

## Effect of *Aspergillus niger* extract on production of coumarins in cell suspension cultures of *Angelica archangelica*

DHUHA S. NADIR

Al-Fadhilat Preparatory School for Girls, Baladruz Department of Education, Diyala General Directorate of Education. Ramadhan, Baladruz, Diyala, Iraq. Tel./Fax.: +62-271-637457 ext. 129, email: authors@staff.uns.ac.id

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**Abstract.** Nadir DS. 2022. *Effect of Aspergillus niger extract on production of coumarins in cell suspension cultures of Angelica archangelica*. Biodiversitas 23: 5132-5138. *Aspergillus niger* is one of the most significant microbes employed in biotechnology and has been used for many years to make citric acid and extracellular enzymes. It is also utilized for waste treatment and biotransformation. In the last 20 years, *A. niger* has become a crucial transformational host to overexpress food enzymes. Different concentrations of *A. niger* extract were used to investigate their effects on the production of coumarin, both in the liquid medium and the cells, of batch cultures of *Angelica archangelica* L. Liquid Murashige and Skoog (MS) medium enriched with 2.0 mg.L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 0.5 mg.L<sup>-1</sup> Kinetin (Kin), was used as a growth medium for batch cultures of *A. archangelica* incubated for different incubation periods (7, 14 and 21 days). The results showed that 3.053 mg.g<sup>-1</sup> was the highest coumarin concentration, obtained from the nutritional medium drawn from cultures treated with 2.0 mL.L<sup>-1</sup> of *A. niger* after 21 incubation days. However, 2.055 mg.g<sup>-1</sup> was the maximum coumarin concentration reached in the harvested cells after 21 days of incubation when the cultures were treated with 2.0 mL.L<sup>-1</sup> of *A. niger* extract. This study was aimed at performing extraction, identification, and estimation of coumarins from cell suspension cultures of *A. archangelica* and also investigating the effects of *A. niger* extract, at different concentrations, for various incubation periods on the production of coumarins. These concluded that *A. niger* extract affected coumarin accumulation in the medium and cells from the cultures of *A. archangelica*. A dose of 2.0 mL.L<sup>-1</sup> of *A. niger* extract as a biotic elicitor was found to have the best elicitor concentration, and 21 days of incubation was the optimum period for the elicitor concentration to produce the highest coumarin content in batch cultures. Therefore, these results may increase coumarin production by using different fungal extracts as biotic elicitors in cell suspension cultures.

**Keywords:** *Angelica archangelica*, *Aspergillus niger*, batch cultures, coumarins

### INTRODUCTION

*Angelica archangelica* L. is a perennial herb in the Apiaceae family with greenish-white flowers and large compound pinnately compound leaves (Maurya 2017). It is native to Syria and is also found in west Asia and many parts of Europe (Budniak et al. 2022). It was traditionally used as a medicinal herb for treating headaches, fevers, and toothaches and as a spice (Kowal et al. 2017). It is an important medicinal plant due to its high content of vitamins and minerals (Chandara and Saklani 2017). In addition to its content of secondary metabolites such as essential oils, alkaloids, furanocoumarins, terpenes, and phenolic compounds (Begum et al. 2022), which give it medical and pharmaceutical importance and antimicrobial, antifungal, and anticancer properties (Kaur et al. 2020). Plants produce many secondary metabolites, which are of major importance in the pharmaceutical, food, cosmetics, and agricultural industries (Alamgir 2017a). However, it isn't easy to obtain most of these compounds at the industrial level because of the complexity of their biosynthetic pathways and their complex structures (Siatka 2019). Therefore, cell and tissue culture techniques are now used as an alternative, which is a valuable tool for producing secondary metabolites in the required quantities

in a friendly way to the environment and without environmental constraints (Alamgir 2017b).

Cell suspension cultures are one of the important techniques in the plant cell tissue cultures field due to their biological applications as it is used for the analysis of complex plant physiological processes on a cellular and molecular level, ion transport, enzyme induction, defense responses, and gene expression (Motolinía Alcántara et al. 2021). On top of that is the high ability of cell suspensions to perform physiological processes in the liquid nutritional medium to which suitable physiological regulators were added. Therefore, cell suspension cultures can be used to produce the secondary metabolites produced by whole plants (Sello 2017). Synthesis and accumulation of secondary metabolites are affected by elicitors when plants are exposed to them (Amiri et al. 2020). Elicitors are chemical compounds from biotic or abiotic sources which can stimulate stress responses in plant cells, leading to an increase in synthesis and accumulation of secondary metabolites (Ariningsih et al. 2003; Nagella 2022). The major factors determining the effects on the production of secondary metabolites are type and concentration of elicitor, duration of exposure to elicitor, and cell line (Makowski et al. 2020).

Biotic elicitors such as fungi, considered the most important biotic elicitors, are used in plant cell and tissue

cultures because they stimulate plant cells to perform secondary metabolism to produce compounds such as phenols. However, *Aspergillus niger* was used as an abiotic elicitor by other researchers (Asaf et al. 2018). In addition, fungi play an important role in biotechnological fields, like the production of drugs, chemicals, and enzymes (Salehi et al. 2019). Coumarins considered a large group of phenols, fall within the category of plant secondary metabolites that constitute 150 different species distributed among 30 different plant families. Their production occurs at high levels in the following plant families: Umbelliferae, Caprifoliaceae, Rutaceae, Oleaceae, Clusiaceae, and Apiaceae (Nascimento et al. 2019). Coumarins are found in leaves, fruits, roots, seeds, flowers, and stems. Coumarins are characterized by their smell, similar to new straw (under 2020). Coumarins recently attracted the attention of chemists and pharmacists because they showed that they have antioxidants, anti-inflammatory, anticancer, anticoagulation, and anti-hepatitis (Singh et al. 2016). Coumarins are also used in cosmetic preparations, industrial additives, and their derivatives to improve tobacco smell and taste (Sharifi-Rad et al. 2021).

This study aimed to perform extraction, identification, and estimation of coumarins from cell suspension cultures of *A. archangelica* and investigate the effects of *A. niger* extract, at different concentrations, for various incubation periods on the production of coumarins.

## MATERIALS AND METHODS

### Preparation of seedlings

Seeds of *A. archangelica* were sterilized by washing under running tap water for 15 min in order to eliminate the soil particles, then immersed in sodium hypochlorite solution (the percentage of active chloride is 6%) at a ratio of one volume of the sterilizing material to one volume of sterilized water for 15 min with shaking. The seeds were then washed 3 times with sterilized distilled water, at an average of 5 min each time, to remove the residual of the sterilizing material. Finally, they were sterilized according to the method described by Al-Mahdawe et al. (2018).

### Callus induction

Separated leaves from sterilized seedlings of *A. archangelica* were used as explants for callus induction (Ahmad et al. 2020a). The solid Murashige and Skoog (MS) medium was used for callus induction and maintenance after being enriched with an amount of 2.0 mgL<sup>-1</sup> 2,4-D combined with 0.5 mg.L<sup>-1</sup>kinetin. Cell suspension cultures derived from callus were used to initiate cell suspension cultures derived from callus and to grow cells in liquid nutritional media in Erylimyer flasks of 250 mL (Chimdessa 2017).

### Isolation, identification, and growth of *Aspergillus niger*

*Aspergillus niger* was isolated from the air by the method reported by Checinska et al. (2015). The hyphae were 2.5 to 8 mm wide, septate, hyaline, and branch at an acute angle, either in the shape of a tree or a fan. The

conidial head may be biseriate, radiating, have conidia in chains, or be detached and distributed, and the stipes may resemble zygomycetes' hyphae; Conidia that are single or in pairs can resemble yeast cells. Common clinical isolates of *Aspergillus* spp. can typically be isolated in culture and identified phenotypically quickly and easily. However, culture is frequently characterized as sluggish, leading to misunderstandings about its value for *Aspergillus* detection. *Aspergillus fumigatus* develops quickly. The typical velutinous, grey-blue-green colonies and uniseriate conidial heads grow within 24-48 hrs on both fungal media and the usual sheep blood agar used for bacterial culture. When colonies were measured on malt extract agar and Czapek yeast agar after seven days of incubation at both 25°C and 37°C, it was found that the growth rates of other aspergilli linked to invasive aspergilloses, like *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus*, were similar to those of *A. fumigatus* (Keller et al. 2005).

### Preparation of the important materials (*Aspergillus niger* and Initiation of cell suspension cultures)

*Aspergillus niger* was grown after being isolated and identified, as mentioned previously, and its extract was according to the method described by Madhusudhan et al. (2017). Cell suspension cultures were initiated from callus obtained from leave explants of *A. archangelica*, according to Ahmad et al. (2020b) method. The effect of *A. niger* extract on growth and coumarin contents of batch cultures was tested, following the method reported by Ahmad et al. (2020c). Finally, coumarins were extracted and separated by a method described by Rodrigues-Delgado et al. (2001).

### Quantitative estimation of coumarin

Separation and quantitative estimation of coumarins were performed using fast liquid column chromatography (FLCC), type by Shimadzu 10AV-LC Japan, supported with tow delivery pumps model LC-10A. Shimadzu and metallic separation column has the following characteristics (immobile phase C18-DB; length of column 50 mm; internal diameter of column 2 mm; diameter of matrix granules 3 µm). The identification process was carried out according to compound-specific conditions by comparing standard coumarin specimens with coumarins in the selected samples. The standard coumarin sample identification conditions were as follows: (mobile phase (V: V) 80 methanol 20 distilled water; flow speed of mobile phase 1.2 mL. minute-1; 20 µL of the sample were injected; separation was performed at 25°C; type of detector, at a wavelength of 260 nm) (Rodrigues -Delgado et al. 2001).

Identification of coumarins was performed in laboratories of the Ministry of Science and Technology in Baghdad, depending on the retention time of the standard sample. The concentration of coumarins was estimated according to the following equation:

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of the standard}} \times \text{standard} \times \text{number of dilution.}$$

### Statistical analysis

The statistical analysis was performed using the Duncan test with a probability of 0.05. Therefore, the results with the same letters do not show significant differences, which will be referred to as a star (\*) in the table footnote.

## RESULTS AND DISCUSSION

### Effect of different concentrations of *Aspergillus niger* extract on the growth of cells in suspension cultures

The results showed the effect of different concentrations (0.0, 1.0, 1.5, and 2 mL<sup>-1</sup>) of *A. niger* extract (fungus extract) on the growth of cell suspension cultures showed a decline in the average of the biomass of the harvested cells at 21 days of incubation for all used *A. niger* extract concentrations. The size of the harvested cells after 7 days of growth culture on the medium enriched with 1.0 mL.L<sup>-1</sup> fungus extract was 1.1 mL, while the size of the harvested cells reached 3.9 and 3.2 mL after 14 and 21 days, respectively. The harvested cells from the medium to which 1.5 mL.L<sup>-1</sup> fungus extract was added recorded the size of 1 mL after 7 days of incubation, which increased to 2.9 mL after 14 days of incubation, which then declined in the third week to 2.6 mL (Figure 1).

The cells grown on the medium fortified with (2.0 mL.L<sup>-1</sup> fungus extract) revealed the highest values of volume of the harvested cells in the second week of incubation (5.8 mL) and decreased in the third week (5.0 mL). It was observed from these results that there was an increment in the size of harvested cells after 14 days of incubation and that this decreased after 21 days of incubation at all the used concentrations. The cells grown on the medium fortified with (2.0 mL.L<sup>-1</sup> fungus extract) revealed the highest values of volume of the harvested cells in the second week of incubation (5.8 mL) and decreased in

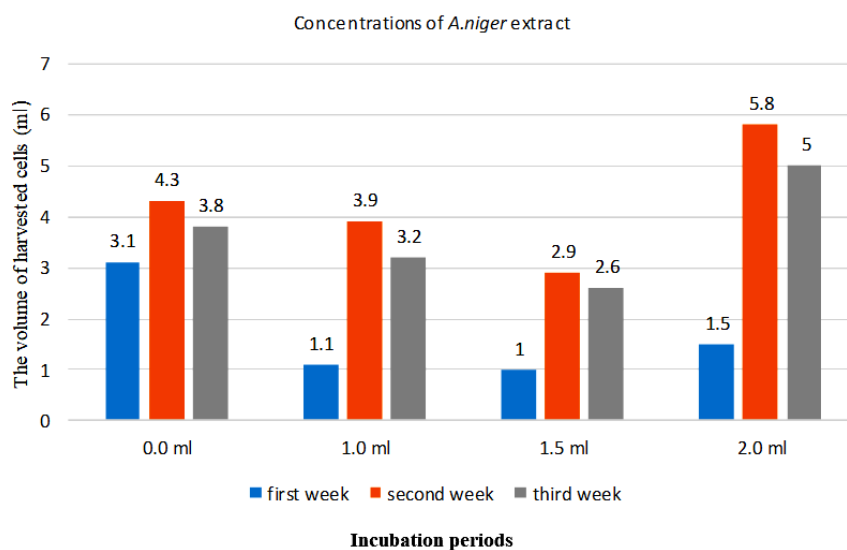
the third week (5.0 mL). It was observed from these results that there was an increment in the size of harvested cells after 14 days of incubation and that this decreased after 21 days of incubation at all the used concentrations.

### Identification and quantitative estimation of coumarin

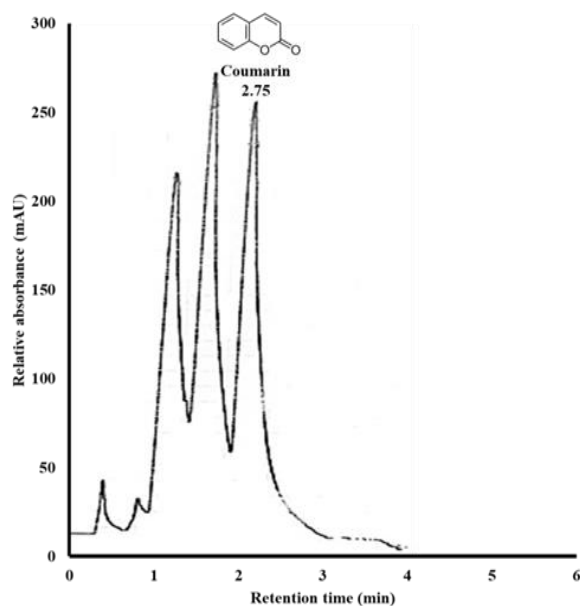
The FLCC profile confirms the presence of coumarins through the nutritional environment of the batch cultures grown on MS medium enriched with growth regulators, which were stimulated by the biological elicitor *A. niger* extract (0.0, 1.0, 1.5, and 2.0 mL.L<sup>-1</sup> *A. niger* extract) incubated for 7, 14 and 21 days (Table 1). In addition, the presence of coumarins was indicated by comparing the retention times of coumarins in the extracted samples with that of the standard coumarins (Figure 2).

Data from statistical analysis of coumarin concentrations in the nutritional medium of different cultures revealed significant variations among different cultures. For example, cultures subjected to 2.0 mL.L<sup>-1</sup> of fungus extract for 21 days showed a concentration of 3.053 mg.g<sup>-1</sup>, which means 5.97-fold compared with 0.841 mg.g<sup>-1</sup> of the control (Table 1).

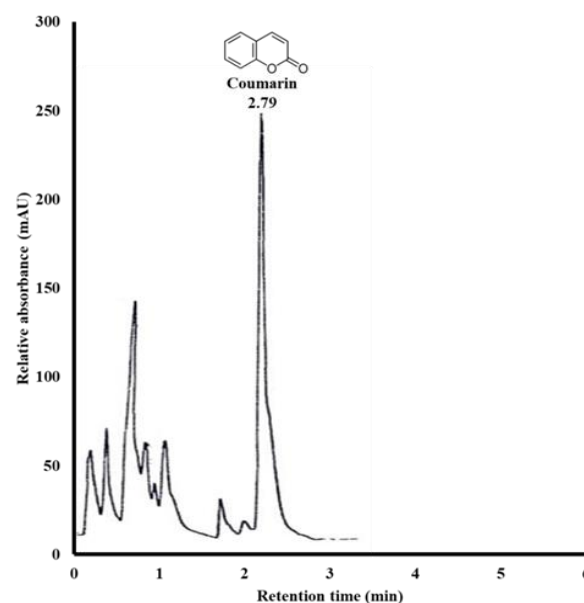
While the highest values of the coumarin in the nutritional medium of cultures exposed to 1.5 mL.L<sup>-1</sup> of *A. niger* extract for 21 days of incubation was 2.889 mg.g<sup>-1</sup>, which is 3.4 times more compared to that of the control (0.841 mg.g<sup>-1</sup>) and reached 2.869 mg.g<sup>-1</sup> in the batch culture, to which 2.0 mL.L<sup>-1</sup> of fungus extract was added for 14 days of incubation, which was 4-fold as much as that of the control (0.709 mg.g<sup>-1</sup>). In general, the coumarin concentration in the liquid nutritional medium increased directly with the increment of the fungus extract at 14 and 21 days of incubation. Therefore, treating *A. archangelica* with 2.0 mL.L<sup>-1</sup> of fungus extract for 21 days of incubation is the most suitable treatment for production in the liquid medium (Figure 3).



**Figure 1.** Effect of different concentrations of *Aspergillus niger* extract on the volume of the harvested cells in the batch cultures during different incubation periods



**Figure 2.** Profile of coumarins using fast liquid column chromatography technique: Profile of standard coumarins



**Figure 3.** Profile of coumarins using fast liquid column chromatography technique: Profile of isolated coumarins from the nutritional medium of the batch cultures

**Table 1.** Retention time and the concentrations of the isolated coumarins from the nutritional medium

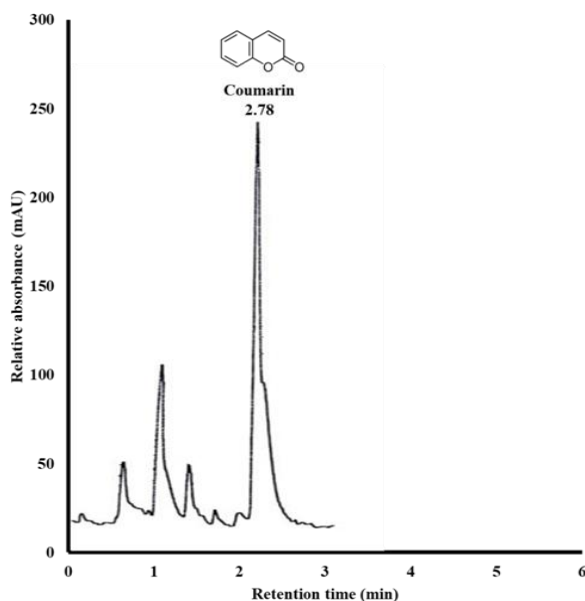
Coumarins source	Day	Coumarins retention time (min)	Coumarins area under the curve (mAU x min)	Coumarins of concentration (mg.g <sup>-1</sup> )
Standard	-	2.75	46563	100
Control	7	2.77	51442	0.511 d
	14	2.74	58781	0.709 b
	21	2.76	40690	0.841 c
The nutritional medium to which 1.0 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.78	40998	0.182 c
	14	2.76	86929	1.717 c
	21	2.79	87904	2.312 b
The nutritional medium to which 1.5 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.77	46628	1.339 b
	14	2.72	48779	2.346 a
	21	2.74	20890	2.889 a
The nutritional medium to which 2.0 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.78	60997	1.103 b
	14	2.73	25226	2.869 a
	21	2.79	40840	3.053 a

Note: \*: See materials and methods

**Table 2.** Retention time and the concentrations of the isolated coumarins from the harvested cells from the batch cultures

Coumarins source	Day	Coumarins retention time (min)	Coumarins area under the curve (mAU x min)	Coumarins of concentration (mg.g <sup>-1</sup> )
Standard	-	2.75	46563	100
Control	7	2.73	30037	0.421 c
	14	2.76	51420	0.507 c
	21	2.74	83613	0.544 b
The nutritional medium to which 1.0 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.77	36174	0.721 a
	14	2.78	40560	1.172 b
	21	2.74	86867	1.314 b
The nutritional medium to which 1.5 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.76	42307	0.071 c
	14	2.77	48449	1.218 a
	21	2.79	47665	1.341 a
The nutritional medium to which 2.0 mL L-1 of <i>Aspergillus niger</i> extract was added	7	2.75	53001	0.679 b
	14	2.79	76433	1.996 a
	21	2.78	50367	2.055 a

Note: \*: See materials and methods



**Figure 4.** Profile of coumarins using fast liquid column chromatography technique (c): Profile of isolated coumarins from the harvested cells of the batch cultures

#### Identification and quantitative estimation of coumarin in the harvested cells

FLCC is used to determine the amount of coumarins. Coumarins are also present in the harvested cells when incubated for different incubation periods (Table 2), as shown by comparing the retention times of the samples with those of standards (Figure 2). Results showed a clear difference in the contents of coumarins in the harvested cells at all incubation periods. The highest concentration of coumarin, obtained from cells of the batch cultures, was recorded at 21 days of incubation ( $2.055 \text{ mg.g}^{-1}$ ). The culture was treated with  $2.0 \text{ mL.L}^{-1}$  fungal extract, which is 3.7 fold more than  $0.544 \text{ mg.g}^{-1}$  of the control (Figure 4).

Followed by concentrations of  $1.5 \text{ mL.L}^{-1}$  and  $2.0 \text{ mL.L}^{-1}$  fungal extract for 21 and 14 days of incubation, respectively, which stimulated the production of coumarins with recorded values of  $1.341 \text{ mg.g}^{-1}$  and  $1.996 \text{ mg.g}^{-1}$  respectively, that were 2.4-fold and 3.9-fold more compared with the control ( $0.544 \text{ mg.g}^{-1}$  and  $0.507 \text{ mg.g}^{-1}$ ).

The results demonstrated that the volume of the harvested cells decreased after 7 days of incubation compared with the volume of the harvested cells after 14 days at all concentrations (Figure 1).

Those could be attributed to the fact that the cell suspension cultures undergo consecutive typical growth periods, which are the lag phase of the slow growth rate, the exponential phase, and the stationary phase, as observed in the cell suspension cultures of the soybean, which pass through the lag phase that lasted from 1-5 days, followed by the log phase that continued for 17 days during which the cells divide rapidly, resulting in an increase in cell density and the growth medium gradually converting to brown color when the cells enter the stationary phase when the rate of cell division declines due to the formation of the secondary metabolites (Ngara et al. 2018; Ramulifho et al.

2019; Mohanlall 2020). Nguyen et al. (2019) reported that the decrease could be due to the accumulation of phytoalexins which are synthesized by the cells of the cell suspension cultures after reacting with the elicitors, some of which suppress the primary metabolism and convert it to the products of secondary metabolism that cause the delay of the cell's growth and reproduction.

Previous data (Ahmad et al. 2020c) showed that the coumarin contents of the batch cultures recorded lower values than those of callus cultures. That can be attributed to the cell culturing system, which depends on the physiological state of the cultured cells that utilize the secondary metabolites. Ramawat and Merillon (2019) reported that the elicitors act by removing the electric charge of the plant cells' plasma membrane to rapidly stimulate a physiological response, leading to changes in the cells' ability to absorb. In contrast, biological extracts affect the production of secondary metabolites by activating the genes involved in producing these compounds or by activating physiological processes that increase the accumulation of secondary metabolites.

That explains the results obtained by the direct increment in the concentrations of coumarin with the increase of the fungus extract concentration for different incubation periods. Which may be related to the stages of growth through which the cell cultures are experienced. Those were reported by Vakil and Mendhulkar (2013) when they studied the effect of *A. niger* extract on the production of andrographolide by cell suspension cultures of *Andrographis paniculata*. They found that the best concentration of andrographolide, which was 6.94-fold higher than the control treatment, was in the cell suspension culture at an incubation period of 10-17 days stimulated by  $1.5 \text{ mL.L}^{-1}$  of *A. niger* extract.

It was observed that the contents of coumarins in the harvested cells were less than those in the nutritional medium drawn from the batch cultures subjected to different *A. niger* extract concentrations at various periods (Table 2). There are various possible explanations for these coumarins' contents, one of which is that some plant species form their secondary metabolites intracellularly and secrete them into the medium afterward. However, in the other types of plants, secondary metabolites are stored in cell vacuoles and not secreted (Isah 2017). This secretion may be due to concurrent cell division and growth and increased biomass, which resembles different stages of the typical growth curve, including the stationary phase where cells do not carry out any division or growth (Ramawat and Merillon 2019; Kriaa et al. 2019). Environmental parameters play a major role in cell cultures (Isah 2017).

Changing color to brown is a prominent feature of these cultures, which may be attributed to increased phenolic compounds. As a result, secondary metabolites are secreted by the cells into the medium due to inadequate culture conditions (Javed et al. 2018). In addition, the cells can secrete coumarins into the medium, as indicated by the results. The results also indicated that the highest concentrations of coumarins were recorded by the culture to which  $2.0 \text{ mL.L}^{-1}$  fungus extract was added as a biotic elicitor for 21 days of incubation. That could be due to the



fact that the elicitors are capable of activating the production of the secondary metabolites that are naturally stored inside the cell (Naik and Al-Khayri 2016). It was reported that increased production of secondary metabolites might occur during both the stationary phase and the last period of the log phase, which could be explained by shifting the primary metabolism to secondary metabolism (Ramawat and Merillon 2019). In addition, the cultures produce substantial coumarin content, as shown in (Tables 1 and 2). Therefore, *A. archangelica* cells partially store or secrete the produced metabolites. Therefore, secondary metabolites from *A. archangelica* cell suspension cultures should be counted together from both medium and cells to maximize the yield.

In conclusion, the *A. niger* extracts affected coumarin accumulation, both in the medium and cells, from the cultures of *A. archangelica*. Furthermore, a dose of 2.0 mL.L<sup>-1</sup> of *A. niger* extract as a biotic elicitor was found to have the best elicitor concentration, and 21 days of incubation was the optimum period for the elicitor concentration to produce the highest coumarin content in batch cultures. Therefore, these results may increase coumarin production by using different fungal extracts as biotic elicitors in cell suspension cultures.

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