used to obtain the best callus vegetative growth. While the maximum phenolics and flavonoids production was obtained from callus grown on M&S media supplemented with 1 mg L^{-1} naphthalene acetic acid, 1 mg L^{-1} kinetin and 12 mg L^{-1} yeast extract as abiotic elicitor.

Key words: *Centaurea pumilio*, callus culture, flavonoids and phenolics, elicitors, UPLC-ESI-MS/MS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plant tissue culture is considered one of the most proficient technology for the production of secondary metabolites, that couldn't be detected in the parent plant or produced in smaller or larger amounts[1].

According to the literature survey of genus *Centaurea*, it had been found that several compounds including flavonoids, phenolics, sterols, triterpenes, sesquiterpene lactones and coumarins were previously isolated, identified and characterized in this genus[2-4]. *Centaurea pumilio* L. (Asteraceae) is an endangered plant growing in Egyptian deserts containing methoxylated flavonoids as major constituents[5].

Flavonoids and other phenolic compounds are well known for their antioxidants, antibacterial, anticancer, cardioprotective and anti-inflammatory activities. Additionally, they enhance the immune system, protect skin from UV radiation and are considered to be interesting candidates for pharmaceutical and medical applications[6,7].

The application of elicitors had been considered as one of the most effective methods to improve the synthesis of secondary metabolites in medicinal plants. Yeast, *Fusarium* sp. and calcium chloride had been used for flavonoids and phenolic elicitation in the family Asteraceae[8], however, elicitation had not been reported in genus *Centaurea*.

Spirulina platensis (a blue-green alga) is considered a source of proteins, polyunsaturated fatty acids and pigments[9-11]. This study aimed to induce and maintain a stable callus culture and to investigate the effect of biotic and abiotic elicitors and also exchange the external plant growth hormones in plant tissue culture media by natural algal extract of *S. platensis* monitoring the effect on the growth and productivity (flavonoids and phenolic content) of callus cultures. Also, phytochemical investigation of callus cultured on 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} kinetin which exhibited highly productive media was carried out to identify their active constituents by UPLC-ESI-MS/MS analysis.

MATERIALS AND METHODS

Study area: A tissue culture study was carried out from January, 2017 to April, 2018. UPLC-ESI-MS/MS study was completed in March, 2019.

Plant material: Aerial parts and seeds of *Centaurea pumilio* L. [Syn. *Aegialophila pumilio* L. Bioss, *Aegialophila pumila* L. Boiss, *Centaurea pumila* L.] family Asteraceae were collected from the north coast region, Egypt in April, 2016. The plant was identified by Prof. Abdel-Halim Abdel-Mogaly, Herbarium of Horticultural Research Institute, Agricultural Research Centre, Ministry of Agriculture, Dokki, Giza, Egypt. A voucher specimen of *C. pumilio* No. 4112-CAIM was kept in the Herbarium of Horticultural Research Institute.

***In vitro* seed germination:** Seeds of *C. pumilio* were washed with running tap water for 2-3 min and rinsed with distilled water. They were sterilized in 70% ethanol (El Nasr Pharmaceutical Chemicals, Egypt) for 1 min, followed by immersion in 5% hypochlorite (Clorox®) for 12 min with shaking followed by three washes in distilled water. The seeds were germinated in previously sterilized Petri dishes containing filter papers Whatman number 1 moistened with distilled water and were incubated at 25±2°C under continuous light using fluorescent white lamps with a 16/8 hrs light/dark period (photoperiod). Germination of seeds was evaluated using seed germination percentage according to the following Eq.:

$$\text{Seed germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total numbers of cultured seeds}} \times 100$$

Callus induction and maintenance: The 2-3 weeks old seedlings were incised and transferred aseptically into M&S medium (Duchefa, Germany) supplemented with different hormonal combinations(Sigma Chemical Co, U.S.A) as 1 mg L^{-1} naphthalene acetic acid (NAA) and 1 mg L^{-1} Benzyl Amino Purine (BAP) (M&S I), 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP (M & SII), 1 mg L^{-1} NAA and 1 mg L^{-1} kinetin (KIN) (M&S III), 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg L^{-1} BAP (M&S IV), 0.02 mg L^{-1} Thidiazuron (TDZ) (M&S V), 2 mg L^{-1} 2, 4 D (M&S VI). Green yellowish, friable and healthy callus was maintained on three types of solid media, (M&S I, M&S II and M&S III).

All media were supplied with 30 g L^{-1} sucrose (Adwic, A.R.E.), adjusted to 5.8 pH and solidified with 8 g L^{-1} agar (Bioworld, USA). Callus was sub-cultured into fresh medium every four weeks and incubated at 25±2°C with 12 hrs photoperiod.

Growth parameters: The effect of phytohormones on the *in vitro* induced calli were evaluated by different growth parameters. Growth dynamics were calculated according to Godoy-Hernández *et al.*[12] as follows:

$$\text{Growth index (GI)} = \frac{\text{Ge} - \text{G start}}{\text{G start}}$$

Where:

Ge : Weight of biomass at the end of generation (final weight)

G start : Weight of biomass at zero time (initial weight)

$$\text{Specific growth rate } (\mu) = \frac{\ln x - \ln xo}{t}$$

Where:

xo : Initial biomass and

x : Biomass at time t

Doubling time (dt) which is the time required for the biomass of a population of cells to double where:

$$dt = \frac{\ln (2)}{\mu''}$$

Growth curves were carried out for calli grown on M&S I, M&S II and M&S III media according to Godoy-Hernández *et al.*[12]. The weight of the biomass from each container was determined before and after drying with time intervals 3, 6, 9, 12, 15, 18, 21, 24 and 28 days for recording the fresh and Dry Weights (DW).

Somatic embryogenesis: After 16-20 weeks of culture on M&S I, pockets of greenish 4th generation embryogenic calli with nodular structures were transferred into the same media. The embryogenic calli were observed for 4-5 months where different stages of embryos were monitored by using a microscope. The embroids were subcultured to hormonal free medium or M&S solid media supplemented with 0.5 mg L^{-1} Indole-3-butyric acid (IBA) (Sigma Chemical Co, U.S.A)or M&S medium supplemented with 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} BAP in a trial for regeneration of the whole plant.

Quantitative determination of total flavonoids and total phenolic contents of callus cultures: About 20 g of 16 weeks old fresh callus grown on (M&S I), (M & S II) and (M & S III) media were extracted according to Al-Gendy *et al.*[13].

Total flavonoids content: Total flavonoids content of callus cultures was evaluated by the aluminium chloride-potassium acetate method[14]. The absorbance of the reaction mixture was measured at λ_{max} 415 nm by using a 6715 UV/VIS (SENWAY) spectrophotometer. Quantitation was done based on the standard calibration curves generated with rutin (6.25-200 µg mL^{-1}) in 80% ethanol (v/v) and quercetin (3.125-200 µg mL^{-1}) in 80% ethanol (v/v)[15].

Total phenolic content: Total phenolic content of calli extracts was determined using the Folin-Ciocalteu colorimetric method[16]. The absorbance was measured at λ_{max} 765 nm. The concentration of phenolic content was expressed as gallic acid equivalent. The calibration curve of gallic acid was constructed by using standard solutions of 40 to 300 µg mL^{-1} in 80% ethanol (v/v).

Elicitation of callus culture: Different concentrations of dried commercial yeast extract *Saccharomyces cerevisiae*, (Holw el Sham, Egypt (at. 8 (Y1), 12 (Y2) and 16 (Y3) mg L^{-1} and calcium chloride obtained from El-Nasr Pharmaceutical Chemicals Company at 0.4 (C1), 0.6 (C2) and 0.8 (C3) mg L^{-1} were added to M&S III before autoclaving. *Fusarium* sp. hyphae, obtained from the Faculty of Science, Zagazig University was washed with distilled water, filtered, dried at 40°C till constant weight and finely powdered before addition to M&S III media at 1 (F1), 2 (F2) and 3 (F3) mg L^{-1} [8].

M&S III media was adjusted to pH 5.8 after the addition of these elicitors and then autoclaved. Control media was performed by substituting the elicitor with distilled water. Fresh and dry weights, total flavonoids and phenolic contents were estimated every 3 days on day 3, 6, 9, 12, 15, 18, 21, 24 and 28 for elicitor treated callus cultures compared with control.

Effect of *Spirulina Aqueous* Extract (SAE) on growth dynamics, total flavonoids and phenolic contents of callus culture: *Spirulina Aqueous* Extract (SAE) was prepared by air-dried *Spirulina platensis* cells (5 g), obtained from the Faculty of Science, Zagazig University, according to Amin *et al.*[17]. This sterilized SAE represented the stock solution source for phytohormones. It was added at concentration 10, 15 and 20% (v/v) to M&S medium without phytohormones after autoclaving. M & S medium supplemented with 1 mg L^{-1} NAA and 1 mg L^{-1} KIN (M&S III) was used as control. Growth dynamics, total flavonoids and phenolic contents were estimated for SAE treated callus cultures compared with control.

Extraction and fractionation: Callus grown on M&S III (500 g) was extracted by 95% ethanol, filtered and distilled off under reduced pressure at 50°C to give 36 g of dark green to a brown viscous residue which was dissolved in methanol: water mixture (1:9) and partitioned against light petroleum,

methylene chloride and ethyl acetate, dried over sodium sulphate anhydrous and concentrated to yield 2, 1 and 1.5 g of light petroleum, methylene chloride and ethyl acetate soluble fractions, respectively.

Ultra performance liquid chromatography-Electro spray ionization-Mass spectrometry (UPLC-ESI-MS/MS): Methylene chloride and ethyl acetate soluble fractions of callus grown on M & SIII were subjected to UPLC-ESI-MS/MS. The ESI-MS positive and negative ion acquisition modes analysis was used. The UPLC-ESI-MS/MS analysis was carried out on XEVO TQD triple quadruple instrument, using UPLC coupled with a Waters Corporation, Milford, MA01757, USA, ion trap mass spectrometer with an ESI source. C18 reversed-phase column (ACQUITY UPLC-BEH-C18, rapid resolution, 2.1×50 mm, 1.7 µm) was used. Mobile phase elution was made with the flow rate of 0.2 mL min^{-1} using gradient mobile phase comprising two eluents where eluent A was water acidified with 0.1% formic acid and eluent B was methanol acidified with 0.1% formic acid. Elution was performed using the following gradient: 10% B (0-0.3 min), 10-90% B (0.3-18 min), 90% B (18-22 min) and 10% B (22-25 min). Run time was 15 min and pressure limits were from (0-12000 psi). The parameters for analysis were carried out using positive and negative ion acquisition modes as follows: Source temperature 150°C, cone voltage 50 eV, capillary voltage 3 kV, desolvation temperature 400°C, cone gas flow 50 L h^{-1}, desolvation gas flow 900 L h^{-1} and collision energy for fragmentation 20, 40 and 55 V. Mass spectra were detected in the ESI positive and negative ion modes between m/z 50-1000. The sample solution (100 µg mL^{-1}) was prepared using HPLC analytical grade solvent of water/methanol, filtered using a membrane disc filter (0.2 µm) then injected (10 µL) automatically using autosampler into the UPLC. The sample mobile phase was prepared by filtering and degassed by sonication before injection. The peaks and spectra were tentatively identified by comparing its retention time (Rt.) and mass spectrum with reported data.

Statistical analysis: All the results in the current study were repeated three times. Calculation of results and drawing the figures were carried out using Microsoft Excel 2010. Each value recorded as the Mean±SD of three samples.

RESULTS AND DISCUSSION

***In vitro* seed germination:** The highest germination percentage of sterilized seeds (40%) was observed after 4 weeks of culture. These results are in concurrence with the reported data in *C. cineraria* seed germination[18].

Induction and maintenance of callus cultures: When M&S media supplemented with different combinations of NAA, KIN and BAP inoculated with explants, callus formation occurred. The results were evaluated in terms of growth parameters. M&S I showed the best callus growth parameters (GI and µ) and the lowest dt as shown in Table 1. Also, both M&S II, M&S III produced green yellowish, friable and healthy callus. So, the cultures were maintained on M&S I, M&S II, M&S III and were followed for 28 days. Fresh and dry weights were determined every 3 days as shown in Fig. 1a and b.

Generally, the best culture media reported for *Centaurea* sp. was M&S medium[18-21]. A similar finding was reported by Yüzbaşıoğlu *et al.*[22] in which the M&S medium supplemented with 0.1 mg L^{-1} NAA and 1 mg L^{-1} BAP, 0.2 mg L^{-1} NAA and 2 mg L^{-1} BAP significantly affected the callus induction in C. *arifolia* but concentration different from current results. Moreover, M&S medium supplemented with 2, 4-D alone was not recommended for callus formation in *C. montana*, compared to M&S medium supplemented with a combination of 2, 4-D and BAP[19]. The M&S III has been used for the first time in callus induction for genus *Centaurea* plants.

According to growth curves and vitality, it was obvious that M&S I media is the best hormonal combination. Additionally, a remarkable increase in the average fresh and

Table 1: Growth dynamics of *C. pumilio* L. callus cultures

Media	Growth index (GI) (mean±SD)	Specific growth rate (µ) g day	Doubling time (dt) (day)
M&S I	5.0±1.2	0.063	11.002
M&S II	4.0±3.5	0.057	12.16
M&S III	3.5±2.6	0.053	13.0
M&S IV	1.4±4.5	0.032	21.66
M&S V	1.1±1.6	0.026	26.65
M&S VI	0.65±3.2	0.017	40.77

M&S: Murashige and Skoog media, M&S I: 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} benzyl amino purine, M&S II: 0.5 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} benzyl amino purine, M&S III: 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} kinetin, g: grams

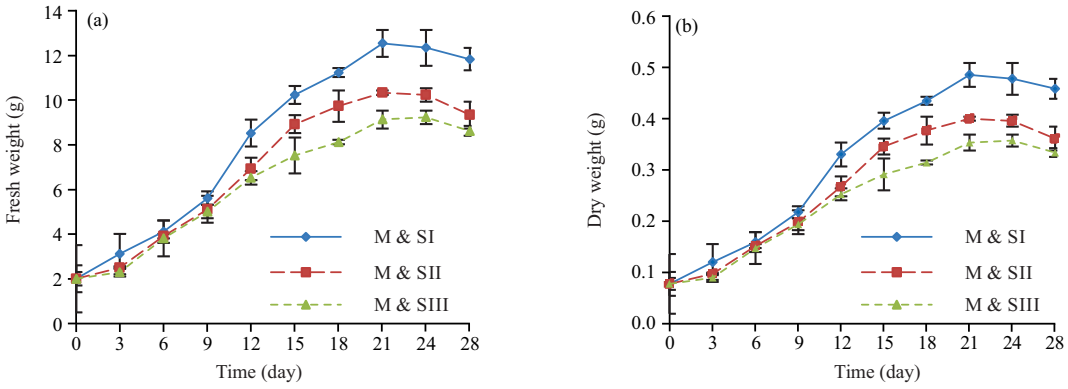

Fig. 1(a-b): Growth curves of *C. pumilio* L. *calli* for 28 days on M&S I, M&S II and M&S III
(a) Growth curve of fresh weight and (b) Growth curve of dry weight

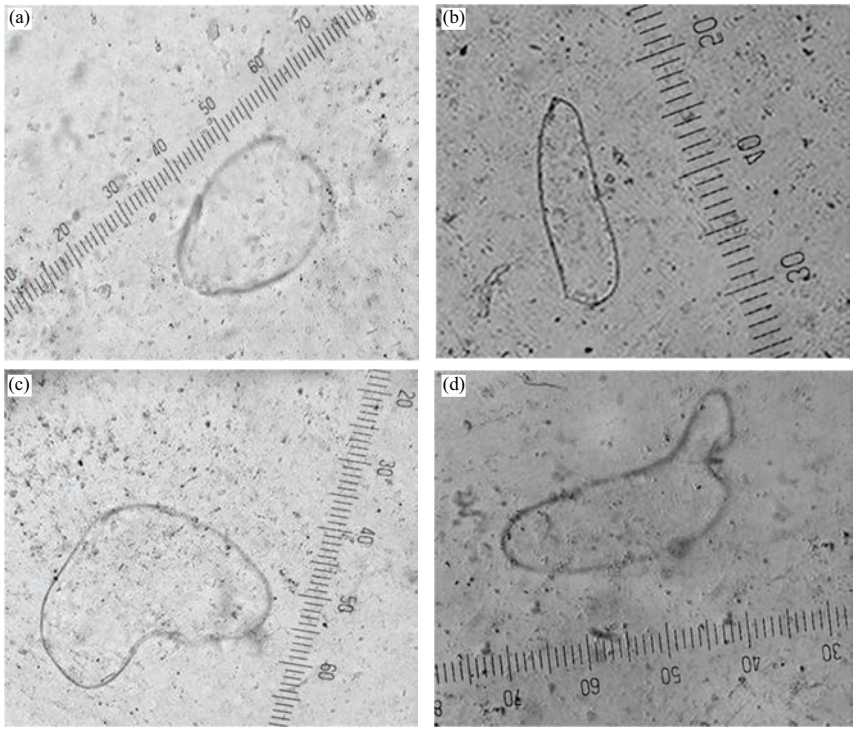

Fig. 2(a-d): Stages of embryogenic callus of *C. pumilio* L.
(a) globular "g" (121.2 µm), (b) Torpedo shaped "t" (315.2 µm) embryos, (c) Heart shaped "h" (290.6 µm) and (d) Cotyledonary embryos (335.6 µm)

dry weight of calli (embryogenic callus) was obtained. However, M&S III produced the lowest fresh and dry weight (non-embryogenic callus) as shown in Fig. 1a and b.

Somatic embryogenesis and plantlets regeneration: Eight weeks old calli grown on M&S I showed pockets of light greenish-yellow calli tended to be embryogenic callus initiation. Embryogenic callus maintained on M&S I showed somatic embryos as globular (121.2-140 µm in diameter) in Fig. 2a, torpedo-shaped (315.2-430 µm in length) in Fig. 2b and heart-shaped (290.6-420.3µm in diameter) as in Fig. 2c. Mature embroids successfully germinated into cotyledonary form embryos (321.2-335.6 µm) as shown in Fig. 2d. Al-Gendy *et al.*[8] reported that M&S medium supplemented with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP was the best for somatic embryogenesis formation in other Asteraceae plants, similar to the current finding in media combination for embroid formation but with different concentration.

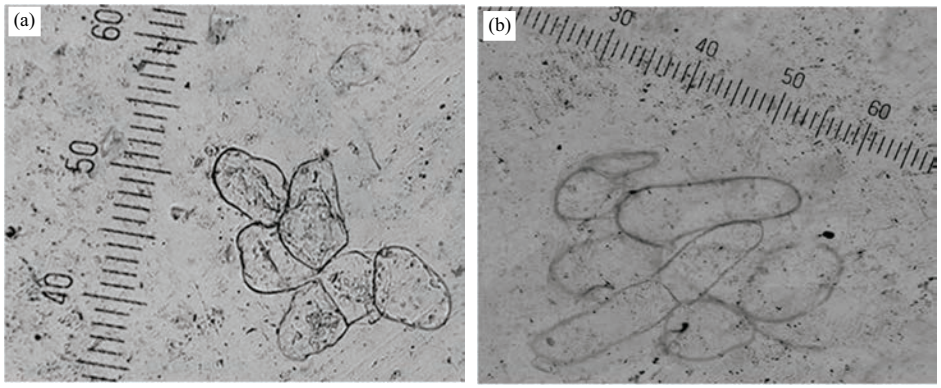

Fig. 3(a-b): Abnormalities in callus culture of *C. pumilio* L.
(a) Fused globular (175-188 µm) and (b) Fused globular and torpedo shaped embryos (399-410 µm)

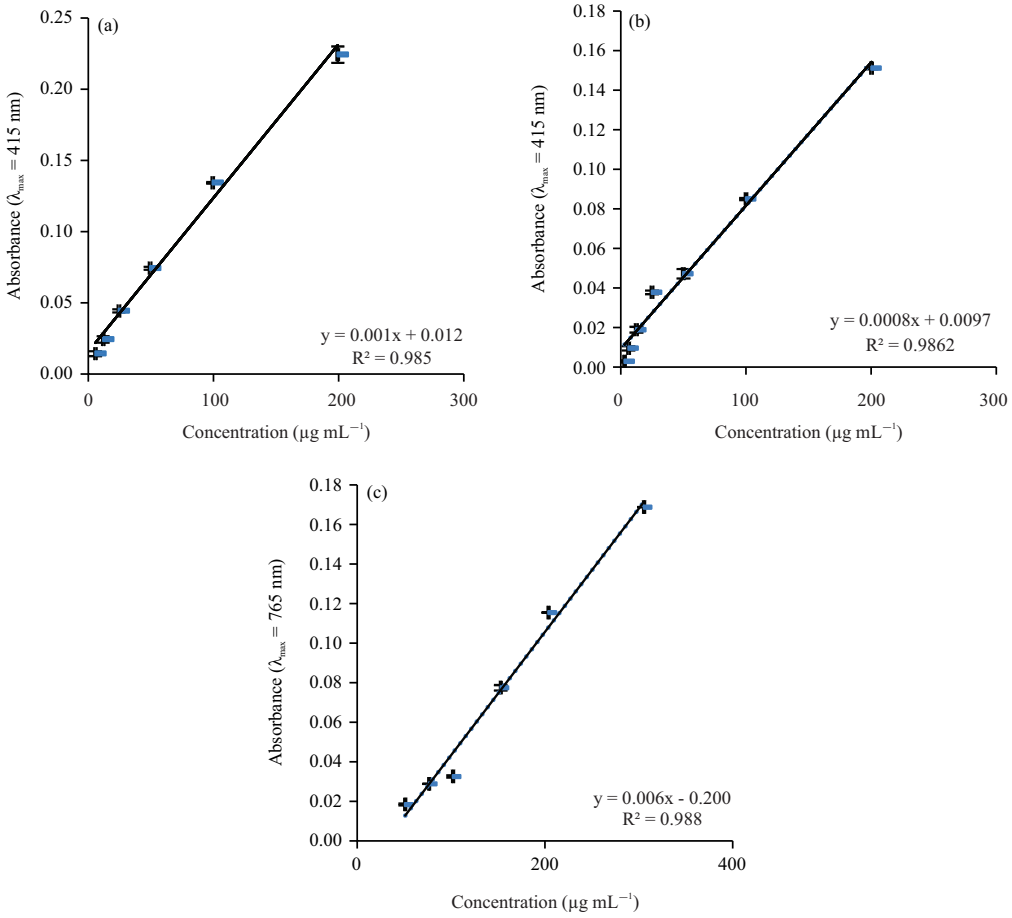

Fig. 4(a-c): Calibration curves for standard flavonoids and phenolic compound
(a) Rutin, (b) Quercetin and (c) Gallic acid

Several trials were carried out for complete plant regeneration but unfortunately, dwarfed undifferentiated plantlets were produced. Abnormal embroids were observed by microscopical examination as fused globular shaped (175-188 µm in diameter) as in Fig. 3a and fused globular and torpedo-shaped (399-410 µm) embryos as shown in Fig. 3b, which may explain the difficulty of production of healthy differentiated plantlets.

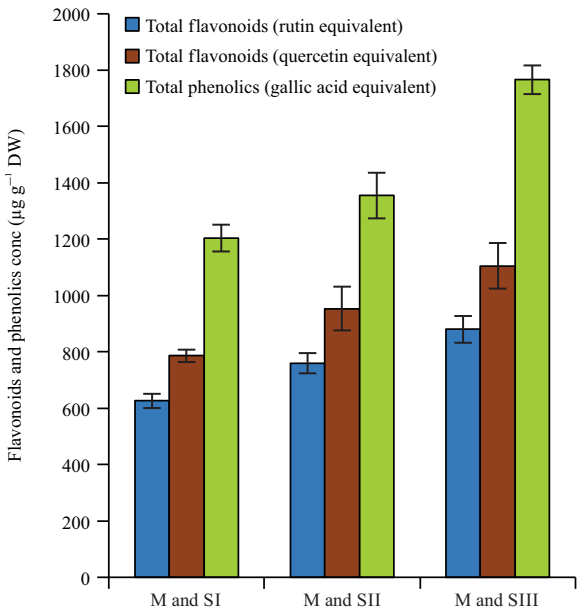

Fig. 5: Total flavonoid and phenolic contents of *C. pumilio* L. 16 weeks old calli growing on M&S media supplemented with different hormonal combinations expressed as rutin, quercetin and gallic acid equivalents

Somatic embryos also failed to mature in *Iphiona mucronata*[13] and *Stevia rebaudiana*[23] belongs to the same family Asteraceae. According to Canhoto *et al.*[24], the most common abnormalities in culture are embryo fusion. Fused globular and torpedo-shaped embryos were reported in *I. mucronata* embryogenic suspension culture[13], similar to the current finding in *C. pumilio* somatic embryos abnormalities.

Quantitative determination of total flavonoids and total phenolic contents of *C. pumilio* callus culture: Total flavonoids content was determined by aluminium chloride potassium acetate spectrophotometric method and expressed as quercetin and rutin equivalents by reference to calibration curves where $y = 0.001x + 0.012$ and $r^2 = 0.985$ in case of rutin as standard while $y = 0.0008x + 0.0097$ and $r^2 = 0.9862$ in case of quercetin as standard as shown in Fig. 4a and b.

On the other hand, total phenolic content was determined by the Folin-Ciocalteu method are expressed as Gallic Acid Equivalents (GAE) by reference to the standard calibration curve ($y = 0.006x-0.200$ and $r^2 = 0.988$) as represented in Fig. 4c.

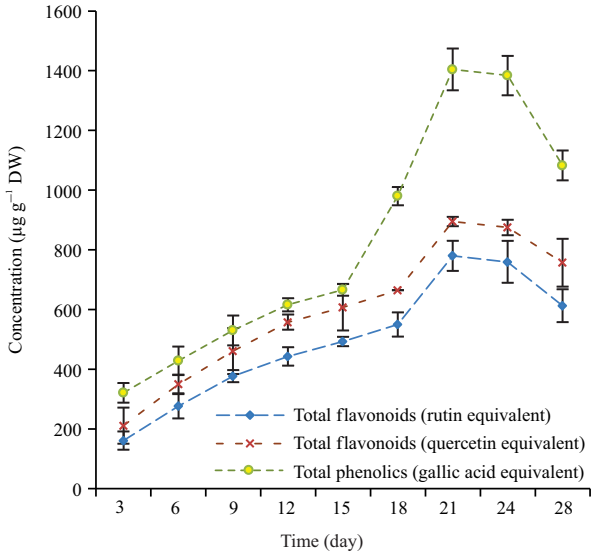

Fig. 6: Time course production of total flavonoid and phenolic contents of *C. pumilio* L. *calli* growing on M&S III expressed as rutin, quercetin and gallic acid equivalents

Callus grown on M&S III gave the highest values of total flavonoid contents (884.8±47.5 and 1108.0±50.3 µg g^{-1} DW) expressed as rutin and quercetin equivalents, respectively, nearly 1.4 times of its content where grown on M&S I media. Moreover, they showed the highest values of total phenolic (1766.6±55.8 µg g^{-1} DW) expressed as gallic acid equivalent, nearly 1.5 times of its content on M&S I media in Fig. 5. Total flavonoids and phenolic production were followed up every 3 days for callus maintained on M&S III media and revealed that the highest concentration of flavonoids and phenolics were detected on the 21st day as shown in Fig. 6.

Elicitation of callus culture: M&S III was selected to be treated with different elicitors as yeast extract, *Fusarium* sp. fungi and calcium chloride. These elicitors were reported in *Artemisia monosperma* callus cultures that belong to the same family Asteraceae[8].

Yeast (Y): Y1 (8 mg L^{-1}) exhibited the highest fresh and dry weight at day 21 approximately 2.2 times more than its control and kept the vitality even after 28 days as shown in Fig. 7a and b. The inverse relationship between yeast concentration and callus growth was observed.

While, Y2 (12 mg L^{-1}) showed the highest flavonoids content after 6 days (2161.6±55.7, 2711.4±60.3 µg g^{-1} DW)

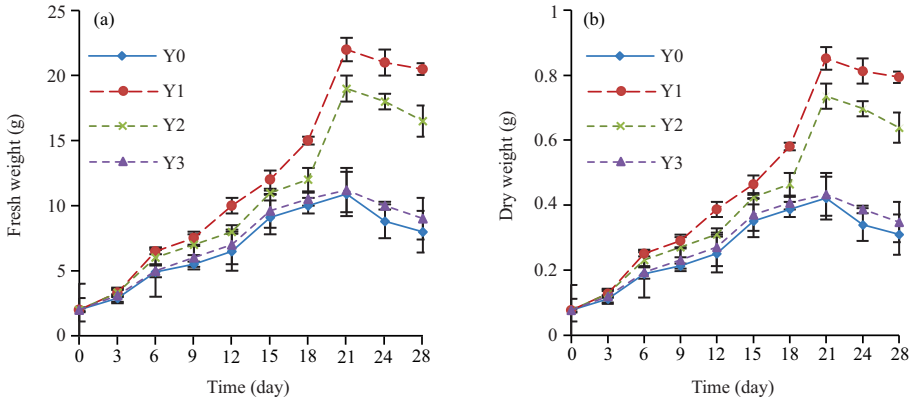

Fig. 7(a-b): Effect of yeast elicitation on growth of *C. pumilio* L. callus culture
(a) Fresh weight and (b) Dry weight

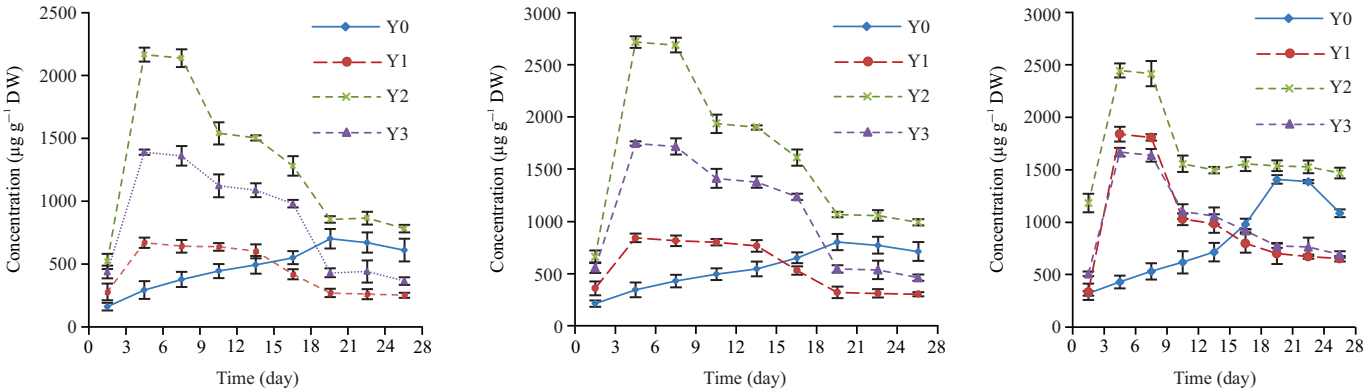

Fig. 8(a-c): Effect of yeast elicitation on flavonoids and phenolics content of *C. pumilio* L. callus culture expressed as rutin, quercetin and gallic acid equivalents
(a) Rutin, (b) quercetin and (c) gallic acid

compared with control (293±30.7 and 343±35.2) expressed as rutin and quercetin equivalents, respectively nearly 7.3 times more than the control (Y0), then flavonoids content started to decrease gradually till 28 days as represented in Fig. 8a and b. Additionally, Y2 showed the highest phenolic content (2440.9±66.7 µg g^{-1} DW) compared with control (429±50.3) expressed as gallic acid equivalent after 6 days of subculture representing 5.6 times more than the control (Y0) and then started to decrease gradually till 28 days as shown in Fig. 8c.

Similarly, the reported data concerning the increase in the production of flavonoids and phenolic after addition of yeast elicitor to callus cultures of *A. monosperma*[8]. Also, Rady *et al.*[25], reported yeast elicitation showed enhancement production of silymarin (flavonolignan) in hairy root cultures of *Silybum marianum*. Yeast elicitor increase production of artemisinin in callus culture of *Artemisia annua*[26].

***Fusarium* sp. (F):** The effect of *Fusarium* sp. elicitor on plant cell growth was shown in Fig. 9. Little difference between treated calli and control was observed in the first days. The fresh and dry weight of treated calli in Fig. 9a and b were increased at day 21 approximately 1.6 times more than its control at concentration 3 mg L^{-1} (F3). Also, F3 showed higher total flavonoids production with yield (1080.8±25.9, 1355.7±30.6 µg g^{-1} DW) compared to control (239±12.9 and 331.7±35.9) expressed as rutin and quercetin equivalents, respectively, nearly 4.5 times of control as represented in Fig. 10a and b and the total phenolics with yield (2394.4±36.6 µg g^{-1} DW) compared to control (343.8±15.2), expressed as gallic acid equivalent, representing 6.9 times more than control as shown in Fig. 10c after 6 days of treatment then started to decrease gradually till the 28th day. Observations were recorded after 6 days of the culture treatment period and then started to decrease gradually till 28 days as shown in Fig. 10.

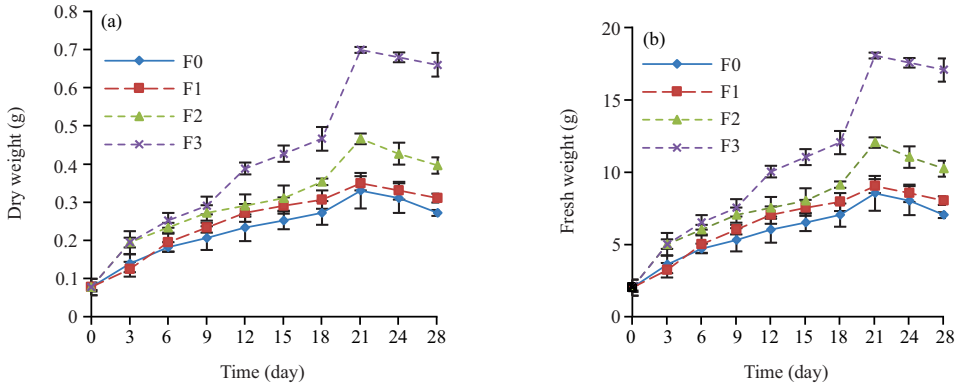

Fig. 9(a-b): Effect of *Fusarium* sp. elicitation on growth of *C. pumilio* L. callus culture
(a) Fresh weight and (b) dry weight

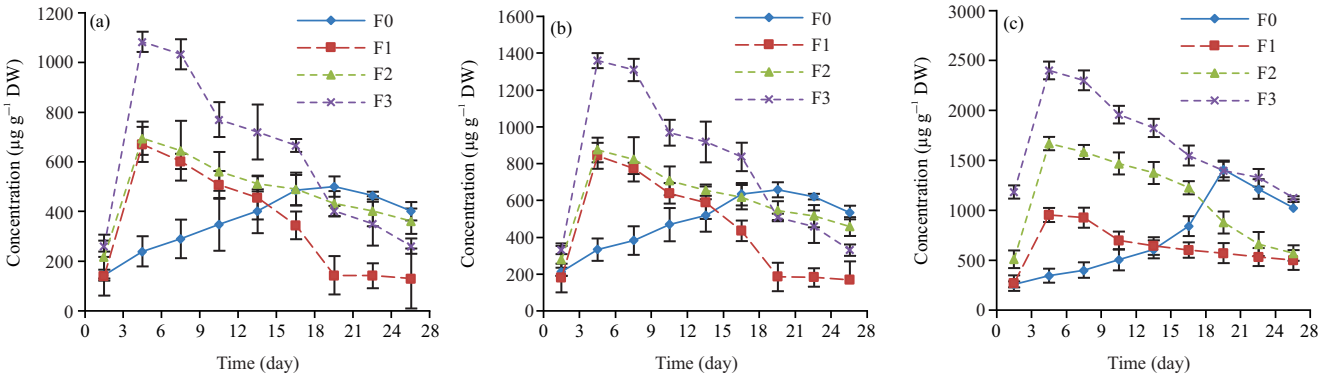

Fig. 10(a-c): Effect of *Fusarium* sp. fungi elicitation on flavonoid and phenolic contents of *C. pumilio* L. callus cultures expressed as rutin, quercetin and gallic acid equivalents
(a) Rutin, (b) Quercetin and (c) Gallic acid, (F0: control, F1: 1, F2: 2, F3: 3 mg L-1 Fusarium sp)

The reported data concerned with *Fusarium* sp. elicitor in the same family, Asteraceae. These results are in agreement with Al-Gendy *et al.*[8], who reported similar results for enhancing flavonoids and phenolic content in callus culture of *A. monosperma* treated with fungal elicitors. Additionally, fungal elicitor enhanced secondary metabolites production as phenolic acids and phenolic diterpenes in callus cultures of *Rosmarinus officinalis*[27]. Another report about the effect of *F. oxysporum* on secondary metabolite production in cell suspensions of *Hypericum triquetrifolium*, which showed accumulation of catechin 3-folds more than its control[28].

Calcium chloride (C): Calcium is essential for plant life and calcium chloride was used as abiotic elicitors in several plant cell cultures. The C3 (0.8 mg L^{-1}) was the best concentration showing the highest fresh and dry weight of callus while other concentrations didn't show any difference from control.

Moreover, C3 showed maximum production of flavonoids and phenolic contents at the 12th day of subculture (770±77.3, 971±60.4 and 1021.5±80.2 µg g^{-1} DW) expressed as rutin, quercetin and gallic acid equivalents, respectively. Flavonoids concentration was nearly 1.7 times as control (C0) as shown in Fig. 11a, b, while phenolics was nearly 2 times as control C0 as shown in Fig. 11c. On the other hand, (C1) and (C2) weren't successful as elicitation tool for induction of flavonoids or phenolics content.

Al-Gendy *et al.*[8] reported that calcium chloride elicitation showed a little increase representing nearly 1.48 and 1.47 times control in flavonoids and phenolic contents, respectively of callus culture in *A. monosperma*. Also, calcium chloride enhanced phenolic acids production in callus cultures of *R. officinalis*[27].

Effect of *Spirulina* aqueous extract (SAE) on growth dynamics: Growth dynamics of callus cultures under the effect

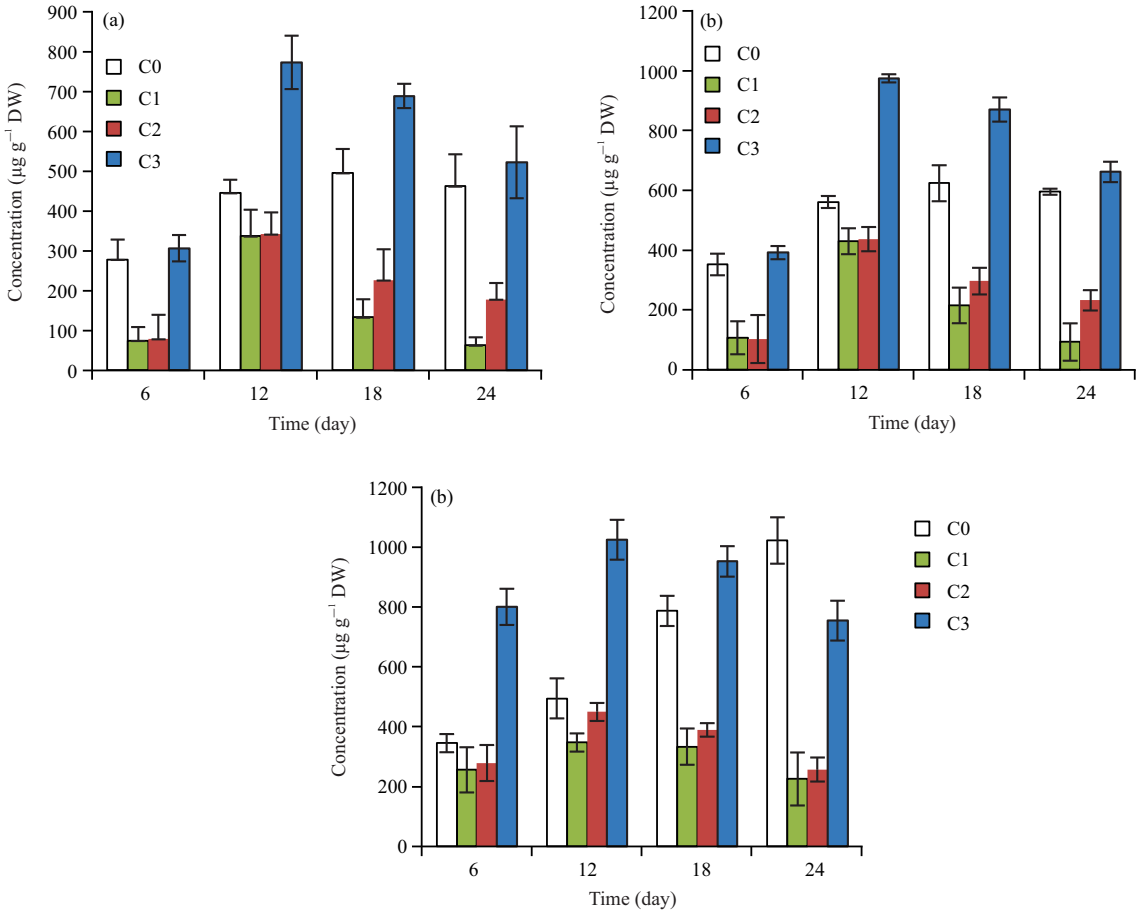

Fig. 11(a-c): Effect of calcium chloride elicitation on flavonoid and phenolic contents of *C. pumilio* L. callus cultures expressed as rutin, quercetin and gallic acid equivalents
(a) Rutin, (b) Quercetin and (c) Gallic acid, (C0: control, C1: 0.4, C2: 0.6, C3: 0. mg L-1 calcium chloride)

Table 2: Growth parameters of *C. pumilio* callus grown for 28 days on hormonal free M&S media supplemented with different concentrations of SAE

Media	Growth index (GI) (mean±SD)	Specific growth rate (µ) g day	Doubling time (dt) (days)
M&S (10%v/v SAE)	2.6±0.96	0.0457	15.1
M&S (15%v/v SAE)	3.3±1.32	0.0520	13.3
M&S (20%v/vSAE)	4.0±3.51	0.057	12.1
M&S III	3.5±4.92	0.053	13.0

M&S: Murashige and Skoog media; M&S III: 1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} kinetin; SAE: *Spirulina* Aqueous Extract, g: Grams, v: Volume

of *Spirulina* aqueous extract (SAE) as substitutes of phytohormones were estimated.

Callus treated with 15% SAE in M&S hormone-free media showed growth parameters nearly equal to those grown on control (M&S III) media. On the other hand, Callus treated with 20% SAE exhibited better growth parameters than control, with a growth index nearly 1.1 times more than (M&S III) media as shown in Table 2.

Total flavonoids and phenolic content estimation of callus cultures under the effect of *Spirulina* aqueous extract: The 20% SAE treated culture M&S media exhibited the highest flavonoids values (1023±30.2, 1297.9±44.6 µg g^{-1} DW) expressed as rutin and quercetin equivalents, respectively which were 1.7 times as control but phenolic content (1482.6±55.4 µg g^{-1} DW) expressed as gallic acid equivalent was nearly equal to untreated culture (control) after 28 days of subculture as shown in Fig. 12.

This is the first report for the use of *S. platensis* as a growth promoter and productivity enhancer for the establishment of *in vitro* callus cultures of genus *Centaurea*.

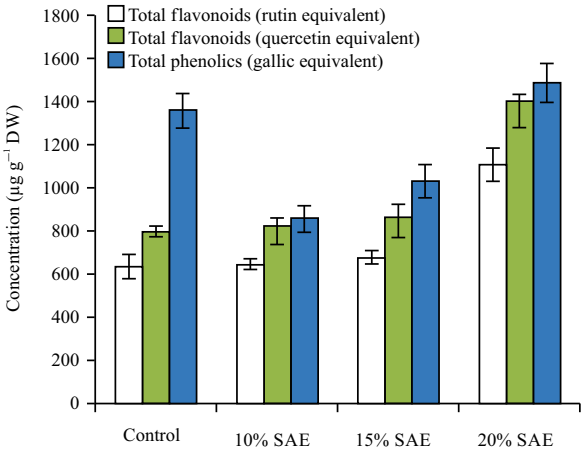

Fig. 12: Total flavonoid and phenolic contents of different concentrations of SAE and control after 28 days of *C. pumilio* L. callus culture

UPLC-ESI-MS/MS analysis: UPLC-ESI-MS/MS of methylene chloride and ethyl acetate soluble fractions of callus grown on M&S III positive and negative ion modes revealed the following.

Methylene chloride fraction of callus culture grown on M&S III: Nineteen compounds were detected, arranged according to retention time (Rt) and summarized in Table 3. The identification was based on a comparison with available literature:

- **Phenolic acids derivatives:** One phenolic acid derivative was tentatively identified as coumaroyl agmatine (1) by the fragment ion m/z 147, correspond to a coumaroyl moiety after losing agmatine residue ([M+H-130]$^+$)
- **Flavonoidal aglycones:** Three flavonoidal aglycones including quercetagetin (3), isorhamnetin (4) and trihydroxy methoxy flavone (6) were recognized by comparing their MS/MS fragmentation pattern with the reported data[29]
- **Flavonoidal glycosides:** Apigenin-7-O-rutinoside (2) showed fragment ion at m/z 271 ([M+H-308]$^+$) (apigenin)[30]
- **Fatty acids and fatty acid derivatives:** Fatty acids as (9), (10), (11), (12), (15), (16) and (19) are characterized by neutral losses of carboxyl or/and water and recognized as behenic, arachidic, linoleic, palmitic, oleic and heptadecanoic acids[31-34], while compound (7) showed losses of two carboxyl group and identified as linoleic acid hydroperoxide[35]
- **Sterols, triterpenes and their derivatives:** Lupenone (8) exhibited a base peak at m/z 189, considered as the characteristic fragment of triterpenoid molecules with a lupane skeleton[36]. Stigmasterol (13), β-sitosterol (14) and 24-methylene cholesterol (17) showed parent ion peaks of ([M+H-H2O]$^+$) at m/z 395, 397 and 381, respectively as unsaturated sterols are characterized by losing water molecule upon fragmentation[37,38]
- **Other classes:** Parthenolide (5), known as sesquiterpene lactone and carvotanacetone (18), known as menthane monoterpenoids, were identified as shown in Table 3

Ethyl acetate fraction of callus culture grown on M&S III: Thirteen compounds were detected, arranged according to their retention time (Rt) and summarized in Table 4:

- **Phenolic acids derivatives:** Chlorogenic acid (2) and its isomer neochlorogenic acid (5) showed two peaks at different retention times and similar molecular ion ([M-H]$^-$) at m/z 353[39]. Additionally, dicaffeoylquinic acid isomers (4) and (7) showed fragment ion at m/z 353 and 191 due to neutral losses of two caffeoyl residues[29]. ESI-MS/MS analysis of cinnamoyl-galloyl-beta-D-glucopyranose (12) exhibited fragment ion at m/z 299 after losing glucose moiety[35]
- **Flavonoidal-O-glycosides:** The key fragmentations utilized for flavonoidal glycosides were the aglycone ion derived from the loss of sugar moiety as laricitrin-3-O-rhamnose-7-O-trihydroxycinnamic acid (6) and isorhamnetin-O- rhamnoside (11) showed fragment ion at m/z 510 and 317, respectively due to loss of rhamnose moiety (-146 Da)[40,41]. Kaempferol-pentosyl hexoside (8) exhibited at m/z 285 appeared after the neutral loss of (162 and 132 Da) for hexose and pentose moiety, respectively[35]. Compound (9) was assigned as kaempferide-glucuronide with an aglycone mass of 301 (176 Da for glucuronide moiety)[42]. While luteolin hexoside (10) and tetrahydroxy-dimethoxy flavanone hexoside (13) characterized by aglycone ion after lysis of hexose moiety (-162 AMU)[43,44]
- **Other classes:** Adenine ribose [(1), nitrogen-containing compound] showed fragment ion at m/z 136 ([M+H-132]$^+$)[35]. O-galloyl arbutin [(3) hydrolysable tannin derivatives] gave fragment ion at m/z 273 correspondings to arbutin[45]

Table 3: Identified major components of methylene chloride soluble fraction of callus cultured on M and S III by UPLC-ESI-MS/MS in positive/negative ionization mode

		Positive mode		Negative mode		
Compound name	Rt	$[M+H]^+$	MS2 fragments (m/z)	$[M-H]^-$	MS^2 fragments (m/z)	References
Coumaroyl agmatine	0.74	277	147 (100), 119	-	-	Abu-Reidah *et al.*[35]
Apigenin rutinoside	14.19	579	271 (100%)	577	269 (100%)	Cao *et al.*[30], Carretero *et al.*[37]
Quercetagetin	18.22	319	301,273,123	317	299, 271, 179	Bakr *et al.*[29]
Isorhamnetin	20.48	317	302 (100%)	315	300, 255, 151	Bakr *et al.*[29]
Parthenolide	21.13	249	203,191,59,43			Avula *et al.*[46]
Trihydroxy methoxy flavone (Hispidulin)	22.11	301	286,187,129	299	284 [M-H-15]	Bakr *et al.*[29]
Linoleic acid hydroperoxide	22.69	-	-	311	223,183	Abu-Reidah *et al.*[35]
Lupenone	25.84	425	355,205,189, 133	-	-	Modugno *et al.*[36]
9-Hydroxy palmitic acid	25.92	-	-	271	255, 225.2 (100%) ([M-H- H_2O-CO]$^-$), 197 ([M-H-C_2H_4]$^-$), 127 ([M-H-C_5H_{10}]$^-$)	Zhang *et al.*[34]
Behenic acid	25.94	-	-	339	321,293,197,183, 170	Eser *et al.*[31]
Arachidic acid	26.84	313	269, 239	-	-	Eser *et al.*[31]
Linoleic acid	27.15	-	-	279	261,235,137,111	Yang *et al.*[32]
Stigmasterol	28.56	413	395([M-H_2O+H]$^+$) 271 ([M-side chain-2H]+), 213([M-side chain-ring D cleavage-H_2O]$^+$), 95,41	-	-	Suttiarporn *et al.*[38]
Palmitic acid	28.87	-	-	255	237, 212, 183, 117	Yang *et al.*[32]
Oleic acid	28.9	-	-	281	237,111,59	Yang *et al.*[32]
24-Methylene cholesterol	29.33	381[M+H–H_2O]$^+$	297 ([M+H-C_6H_{12}-H_2O]$^+$), 255, 203, 161			Carretero *et al.*[37]
Carvotanacetone	30.23	153	137, 109, 107, 81, 80, 79	-	-	Joshi and Pai[47]
Heptadecanoic acid	30.83	271	253([M+H-H_2O]$^+$), 242, 227,214, 141.9, 205, 147,121	-	-	Butovich *et al.*[33]

Rt: Retention time, M+H: Protonated molecular weight, M-H: Deprotonated molecular weight, MS^2: Second mass fragmentation

Table 4: Identified components in the ethyl acetate soluble fraction of callus cultured on M and S III by UPLC-ESI-MS/MS in positive/negative ionization mode

		Positive mode		Negative mode		
Compound name	Rt	$[M+H]^+$	MS2 fragments (m/z)	$[M-H]^-$	MS^2 fragments (m/z)	References
Adenine ribose	0.74	268	136 (100%)	-	-	Abu-Reidah *et al.*[35]
Chlorogenic acid	0.81			353	191,179	Choi *et al.*[39]
O-galloyl arbutin	1.18	425	273 (100%), 45.4	-	-	Abu-Reidah *et al.*[45]
Dicaffeoylquinic acid	1.18	-	-	515	353,191	Bakr *et al.*[29], Abu-Reidah *et al.*[35]
Neochlorogenic acid	1.28	-	-	353	191,179	Choi *et al.*[39]
Laricitrin-3-O-rhamnose-7-O-trihydroxycinnamic acid	1.51	657	510,348	655	509, 347, 329	Downey and Rochfort[41]
Dicaffeoylquinic acid isomers	1.6	-	-	515	353,191	Abu-Reidah *et al.*[35]
Kaempferol-pentose hexoside	1.81			579	285 (100%), 179	Abu-Reidah *et al.*[35]
kaempferide-glucuronide (4'-Methyl kaempferol glucuronide)	3.31	477	301, 286	475	299, 284	Blazics[42]
Luteolin-O-hexoside	3.7	-	-	447	285 (100%), 267, 241	Olennikov *et al.*[44]
Isorhamnetin rhamnoside	4.88	463	317, 301	-	-	Iwashina *et al.*[40]
Cinnamoyl-galloyl-beta-D-glucopyranose	7.7	463	301, 150	461	315, 299,163,152	Abu-Reidah *et al.*[35]
Tetrahydroxy-dimethoxyflavanone-hexoside	10.08	-		509	346, 329	De Rosso *et al.*[43]

Rt: retention time; M+H: protonated molecular weight; M-H: deprotonated molecular weight.; MS2: second mass fragmentation.

CONCLUSION

Callus cultures of *C. pumilio* (Asteraceae) were successfully established under different conditions using M&S medium, where M&S I showed the best growth culture media and M&S III best productivity culture media. Small non-differentiated plantlets were produced from embryogenic callus cultures on M&S I without further proliferation. Elicitation of callus culture using yeast and *Fusarium* sp. fungi as biotic elicitors and calcium chloride as abiotic elicitor enhanced the flavonoids and phenolic content production. Yeast extract was favoured compared to *Fusarium* sp. and calcium chloride for the high productivity and maintaining the viability of the culture. SAE could substitute phytohormones with an increase in growth parameters as well as total flavonoids and phenolic contents and might suggest the use of *S. platensis* as a cheaper substitute instead of phytohormones. To the best of our knowledge, there is no report for elicitation in the genus *Centaurea*. Moreover, thirty-two compounds with different classes identified in methylene chloride and ethyl acetate soluble fractions of callus cultured on M&S III by positive and negative mode of UPLC-ESI-MS/MS analysis for the first time in genus *Centaurea* callus culture.

SIGNIFICANCE STATEMENT

This study discovered the best condition for callus induction and maximum production of phenolics and flavonoids in *Centaurea pumilio*. Phytoconstituents of the callus were tentatively identified for the first time in genus *Centaurea* via UPLC-ESI-MS/MS analysis. This study will help the researchers to identify secondary metabolites *in vitro* tissues of related plants.

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