JOURNAL OF NUTRITION FASTING AND HEALTH

Quercetin Production and Phenylalanine Ammonia Lyase Activity Enhancement by Putrescine and Benzyl Amino Purine *in Vitro* Culture of *Achillea Millefolium* Linn

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ARTICLEINFO

Article type:
Research Paper

Article History:
Received: 28 Jun 2020
Accepted: 27 Oct 2020
Published: 20 Nov 2020

Keywords:
Achillea millefolium Linn.
Benzyl amino purine
Putrescine
Quercetin

ABSTRACT

Introduction: Quercetin is observed in abundance in *Achillea millefolium* Linn and it has been employed for the treatment of diseases, infections, as well as health issues. This study examines the individual and combined impact of putrescine and benzyl amino purine (BAP) with the stimulation of quercetin production and the activity of phenylalanine ammonia-lyase (PAL) in *Achillea millefolium* Linn cultures.

Methods: Seedlings with four leaves were cut and transferred to MS basal medium (Murashige and Skoog Basal Medium). At the next stage, different concentrations of putrescine were applied. Quercetin was identified through using HPLC, and the assessment of PAL activity was completed by accounting for the level of cinnamic acid produced at 290 nm. Different experimental data were analyzed after collection using SAS software and Duncan's test was employed to compare the means.

Results: With the addition of putrescine, the quercetin production, BAP, and PAL activities were remarkably affected. Moreover, quercetin production and PAL activity were enhanced through stimulation applied using putrescine with varying concentrations. When various concentrations of putrescine were applied, the fact was illustrated that 2 mg/L and 1 mg/L experienced the highest amount of quercetin and PAL activity, respectively.

Conclusion: This particular finding suggests that combining elicitors and plant growth regulators is a beneficial strategy adopted in enhancing PAL activity and the production of quercetin for *in vitro* cultures of *A. millefolium.*

► *Please cite this paper as*:
Behdarvand Askar S, Afshari H, Oraghi Ardebili Z. Quercetin Production and Phenylalanine Ammonia Lyase Activity Enhancement by Putrescine and Benzyl amino Purine in Vitro Culture of Achillea Millefolium Linn. J Nutr Fast Health. 2020; 8(4): 264-272. DOI: 10.22038/jnfh.2020.49980.1275.

Introduction

Being employed for the treatment of diseases, infections, and health issues, secondary metabolites are widely found in medicinal plants (1, 2) *Achillea millefolium* Linn. is included in the Asteraceae family and commonly known as milfoil or yarrow (3). The valuable active ingredients of this plant are salicylic acid, isovaleric acid, asparagine, sterols, flavonoids, coumarins, and tannins, etc. This plant has significant medicinal qualities and it is used in a variety of herbal tea to treat headaches, hepato-biliary and gastrointestinal problems and externally applied in the form of ointments and lotions to cure possible skin inflammations, injuries, scars, and abrasions. Some of these herbal and medicinal compounds are used as a haemostatic for the treatment of colds and flu (4-8). Bioactive and valuable compounds can be generally produced by adhering to some strategies including plant cells, tissues, and organ cultures (9). Additionally, secondary metabolites and their metabolic pathways may be investigated *in vitro* cultures creating the optimal conditions in producing these metabolites (10, 11). Plant tissues and suspension cultures are often approached by *in vitro* research and the studies conducted in this regard provide directions to produce secondary metabolites for commercial purposes (12). There are indeed numerous studies in which researchers have attempted to and concentrated on increasing the production of secondary metabolites by means of medium optimization. For example, we can refer to plant growth regulators, cell immobilization, the selection of cell lines, metabolic engineering, precursor feeding, and elicitation (13).

As mentioned above, for *in vitro* systems, elicitation is among the crucial strategies

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selected to improve the quantity and the amount of secondary metabolite. Moreover, this strategy contributes to the reduction of production costs (9, 14-16). The biotic and abiotic elicitors generally applied to stimulate the further development and composition of the secondary metabolites are tremendously helpful in reducing the time taken during the process in increasing and enhancing the volumetric productivity (16). We may point to several reports and cases where elicitors have actually tried to increase the level of rosmarinic acid discharge (17), the number of phenolic compounds (18), thebaine (19), and tanshinine (20). Furthermore, there are more cases similar to these studies. Among the bioactive compounds observed in the biochemical and physiological processes, we can refer to polyamines (i.e. spermidine, putrescine, and spermine). These compounds are composed of synthesized protein, replicated DNA, divided cells, etc. also observed in the reaction in connection with the abiotic stress, the regulation of rhizogenesis, senescence, embryogenesis, fruit ripening, and floral development (18, 21).

Some species such as *whitania somnifera* (22*)* and *Glycine max* (23) have been shown in conditions where they were improved by the exogenous function of polyamines concerning the positive effect they elicited on their micro-propagation. Nevertheless, there are not many studies consistently reporting the positive effect of exogenous polyamines on the quantity and quality of secondary metabolites. For the same reasons, this research investigates the impact of polyamine (putrescine) on the produced quantities of quercetin and the phenylalanine Ammonia Lyase (PAL) activity as a plant growth regulator, 6-Benzyl Amino Purine (BAP), and an elicitor. Quercetin belongs to the flavonoid family and it is one of the most plant pigments (24). According to the IUPAC system, nomenclature for quercetin is 3, 3′, 4′, 5, 7-pentahydroxyflvanone with an antioxidant role specifically (24, 25). PAL is an enzyme catalyzing the conversion of the phenylalanine to form trans-cinnamicacid (26). This enzyme is the main enzyme in the phenyl propanoid pathway playing the key role in the biosynthesis of phenolic materials, i.e. phenols with antioxidant action. besides, this study approaches this investigation for the first time in *A. millefolium* cultures.

Materials and Methods

Plant material

We obtained the seeds of A. millefolium from Pakan Bazr, a company located in Isfahan province in Iran. By using about 1% benomyl fungicide and 1.5 (w/v) sodium hypochlorite solutions for 7 minutes, the seeds were surface-sterilized in this manner. Besides, three drops of tween 20 for 1 min were applied and the seeds were then rinsed in sterile distilled water at three different times. The MS basal medium (Murashige and Skoog medium) (27) was used to germinate the seeds after solidification with 7 g/L agar. Then, the seeds were preserved in the growth chamber at 21 ± 3 ºC with a photoperiod lasting using cool white fluorescent light for 16 h. Each experiment was replicated at least three times and a control group was considered to compare the treatments with the culture medium of the control group with any additives.

Figure 1. Germinated seeds with leaves of *A.millefolium* in the MS culture medium.

Elicitation with putrescine and using BAP

Seedlings with four leaves were then cut and transferred to MS basal medium (Fig. 1). As the next step, different concentrations of putrescine were applied including 1, 2, and 4 mg/L. This procedure was followed one month after

germination. This procedure is aimed at identifying the most effective concentration of putrescine to induce the quercetin biosynthesis and enhance PAL activity. The BAP was added to the concentrations of 0.5 and 1 g/L to observe the effect of BAP on quercetin biosynthesis and PAL activity. Accordingly, to stimulate the studding and the synergic effect of putrescine and BAP, we added 1, 2, and 4 mg/L putrescine to cultures with 0.05 and 1 mg/LBAP (28).

Sample preparation and HPLC analysis of quercetin

Following the methanol method developed and described by Chandrappa, Govindappa (29) for such cases, quercetin was extracted by a modified method. Then, about 1 g of samples (stem and leaves) was powdered. About 10 mL of methanol was added to these samples in the next step. The samples containing solvent were taken to the Erlenmeyer flask and put in a shaker at 140 rpm after being wrapped in an aluminum foil for 48 h. Approximately 48 hours later, we centrifuged the extract at 3000 g at 25 ºC for 15 minutes. Following the same procedure, the supernatant was collected and preserved for 24 hours in the dark at room temperature to allow methanol to evaporate. A waters HPLC system was employed to perform the HPLC analysis (UV Visible Detector). The standards used in this study are combinations of quercetin dehydrate with molecular mass 338.27 M. To plot the calibration curve, different concentrations of the standard were prepared (150, 300, 450, and 750 ppm) and injected in HPLC column (30). Then, 20 µL injections of the sample were done in the C18 reverse-phase Waters column (4.6 × 250 mm). The mobile phase is comprised of two solvents including water (Solvent A) and acetonitrile (Solvent B) (30:70 v/v). With a flow rate of 1 mL/min, quercetin was eluted and 210 nm was recorded for the chromatograms. Quercetin was identified by using standards and comparing the retention time. Accordingly, the standards of external calibration were performed for the quantification.

Determination of phenylalanine ammonia-lyase (PAL) activity

Based on the process explained by Beaudoin-Eagan and Thorpe (30), the measurement of the PAL activity was completed. In summary, according to the protocol, 6 µmol of phenylalanine, 500 µmol of Tris-HCI buffer (pH= 8.01), and 100 µmol of the extracted enzyme were mixed. Moreover, the spectrophotometric assessment of PAL activity was completed by accounting for the level of cinnamic acid produced at 290 nm. This produced amount was indicated as the microgram of cinnamate per min per gram fresh weight (µgCin/min/g/fw) (30).

Statistical analysis

Different experimental data were analyzed after collection using SAS software and Duncan's test was used to compare the means. The charts were drawn and evaluated with Excel software.

Results

Effect of putrescine on quercetin production and PAL activity

Fig. 2 shows the HPLC chromatogram related to the quercetin extracted from samples. The results demonstrated that PAL activity and the amount of produced quercetin had a significant impact concerning different putrescine concentrations. The amount of quercetin produced by seedlings increased once putrescine was added to the culture medium. In this way, the highest level of quercetin (0.31 µgCin/min/g/fw) was obtained equal to 2 mg/L putrescine concentration that was the best concentration to stimulate quercetin production. This amount was obviously higher than that of the control, 1, and 4 mg/L putrescine treatments. The seedlings treated with 2 mg/L putrescine led to a quercetin yield 1.4 fold higher than the control. Additionally, the impacts of putrescine on the produced amount of quercetin were observed to be dose-dependent. The increase in the putrescine concentration from 2 to 4 mg/L caused a 1.26-fold decrease in the amount of the produced quercetin. According to the results of Fig. 3, dose-dependent putrescine concentration shows that any dose of putrescine concentration compared to the control group column (d) led to an enhanced level of quercetin production. However, column (a) shows that the best putrescine concentration to stimulate quercetin production is 2 mg/L, and compared to column (b), it showed no linear relationship between increasing the concentration of putrescine and increasing quercetin production. Finally, it can be said that these results indicated the saturation of 2 mg/L putrescine to the quercetin production.

The putrescine treatment significantly induced the PAL activity of seedlings. Given the PAL activity, the efficient concentration of putrescine was observed to be 1 mg/L. Thus, 1 mg/L putrescine marks the highest PAL activity (2.48 µgCin/min/g/fw) column (a) obtained in this study. This activity was about 1.40 fold higher than that of the control column (d). Likewise, as their effects on the amount of quercetin, the impacts of putrescine on PAL activity were observed to be dose-dependent. As shown in Fig. 4, with an increase from 1 to 2 and 4 mg/L in the concentrations of putrescine, PAL activity decreased to 1.26 and 1.27-fold, respectively.

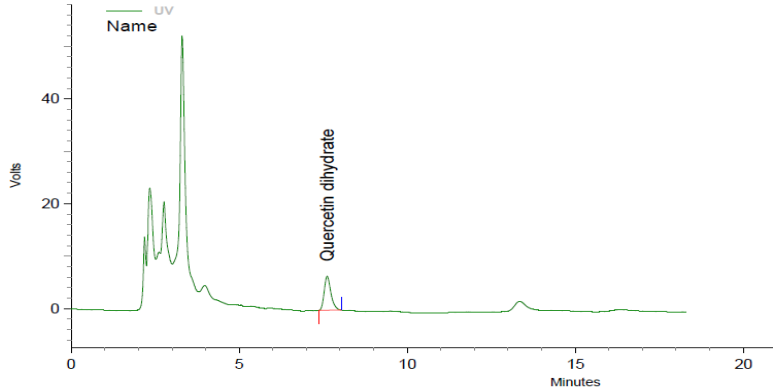

Figure 2.HPLC chromatogram of extracted quercetin from *in vitro* culture of *A.millefolium*.

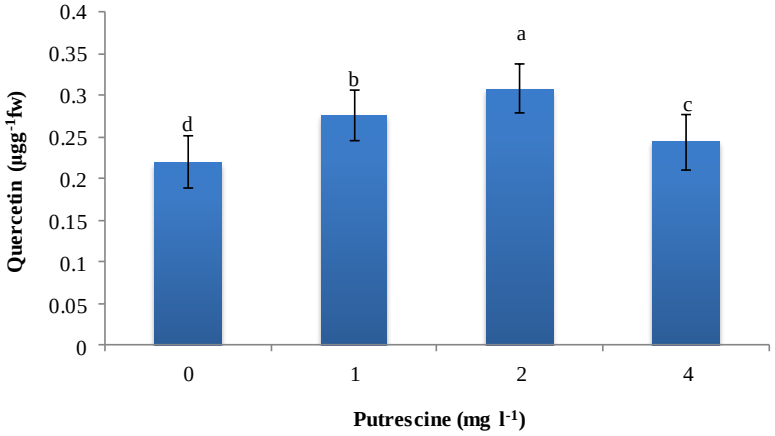

Figure 3. Effect of putrescine on thequercetin production in *in vitro* culture of *A. millefolium*.Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

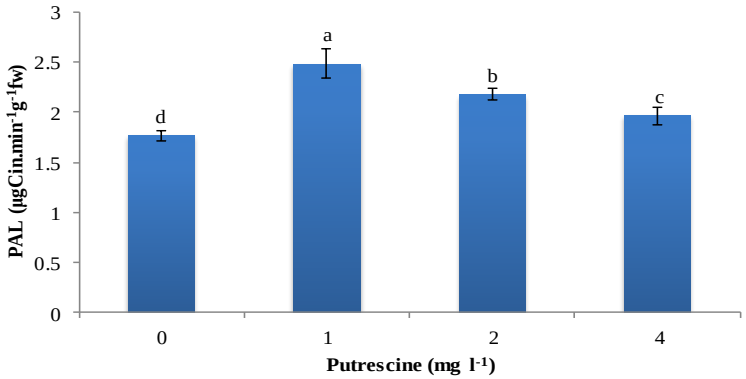

Figure 4. Effect of putrescine on PAL activity in *in vitro* culture of *A. millefolium*.Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

Effect of BAP on quercetin production and PAL activity

The results of Fig. 5 revealed that the BAP different concentrations ($p \leq 0.05$) had a significant impact on the amount of quercetin and PAL activity. BAP application at the culture medium caused the seedlings to increase the yield of quercetin. Seedlings treated with 0.5 mg/L BAP showed a level of quercetin (0.24 µgCin/min/g/fw) indicating an amount that significantly higher than the control column (b). However, this increase in the quercetin yield did not reach statistical significance. Thus, it is worth noting that the BAP addition that was in low concentrations did not show any significant impact on quercetin yield.

The treatment of seedlings with 1 mg/L BAP had the highest yield of quercetin (0.31 µgCin/min/g/fw). Clearly, these levels are significantly higher than 0.5 mg/L BAP and that of the control. Based on the information provided in Fig. 5, when the seedlings were treated with 1 mg/L BAP, they indicated the quercetin yields that were 1.29 fold higher than control. There was a significant effect on the PAL activity of seedlings treated with a different concentration of BAP. The most effective BAP concentration on PAL activity was recorded to be 1 mg/L. Moreover, the highest level of PAL activity (2.26 µg Cin/min/g/fw) was achieved at 1 mg/L BAP and this level was 1.09 and 1.14-fold higher than the control and 0.5 mg/L BAP (Fig. 6).

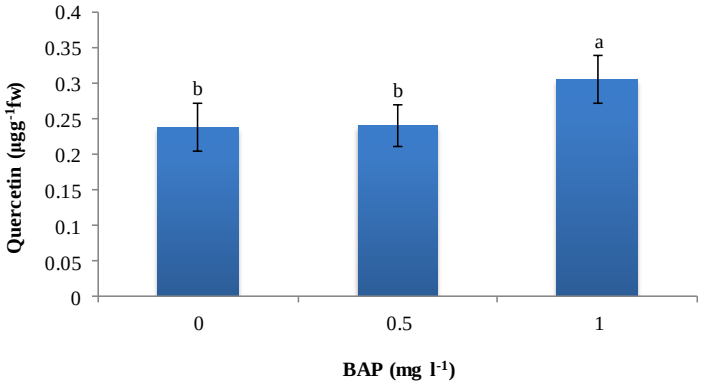

Figure 5. Effect of BAP on quercetin production in *in vitro* culture of *A. millefolium*.Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

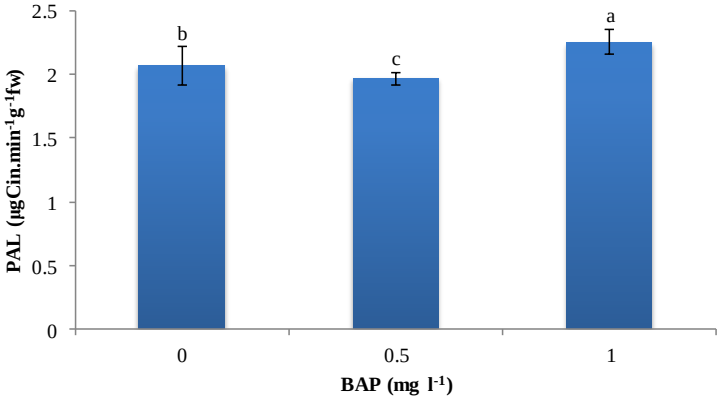

Figure 6. Effect of BAP on PAL activity in *in vitro* culture of *A. millefolium*.Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

Synergistic effects of putrescine and BAP on quercetin production and PAL activity

One of the benefits offered by the present research work is that the impact of BAP the synergic effect of putrescine was examined on PAL activity and quercetin production. Arguably, it was found that the seedlings treated with 2 mg/L putrescine and 1 mg/L BAP showed (0.48µgCin/min/g/fw) quercetin that was 1.55, 1.70, and 5.22 fold higher than 2 mg/L putrescine, 1 mg/L BAP, and control. Thus, the

use of putrescine combined with BAP led to an increase in the quercetin level (as observed in Fig. 6). The highest level of PAL activity was recorded to be 2.83 (µgCin/min/g/fw). This high activity was observed in seedlings merely treated with 1 mg/L putrescine and was 1.77 fold higher than the control. As illustrated in Fig. 7, after applying 1 mg/L putrescine with 1 mg/L BAP, the activities of PAL showed (2.68 µg Cin/min/g/fw), that was 1.38 and 1.68-fold higher compared to 1 mg/L BAP and control.

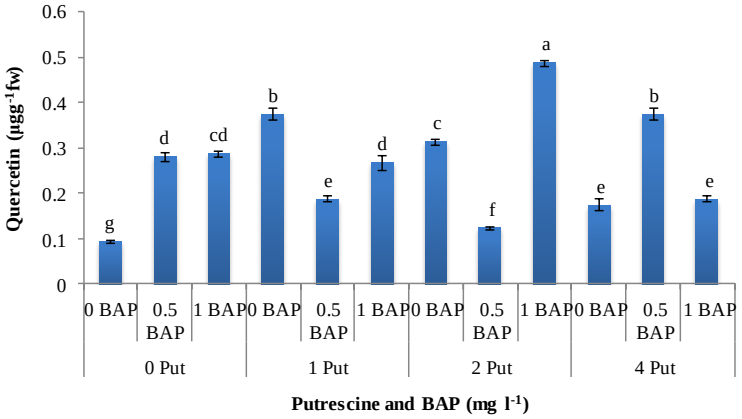

Figure 7. Effect of putrescine and BAP on quercetin production in *in vitro* culture of *A. millefolium*.Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

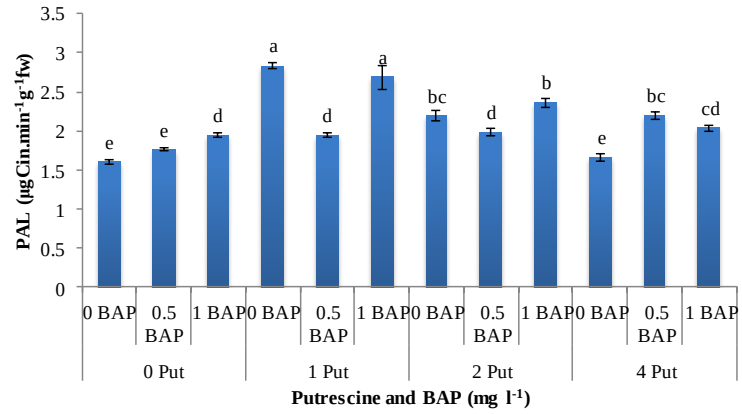

Figure 8. Effect of putrescine and BAP on PAL activity in *in vitro* culture of *A. millefolium*. Values followed by different letters in each trait are significantly different a $p \leq 0.05$.

Discussion

The accumulation of quercetin has been often stimulated by abiotic elicitors like β-phenylalanine in the cultures of *Gossypium* cultivar (31), *Balantiesa egyptiaca* (32), *Citrullus colocynthis* (Linn.), Schrad (33), and *Cassia Angustifolia* (34). In the same line of enquiry, different elicitors (biotic and abiotic) have been used by the researchers over the last few years. The objective of this application has been to cause the plants to increase the production of secondary metabolites. Concerning the functions of plants, the PAL activity improved with elicitor treatment for infection and wounds (35). PAL plays a key role in the phenylpropanoid pathway. It is necessary to activate PAL to allow for the synthesis of the phenolic compounds and improve the plant defense in response to the biotic and abiotic stresses (36). Several researchers have reported the application of various biotic or abiotic elicitors like the yeast extract, methyl jasmonate, and salicylic acid to stimulate the PAL activity. In this connection, we may refer to the cultures of *Solenostemon scutellarioides* and *Salvia miltiorrhiza* (36). Chakraborty (37) demonstrated that chitosan treatment induced the PAL activity in the suspension culture of *Cocos nucifera*. Furthermore, an increase was

observed in PAL activity in cases using pathogenic fungus *Fusarium oxysporum* f. sp. *Albedinid* to inoculate the seedling roots of *Phoenix dactylifera* (38).

As a regulator of plant growth, BAP is among the crucial elements leaving an impact on the formation of metabolite (39). The activities of enzymes such as auxins and cytokinins involved in the production of secondary metabolites are altered about the concentration and kind of regulators used to grow plants (40). Thiruvengadam and Chung (18) noted that the accumulation of flavonols, hydroxybenzoic, and hydroxycinnamic acid derivatives in *Cucumis anguria* L. culture were significantly affected by BAP activities. In this manner, Coste, Vlase (41) also stated that 4 mg/L BA improves the hypericins production in *Hypericumm aculatum* and hyperforin in *H. hirsutum*.

In the latest works in this line of research, scholars have investigated the synergic impact of elicitors, minerals, and precursors on the secondary metabolites. The researchers did not take advantage of the various combinations of elicitors and plant growth regulators in such cases. Amdoun, Khelifi (42) noted that Ca^{2+} (11.1 mM), $H_2PO_4^-$ (6.06 mM), and the ratio of nitrate/calcium (62.5 mM/11.1 mM) caused an increase in the alkaloid level when it was combined with 10 m Mjasmonic acid in *Datura stramonium* culture. In cell cultures of *Artemisia annua,* the highest level of artemisinin accumulation was achieved on the supplementation of methyl jasmonate as an elicitor and mevalonic acid as a precursor (43). Likewise, in the cell suspension culture of *Papver bracteatum,* Zare, Farjaminezhad (19) examined the synergistic impacts of methyl jasmonate and L-tyrosine on the production of thebaine. The results of their study demonstrated that 100 µM methyl jasmonate with 2 mM L-tyrosine affects the thebaine yield by increasing the yield of cells up to 84.62 mg/L.

Conclusions

In sum, the present research recommended an efficient way of enhancing the yield of quercetin and PAL activity for the first time for *in vitro* cultures of *A. millefolium.* It was found that putrescine, BAP, and their dosages affect the quercetin production and PAL activity in cultures. The stimulation of quercetin production through the plant growth regulators (e.g. BAP) and the synergistic quality of elicitors (e.g. quercetin) can be considered as the other useful finding of this research. This particular finding suggests that combining elicitors and plant growth regulators is a beneficial strategy adopted in enhancing PAL activity and the production of quercetin for *in vitro* cultures of *A. millefolium*.

Acknowledgments

The authors thank the Islamic Azad University for financial support.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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