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Comparison of the content of active compounds in cell suspensions and aqueous extracts of *Rosmarinus officinalis* L. with those in the Endophytic fungus *Alternaria alternata*

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ABSTRACT

The current study was carried out to detect the active compounds in both the aqueous extract of a *Rosmarinus officinalis* L. plant and the extract of cell suspensions and compare with the extract of the endophytic fungus *Alternaria alternata* isolated from the leaves of a R. officinalis plant. Callus was induced from the leaves of rosemary plants, and the highest induction of callus formed reached 100% when treated with a concentration of 1-Naphthaleneacetic Acid (NAA)2.0 mg. L^{-1}, compared to the control treatment, which had an induction rate of 0%. the highest fresh weight of callus was 1.628 gm in the presence of NAA. The callus induced in the presence of NAA was charactrized by its fragile texture. cell suspensions were also obtained from it. fungus extract was superior in its content of active compounds to the aqueous extract and cell suspension cultures at 21 days. The total alkaloid content which were 6.65%, phenolic content were 117.25 mg.gm, flavonoid content were 104.35 mg.gm, total terpenoid content was 6.65%, total steroid content was 6.05% and the concentration of rosmaric acid was 154.7%. The results of the current study confirm the single receptor of endophytic fungi isolated from medicinal plants as one of the important sources of effective compounds.

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1. INTRODUCTION

Rosmarinas officinalis L. is a plant belonging to the Dicotyledon class Tubiflorae Lamiacea. It is an aromatic plant widely used in foods, endemic to the Mediterranean regions of Europe and North Africa, and cultivated in Spain, Italy, France, Algeria, Morocco, and Portugal [1]. R. officinalis plant contains many phenolic compounds, some of which are effective antioxidants due to their phenolic hydroxyl groups. These compounds also have many other beneficial effects, such as antimicrobial, antiviral, anti-inflammatory and anti-cancer activities and are known to be an effective chemopreventive agent [2]. The rosemary plant is also widely used as a culinary spice, and its fragrance is commonly utilized in soap and cosmetics, Rosemary leaves contain 1.0-2.5% essential oils, characterized by being colorless and sometimes has a pale-yellow color with a distinctive Fragrant aroma [3]. *R. officinalis* plant is one of the most famous medicinal herbs rich in polyphenols (such us carnosic acid and rosmarinic acid) and flavonoids [4]. Rosmarinic acid is an ester of caffeine acid 3,4-dihydroxyphenyllactic acid [5]. This compound extracted from the rosemary plant is well known in the genus Salvia. It also exhibits several biological and pharmacological activities such as anti-inflammatories and anti-allergic reactions [6]. Metabolic compounds play a multifunctional role in plant growth and development processes, inhibition of reactive oxygen species (Ros), and response to environmental stresses [7].

In addition, in the past decades, several experimental studies along with some clinical trials have shown that essential oils have powerful antioxidant, immunomodulatory, anti-inflammatory, and antimicrobial properties, which offer promising ways for the synthesis and development of new drugs [8]. Concerning the high production of these active compounds, there is a need to employ tissue culture technology such as the use of callus cultures or cellular suspensions, to increase the productivity of plants from these substances [9]. Cellular suspensions represent a good system for producing all primary and secondary metabolic products. They consist of a group of cells derived from fragile calluses, and these cells can divide and carry out various metabolic activities.The fragile, incoherent, and fast-growing callus is preferred in the establishment of cellular suspension cultures because its cells are easily disintegrated into monocytes by stirring in a liquid nutrient medium [10]. The success of this technology depends on the density of the transplanted cells and the type of agricultural medium [11]. Endophytic fungi are a type of relationship between certain types of fungi and plants where fungi coexist within plant tissues without causing any harm [12]. They grow within or between plant cells and spend their entire life or part of their life cycle without causing any damage or phenotypic changes in plant cells [13]. The term endophytes is derived from two Greek words (endo = endon) meaning inside and (phyte = phyton) meaning plant. This relationship was first referred to by De and Barry (1866). According to the hypothesis of the coevolution of internal plant fungi proposed by [14] showing that endofungi help plants in chemical defense against various pathogens by producing biologically active secondary metabolic compounds, these substances were also found to be similar to those produced by plants [15]. Many of these compounds produced by endophytic fungi have a variety of pharmaceutical applications and are used in the production of antibacterial ,antifungal ,anti virall, antioxidant, antidiabetic, anti-inflammatory, and many other compounds [16]. Therefore, the current study aimed to compare the content of active compounds in cell suspensions and aqueous extracts of *Rosmarinus officinalis* L. with Endophytic fungus *Alternaria alternata*.

2. METHOD

This study was conducted in the Mycology Laboratory and the Plant Cell and Tissue Culture Laboratory of the Department of Biology at the College of Education for Pure Sciences, Diyala University from January,2023 to July, 2023. It included each of the following steps:

2.1. *Rosmarinus officinalis* L. plant

Rosmarinus officinalis L. plants were obtained from the nurseries of the city of Baquba. The plants were placed in plastic bags and transported to the laboratory to prepare the aqueous extract of the plant and the third group for cultivation on Murashige and Skoog medium (MS) to induce callus and cultivation of cellular suspensions. The plants were washed to remove the dust, dried and then part of the leaves were ground with an electric mill to turn them into powder. The powder was stored in the refrigerator at 4°C until use for the preparation of the aqueous extract of the rosemary.The method described in [17] was followed to prepare the rosemary leaves extract.

2.2. Establishment of callus farms

The separated leaves of the rosemary plant were used after sterilization. They were washed with running tap water and and gently shaking them for 20 minutes. The leaves were then sterilized using 70% alcohol for 30 seconds, followed by a solution of sodium hypochlorite (NaOCl) with an effective chlorine content of 6% .The NaOCl solution prepared by mixing 3 parts sterile material with 7 parts sterile water for 15 minutes with shaking [9]. The plant parts were then washed with sterile distilled water three timesm for five minutes each time to remove any traces of sterilizing agent. The sterile leaves were cut to a length of 1 cm after removing the affected limbs by the sterilization process and the sterile parts were transported on a surface of 20 ml of MS medium [18] steel reinforced with 1-Naphthaleneacetic Acid (NAA) growth regulators at a concentration of (0.0, 1.0, 1.5 and 2) mg. L1 - Overlapping Benzyladenine (BA) at concentrations (0.0, 1.0, 2.0, and 3.0) mg. L1-. Ten plant parts (frequencies) were used for each hormonal treatment. The samples were kept in the growth room under previously mentioned conditions with a light intensity of 2000 lux, and the changes in the plant part were followed up until the original shape disappeared and callus formation occured.

The fresh weight of the callus was determined by calculus by calculating the difference in the weight of glass bottles and their contents before and after removing the part sections. The response ratio and intensity of response to callus induction were determined based on the theoretical scale (Table 1) which theoretically divides callus into several levels (-, +, ++, +++), as the callus gives the value and then collected and divided by the frequency to obtain the intensity of the response[19]. Observations on callus color and texture were also recorded during the 30-day growing period.

Table 1. Theoretical Scale of Response of Plant Parts to Callus Induction

Symbol	What the symbol refers to
-	Lack of response
+	Poor response and callus development at the cutting tips
++	Average response up to 50%
+++	High responsiveness the pieces lose their shape and turn into a full callus cut up to 100%

2.3. Sustaining Callus Farms

A fortified callus induction medium containing 2.0 mg. L1- NAA was used for the callus induced from the leaves to maintain and encourage growth. The period required for the maintenance of the callus was determined based on the change in callus color and the appearance of brown spots, which were associated with the cracking of the middle. During the maintenance, the dead spots were removed from the callus, and the remaining callus was divided into pieces, transferred to glass bottles, and kept under the same induction conditions as previously mentioned.

2.4. Establishment of cellular suspensions farms

crisp callus derived from leaves was used to create cellular suspensions by transferring approximately 1 g of callus pieces to 250 ml glass flasks containing 50 ml of MS liquid medium fortified with the best concentration of growth regulators to induce callus (2.0 mg. l1-NAA) by 11 bis. The samples were incubated in a shaker at a speed of 150 cycles / minute and a temperature of 28 ° C under 16 hours light and 8 hours of darkness. After 24 hours, the culture from two flasks was passed through a sterile microsieve with a hole size of 46 µm (plan Genet. Manipulation on Lab. Nott. U.K.), to isolate single cells and get rid of the collected cell masses. These cells were the re-suspended in a new nutrient medium fortified with the same concentrations of growth regulators. After allowing the cell suspension to stabilize in the culture cabin for two hours, the liquid medium was carefully poured off without losing any of the stagnant cells [20] The culture was then returned to the incubator for the purpose of growing under the same previous conditions. Active compounds were also detected, rosmarinc acid was determined in cellular suspension by 3 decanters per (7, 14, and 21) days.

2.5. Study of the growth curve of cellular suspension cultures

A 1.0 ml sample was withdrawn from the cell suspensions culture after (24, 96, 168, 240, 312, 384, 456 and 528) hours of filtration as well as construction density (zero phases) and placed on a hemositeometer, to calculate the number of cells and increase their numbers with the age of the culture and the activity of its division and determine the density of the cell suspension culture at each age stage by calculating the number of cells in 1 ml and estimating the total number of cells in the entire culture and these cultures were followed up to identify the path of their growth [21].

2.6. Isolated and Diagnosis of *Alternaria alternata*

The previously identified isolate of the endophytic fungus *Alternaria alternata* obtained from a rosemary plant and preserved in the Mycology Laboratory-College of Education for Pure Sciences - University of Diyala, was used to prepare the fungal extract . The extract of the endophytic fungus *A. alternate* was prepared according to the method described in [22] and [23].

2.7. Determination of Active Compounds

The active compounds were estimated in the aqueous extract of rosemary plant, the cellular suspension extract of the rosemary plant, and the extract of endophytic fungus *A. alternate*. The following methods were used for analysis: total alkaloids: determined using the method described in [24] , the total phenolic content: measured according to the procedure outlined in [25], the total flavonoids : detected using aluminum chloride chromatography [26] , terpenes content : calculated by the method described in [27] , total steroid content measured using the method outlined in [28],the method described by [29] was followed to determine rosmarinic acid content.

3. RESULTS AND DISCUSSION

3.1. Induction of callus from *Rosmarinus officinalis* L. leaves

The results of Figure (1) indicate that callus induced from cutting the leaves of rosemary plant Rosmarinus officinalis L. show that the leaves grown on the medium of MS supplemented with growth regulators outperformed their ability to induce callus compared to their peer in the treatment of comparison. The images in the figure showing callus induced from cutting the leaves of the rosemary plant showed an increase in the induction and soft weight of callus as a result of stimulating cells to divide due to the addition of different concentrations of the growth regulator NAA+ added to the growth regulator BA, and the images show that the callus formed had a soft and dense texture and a dark green-reddish color, the highest induction rate was 1.628 gm when adding concentrations of 2 mg/L NNA+ and 0 mg/L BA+, and this was confirmed by the results of statistical analysis and charts, while the comparison treatment did not show any response Figure (A), the shape of the leaf cuttings growing on the medium became 0 mg/L NAA, 2 mg/L BA (B), 1 mg/L NAA and 1 mg/L BA (C) where the callus tissue was formed in the cut areas and led to a change in the shape of the parts completely, and the leaves went in the direction until they were completely transformed into callus fabric after 30 days, the shape (D) was characterized by its light green color and solid texture, while the shape (E) was characterized by its light green reddish color and fragile texture, and this is fully consistent with what Nader (2021) [9]found in her study. [30] stated that the increase in the soft weight of induced callus occurs as a result of the increase in cell division, as well as the hormonal interaction between the ratios of auxin and cytokinin concentrations, as auxin has an important role in the inducement of callus through its effect on increasing the effectiveness of cells to build basic substances for growth [31].

The results of Figure (2) also show the induction ratio of callus derived from sterile and separated leaves from rosemary plant. The diagram reveals that the highest percentage of Callus formation occurred with a concentration of 2 mg/L of NAA+ , while the lowest percentage of occurred with 0mg of NAA+. A constant concentration of NAA with varying concentrations of BA+ was used. The results show that the change of concentration has an effect on the induction ratio, where the smaller size appears - 0% inductance, while the percentage increases with increasing volume, reaching 25% for volume + and reaching 100% for volume +++. The results of the statistical analysis indicate that there are statistically significant differences between the averages, and the highest induction rate can be obtained by 0.430 when the concentration of both NAA is 0% and BA is 2%.

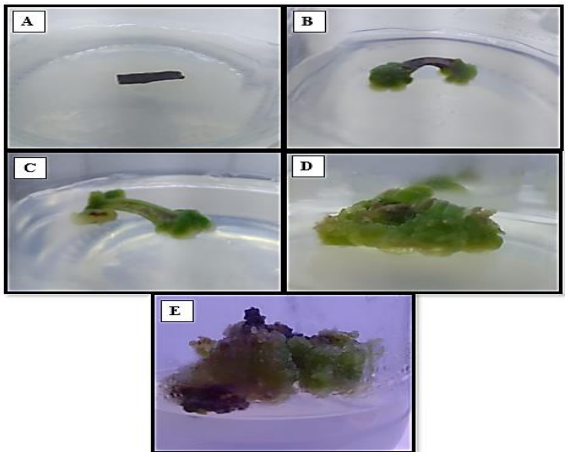

Figure 1. Callus induced from the leaf cuttings of rosemary *Rosmarinus officinalis* by adding different concentrations of growth regulator NAA+ to the growth regulator Benzyladenine (BA). (A): Control treatment no response at concentrations of 0 mg/L NAA+ and 0 mg/L BA. (B): Induction ratio 0.430 at concentrations of 0 mg/L NAA and 2 mg/L BA. (C): inductance ratio 1.424 at 1 mg/L NAA and 1 mg/L BA 0 (D): Inductance ratio 0.9 at 1.5 mg/L NNA + and 2 mg/L BA+. E: Induction ratio 1.628 at concentrations of 2 mg/L NNA + and 0 mg/L BA+.

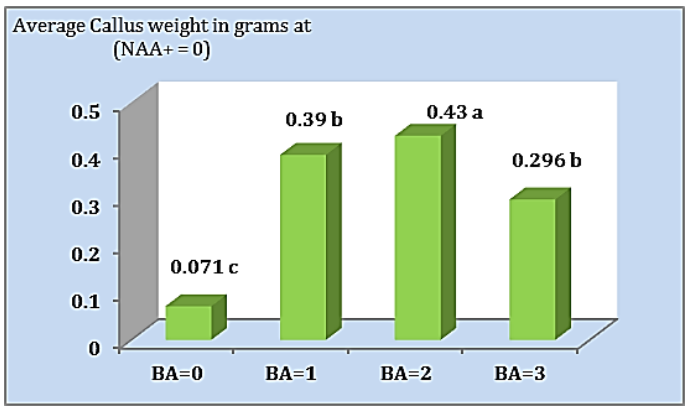

Figure 2. Percentage of callus induction derived from sterile and separated leaves of rosemary *Rosmarinus officinalis* L. at the concentration (NAA+ =0) and different concentrations of BA .

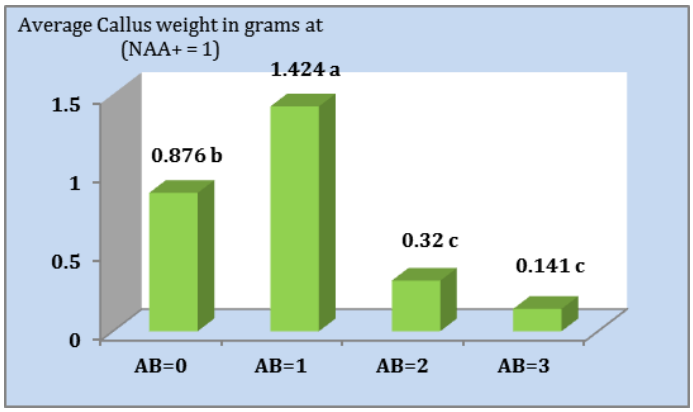

Figure 3. Percentage of callus induction derived from sterile and separated leaves of rosemary *Rosmarinus officinalis* L. at concentration (NAA+ =1) and different concentrations of BA.

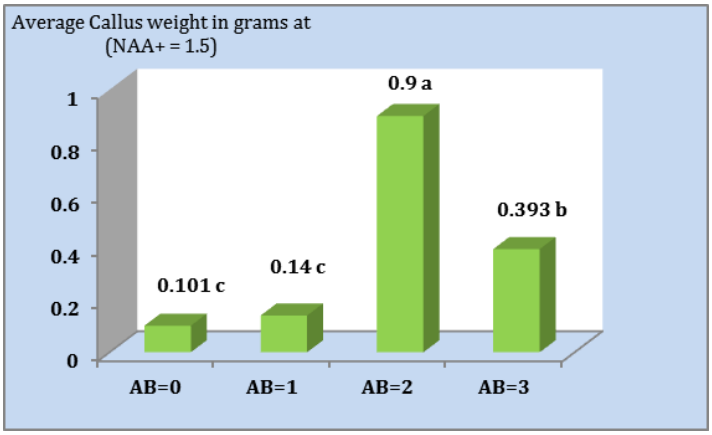

Figure 4. Percentage of callus induction derived from sterile and separated leaves of rosemary *Rosmarinus officinalis* L. at concentration (NAA+ = 1.5) and different concentrations of BA.

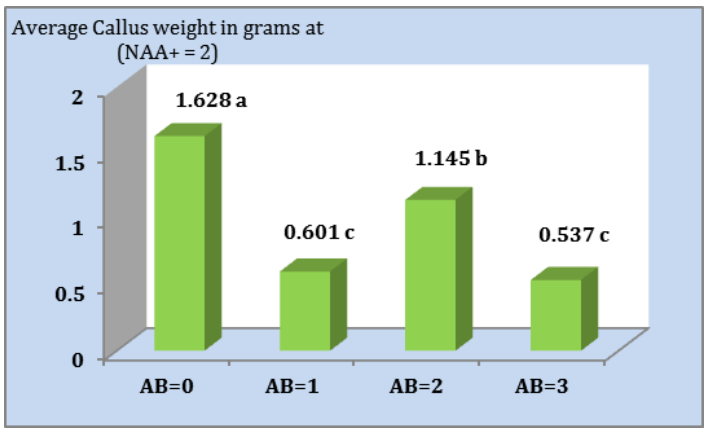

Figure 5. Induction ratio of callus derived from sterile and separated leaves of rosemary *Rosmarinus officinalis* L. at concentration (NAA+ = 2) and different concentrations of BA.

3.2. Cell suspensions of rosemary plant

The results confirmed that the liquid MS induction medium supplemented with 2.0 mg. L1-NAA was highly suitable for creation cellular suspension cultures derived from *Rosmarinus officinalis* L. This medium promoted cell growth and initiated the first cell division within 24 hours of suspension. The density of the cell suspension culture increased progressively, reaching (29.5 × 10 5) cells/cm3 after 384 hours as shown in Table (2) ..

Cell suspension culture represents a recent trend in plant biotechnology as a model system not only for callus acquisition, but also for monitoring the division, expansion, and specialization of single cells [11]. The motivation for adopting the technology of creating cell suspensions derived from callus lies in determining the critical density required for cell division and subsequent growth, following up the behavior of cells in their division, trying to overcome the difficulties of callus inductions, as well as establishing closed suspended cell cultures to know the ability of these cells to produce effective compounds. This study indicated the success of MS liquid-fortified medium at a concentration of 2.0 mg. L1- NAA + 0.0 mg. L1-BA in the creation of cellular suspensions and cell retention of vitality.

Table 2. Growth rate of cell suspension cultures of *Rosmarinus officinalis* L**.**

Total number of cells (x 10^5)	Duration (hourly)
8.5 c	24
14.5 c	96
20.5 b	168
27.3 a	240
28.2 a	312
29.5 a	384
27.6 a	456
27.5 a	528

* Values that have similar letters do not have significant differences between them with statistical significance.

3.3. Detection of Active Compounds in *Alternaria alternata* Extract, Aqueous Extract and Cellular Suspensions Cultures of Rosemary Plant

The results in Table (3) show that the extract of the endophytic fungus *Alternaria alternata* outperformed both the aqueous extract and the cellular suspension extract of rosemary plant in terms of alkaloid content, with a concentration of 6.55 mg. g In comparison, the aqueous extract and the cellular suspension extract at 21 days of age contained 4.92 mg/g and 4.5 mg/g, respectively. The highest total phenolic content was found in the in *A. alternata* extract , at 117.25 mg.gm, followed by the aqueous extract of rosemary, which had 90.08 mg.gm . The cellular suspension extract at 21 days had a phenolic content of 75.9 mg.gm.

The results showed that *A. alternata* extract outperformed the total flavonoid content over aqueous extract and 21-day-old suspensions for rosemary plant, as the flavonoid content of fungi extract was 104.35 mg. gm, while the content of aqueous extract and cellular suspensions at 21 days of age was 33.45 mg. gm and 50.9 mg.gm As shown in the table, the superiority of *A. alternata* fungi extract over aqueous extract and suspensions cultures at the age of 21 days for rosemary plant, as the total concentration of terpenes of fungi extract was 6.65%, while the content of aqueous extract and cellular suspensions at the age of 21 days was 1.29% and 3.6%, respectively, the fungi extract with its total steroid content outperformed the aqueous extract and suspensions cultures at the age of 21 days, as the extract content of total steroids reached 6.05%, while the content of aqueous extract and suspensions Cellular at the age of 21 days 1.19% and 3.0% respectively, the results also show that the internal fungus *A. alternata* isolated from the leaves of the rosemary plant the ability to produce rosmaric acid, which is a characteristic of this plant, where the results show the superiority of the extract of the fungus *A. alternata* on the aqueous extract and cultures of cellular suspensions at the age of 21 days for the rosemary plant in its content of rosemaric acid, which was, as the concentration of rosmaric acid was 154.7%, while its concentration was in the aqueous extract and cellular suspensions By 21 days of age, rosemary plants are 92.5% and 75.9%, respectively.

Table 3. Determination of Active Compounds in Aqueous Extract and Cellular Suspensions Cultures of Rosemary Plant and *Alternaria alternata* Extract

Compound	Fungus extract	Cellular suspensions at 21 days of age	Aqueous extract
Total phenolic content (mg/gm)	117.25	75.9	90.08
Total flavonoid content (mg / gm)	104.35	50.9	33.45
Total alkaloid content %	6.55	4.5	4.92
Total terpenoid content %	6.65	3.6	1.29
Total steroid content %	6.05	3.0	1.19
Rosmaric acid content %	154.7	75.9	92.5

This finding confirms the hypothesis that shows the possibility of endogenous fungi produce active compounds similar to the plant with which they coexist, but in larger quantities, as proven by several studies, including [32], [33], [34]. In [35]pointed out that the endophytic fungi are an important source for the production of alkaloid compounds with multiple biological efficacies and that the alkaloids produced by internal fungi have a role in plant protection as [36] pointed out that a series of compounds were isolated that were similar in the host plant and the fungus symbiosis with it, and this is confirmed by our current study that both the plant and the internal fungi living with it had the same active compounds but in different concentrations. In a research made by Linh et al. [37] that studied the cell and tissue cultures of *Catharanthus roseus* on a large scale as an alternative strategy to improve the production of secondary compounds, he created cellular suspension cultures from this plant's callus of good quality and quantity to improve total alkaloids, and the low alkaline content in the culture was compensated using internal fungi isolated from the local rose plant, which significantly enhanced the accumulation of alkaloids such as vinplastine and vincristine.

Many studies have demonstrated that endophytic fungi can produce high concentrations of phenolic compounds. For instance, [38] showed that the extract of endophytic fungi contains a large amount of phenols compared to the plant from which they were isolated. [39] Reported that *Alternaria alternata* extract contained secondary metabolites such as phenols. Several studies have shown that the aqueous extract of rosemary is rich in active compounds [40], [41]. Given that the plant produces these active compounds, it is natural that the cellular suspensions might also contain high concentrations of these active compounds, which can be higher than their concentration in the plant or plant extract, as confirmed by the current study in that the concentration of steroids was in cellular suspensions higher than in the aqueous extract. These result confirms the importance of cellular suspension technology for obtaining active compounds with different therapeutic properties in large quantities and in shorter time compared to the plant's natural life cycle. The current results are the first of their kind in terms of recording the ability of the internal fungus *A. alternata* to produce rosmaric acid, identifying this fungus as a rich source of this acid compared to the rosemary plant. It is also known that the rosemary plant is an important source of rosmaric acid and that its aqueous extract is rich in this acid, and this is confirmed by the results of the current study, which is consistent with the results of the study of [42] The results of the study also showed the possibility of producing rosemaric acid in cellular suspensions cultures for rosemary plant, and this is consistent with [43] which showed the possibility of producing rosemaric acid by cellular suspension cultures from rosemary plant leaves.

4. CONCLUSION

The results of the current study highlight the promising potential of endophytic fungi for producing secondary metabolic compounds with various therapeutic properties. The study demonstrated that the extract from the endophytic fungus had a superior content of active compounds compared to the rosemary plant from which it was isolated, and even exceeded the amount of active compounds found in the cellular suspension of the rosemary plant. This underscores the importance of using endophytic fungi to obtain secondary metabolic compounds in large quantities, in a shorter period, and at a lower cost.

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