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Formononetin Production by Large-Scale Cell Suspension Cultures of *Medicago sativa* L.

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Abstract: In this study, calli of *Medicago sativa* L. cv. Elçi (alfalfa Elçi) were inoculated in cell suspension culture and analyzed for aggregate assay, cell viability test, total phenolic content assay, DPPH free radical scavenging activity and formononetin assay by means of High-Performance Liquid Chromatography (HPLC). Hypocotyl, cotyledon and apical meristem explants were taken from 15-day-old aseptic seedlings and germinated in MS medium. 10 g calli were grown for each explant and then transferred into cell suspension culture. The highest cell viability rate, which was 75%, and the highest DPPH free radical scavenging activity with 51.36% was measured in 1000 mL cell suspension culture, while the highest total phenolic content, i.e. 40.2 mg/g, was quantified in 250 mL cell suspension culture. In accordance with the findings of the study, the production of formononetin was higher in the calli derived from cell suspension cultures than in herb samples of *M. sativa*. Moreover, in 1000 mL cell suspension culture, 4.99 mg/g of formononetin concentration was quantified, which scored the highest. In large-scale cell suspension cultures of *M. sativa*, it was possible to increase the production of formononetin production. Hence, due to its medicinal significance, a method has been tested to obtain higher amounts of this compound.

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1. INTRODUCTION

Medicago sativa L. (alfalfa) is an important feed plant in all over the world with its ability to adapt to different climates and its high feed efficiency and quality. *M. sativa* spreads in world's temperate regions (such as the USA, Southern Canada, Europe, China, South America and South Africa). In general, it shows spreading characteristics up to 2400 m. It is more resistant to drought compared to other feed types.

The plant has been used as a herbal supplement for increasing strength and energy, detoxifying blood, fighting against infections and treating anemia. *Medicago* is also known as a model plant because of its ability to regenerate *in vitro* in plant tissue culture (Erişen, 2006). *M. sativa* is also reported to reduce the menopausal symptoms in women by increasing the levels of estrogen (Gülen, 2013; Çölgeçen et al., 2014; Franciscis et al., 2019). *M. sativa* is rich in flavonoids, too. The term "flavonoids" is derived from the Latin word "flavus," meaning yellow. The basic flavonoid structure consists of 15 carbon atoms (C_6-C_3-C_6 system) and 2-phenyl benzopyrone (diphenylpropane). Additionally, flavonoids are polyphenolic compounds

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(Kahraman et al., 2002). They are found in all organs of the plants such as flower, leaf, root, stem, seed and fruit (Işık, 2005). Although the flavonoids have been studied for many years, their biological activities and cellular mechanisms have not yet been fully elucidated. However, studies have shown that they have free radical-scavenging (antioxidant) effect, cardiovascular and liver protective role, antiviral, and as recently suggested, anticancer properties (Birman, 2012).

Analyzed in this study, formononetin is an isoflavonoid. Clinical trials have shown that isoflavonoids reduce menopausal symptoms by increasing estrogen levels. It is also known that Asian women are less likely to have estrogen-dependent cancers due to high soybean-based nutrient intake with isoflavonoid content. Formononetin inhibits tumor growth and is anti-allergic (Evcimen & Aslan, 2015; Franciscis et al., 2019, Tay et al., 2019). From this research, it was aimed to inoculate *M. sativa* calli in cell suspension culture. Also, it was analyzed for aggregate assay, cell viability test, total phenolic content assay, DPPH free radical scavenging activity and formononetin assay by means of HPLC.

2. MATERIAL and METHODS

2.1. Plant Material

M. sativa was grown in the trial garden of Department of Biology of Bülent Ecevit University. Two different herb samples were collected from the trial garden in May and September. The samples with an average size of 40-50 cm were dried in a lyophilizer and stored at -80 ° C.

2.2. Aseptic Seedling Plant

The seeds were sterilized in 96% ethanol for one minute and then transferred to 10% commercial sodium hypochlorite solution for 5 minutes. Then, they were rinsed 3 times in autoclaved distilled water and germinated in hormone-free Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). Hypocotyl, cotyledon and apical meristem explants were taken from 15-day-old aseptic seedlings, and later calli were grown.

2.3. *In vitro* Culture Medium

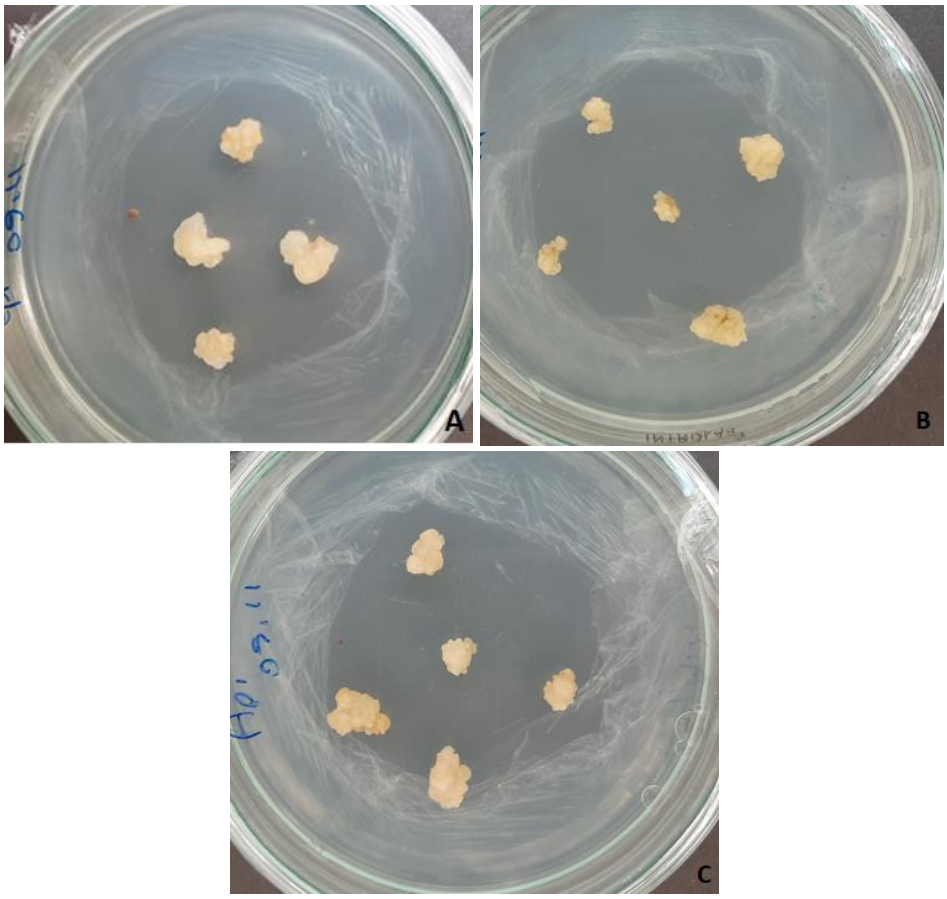
Standard MS medium was used as the callus culture medium. 1.5 mg/L Kinetin, 1.5 mg/L NAA, 0.7 mg/L 2,4-D were added to MS medium as plant growth regulators. All MS media were autoclaved for sterilization with 20 g/L sucrose and 7 g/L agar. Agar-free MS medium + 1.5 mg/L Kinetin, 1.5 mg/L NAA, 0.7 mg/L 2,4 D, 20 g/L sucrose were used for cell suspension cultures. Hypocotyl, cotyledon and apical meristem explants from the 15 days-old aseptic seedlings were germinated in MS medium as 5 explants on each petri dish (Figure 1). The calli were subcultured in every three weeks, and they were stored in the dark at 24 ± 2 ° C. Adequately matured friable calli were grown in cell suspension cultures in 4 different volumes (2.5 g/100 mL, 6.25 g/250 mL, 12.5 g/500 mL and 25 g/1000 mL). The calli that was transferred to the cell suspension cultures were shaken at 180 rpm on the shaker for 20 days. The experiment was repeated 3 times.

2.4. Cell Viability

The calli were shaken for 20 days on the shaker and then filtered with a 200 mesh Sigma-Aldrich Cell Dissociation Kit for 1 min. The filtered cells were then transferred to microcentrifuge tubes, stained with 0.5 mL of 0.2%. Trypan Blue for 20 minutes and then washed with pure distilled water. The washed cells were placed on the microscope slide and covered with cover slips. Cell count was performed by Olympus BX51 Microscope and Olympus SC100 Camera, and Digimizer Image Analysis Software was used for image

processing (Figure 2). Percent viability was calculated by this formula: % viability = (live cell count / total cell count) x 100 (Patel et al., 2009).

Figure 1. Matured calli **A)** hypocotyl, **B)** cotyledon, **C)** apical meristem.



2.5. Extraction

Lyophilized samples were pulverized for extraction. 1 g of each sample was used. They were shaken on the shaker with 100 mL of 80% MeOH at 180 rpm, then filtered. The remaining extract was shaken with 150 mL of 80% MeOH on a shaker at 180 rpm for 24 hours and filtered with filter paper. After filtration, 80% of MeOH was evaporated in a water bath at 45 ºC in the rotary evaporator. After evaporation, the remaining extract was dissolved with 10 mL of 99.9% MeOH.

2.6. Total Phenolic Content Assay

Total phenolic content was determined according to the Folin-Ciocaltaeu method (Wang & Lee, 1996; Ismail et al., 2010; Karimi et al., 2013). The samples were prepared as 1 mg/mL. Each sample (20 µL) was reacted with 100 µL of Folin-Ciocalteu reagent. Sodium carbonate (20% w/v) for 300 µL, and 1580 µL distilled water were then added. The mixture was incubated at room temperature for 30 min. Each reaction was replicated 3 times. The absorbance of each reaction was determined at 765 nm by VWR V-1200 Spectrophotometer. Gallic acid (15.62 mg/L, 31.75 mg/L, 62.5 mg/L, 125 mg/L, 250 mg/L, 500 mg/L and 1000 mg/ mL) was used as a positive control.

2.7. DPPH Free Radical Scavenging Activity Assay

DPPH free radical scavenging activity was measured using the Sanchez-Moreno method (Sanchez-Moreno et al.,1998,1999[a],1999[b]). The samples were prepared as 1 mg/ mL, 0.5

mg/mL, 0.25 mg/mL, 0.125 mg/mL. For comparative purposes, Ascorbic Acid, Butyl hydroxy toluene (BHT), Butyl hydroxy anisole (BHA) solutions were also prepared as 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL. The absorbances of the samples were read at 517 nm by V-1200 Spectrophotometer including 4 cuvettes. Percentage inhibition was calculated with this formula (1):

$$\% \text{ inhibition} = \frac{\text{Ab(control)} - \text{Ab(sample)}}{\text{Ab(control)}} \text{x}100 \quad (1)$$

2.8. HPLC-UV Analyses

Formononetin standard was prepared as 300 ppm, 150 ppm, 75 ppm, 37.5 ppm, 18.75 ppm, 9.37 ppm, and 4.68 ppm dissolved 99.9% MeOH. The formononetin of the *M. sativa* extracts was identified on a Shimadzu 1200 HPLC chromatographic system. Separation was performed using a column (C18 5 µm 250 × 4.6 mm). The samples were run in 70% MeOH with a flow rate of 0.35 mL/min and injection volume of 20 µL. The maximum absorbance of the HPLC was set at 254 nm. Formononetin was monitored in the scheduled multiple reaction monitoring mode (Rodrigues et al., 2014; Krakowska et al., 2018).

2.9. Statistical Analyses

SPSS 13 (SPSS Inc., Chicago, IL, USA) (Snedecor & Cochran, 1967) and Microsoft Office 2010 Excel Software were used in viable cell count, total phenolic content assay, DPPH free radical scavenging activity assay and HPLC quantification. $p<0.05$ was considered statistically significant.

3. RESULTS and DISCUSSION

In the present study, MS3 medium was used as the most successful of previously tested media (Çölgeçen et al., 2014). Çölgeçen et al. (2014), had used hypocotyl, cotyledon, apical meristem, epicotyl and young primary leaf explants taken from seedlings. Due to the low callus growth rate observed in epicotyl and young primary leaf explants, trials were carried out with hypocotyl, cotyledon and apical meristem explants in the study. The rate of contamination in callus and suspension culture media has decreased down to 5% due to use of UV-C lamps in the laboratory. There was no problem in callus production and suspension cultures.

3.1. Aggregate Measurement Results

Friable callus was used in cell suspension cultures. Although calli were distributed in the suspension cultures, their distribution was not homogeneous. Prior to filtration in cell suspension culture media, photographs were taken and aggregate length was measured, yet no significant differences were observed. Generally, aggregates were about 5 mm. The lowest aggregation size was 3 mm and the highest was 7.4 mm (Table 1).

As callus darkening started after the 21st day, 21-day-old yellow friable calli were taken into an agar-free MS media for large-scale cell suspensions of 100 mL, 250 mL, 500 mL, 1000 mL. Callus cells were left in the shaker in the suspension media and stained with 0.2% Trypan Blue for viability assays. In their study on *M. sativa* L. cv. Chaubet, Steward et al. (1999), used 0.375% Trypan Blue for cell viability assays. They followed the viability of cells for 11 days and found that the best result of 80% was achieved on the first day. Cell viability displayed a decreasing trend and dropped to 20% by the end of the 11th day. As the viability in the cells could not be detected at high concentrations of Trypan Blue, 0.2% of it was used in this study. Cell count was made for 20-day-old suspensions and thus the cell viability was determined. There was no significant difference in cell viability among 100 mL, 250 mL, 500 mL and 1000 mL cell suspension cultures. However, the highest cell viability of 75% was obtained in the

1000 mL culture. The lowest cell viability of 62.40% was quantified in 500 mL culture, which could be attributed to the lack of cell homogeneity.

Table 1. Aggregate measurement results (mm) ($p<0.05$).

Extract	Result (mm)
100 mL	4.67±0.1
250 mL	5.18±0.08
500 mL	4.95±0.09
1000 mL	5.81±0.06

3.2. Cell Viability Measurement Results

In cell viability analyses, the best result was obtained as 75% in 1000 mL cell suspension cultures. Percentage viability was calculated as 71.40% in the 100 mL, 67.80% in 250 mL and 62.40% in 500 mL cell suspension cultures (Table 2).

Table 2. Percentage viability (%) ($p<0.05$).

Extract	Percentage viability (%)
100 mL	71.40±4.1
250 mL	67.80±2.2
500 mL	62.40±1.8
1000 mL	75.00±0.9

3.3. Total Phenolic Compound Measurement Results

Comparing May and September herb samples, the best result for total phenolic content was obtained as 44.2 mg/g in the September one. Total phenolic content was measured as 32.2 mg/g in the May sample. In cell suspension cultures, 250 mL sample gave the best result for total phenolic content with 40.2 mg/g. The 100 mL cell suspension culture had the lowest amount of total phenolic compounds (Table 3).

Table 3. Cell suspension cultures, total phenolic contents in May and September herb samples ($p<0.05$).

Extract	Total phenolic content (mg/g)
100 mL	30.2±0.4
250 mL	40.2±0.3
500 mL	32.2±0.3
1000 mL	34.2±0.2
May herb sample	32.2±0.5
September herb sample	44.2±0.1

3.4. Total Phenolic Compound Measurement Results

DPPH free radical scavenging activity of May and September herb samples was higher than that of cell suspension cultures. Although there was no significant difference among the cell suspension cultures, the best result of 51.36% was obtained in the 1000 mL one (Table 4).

Figure 2. Stained cells **A)** 100 mL, **B)** 250 mL, **C)** 500 mL, **D)** 1000 mL cell suspension cultures.

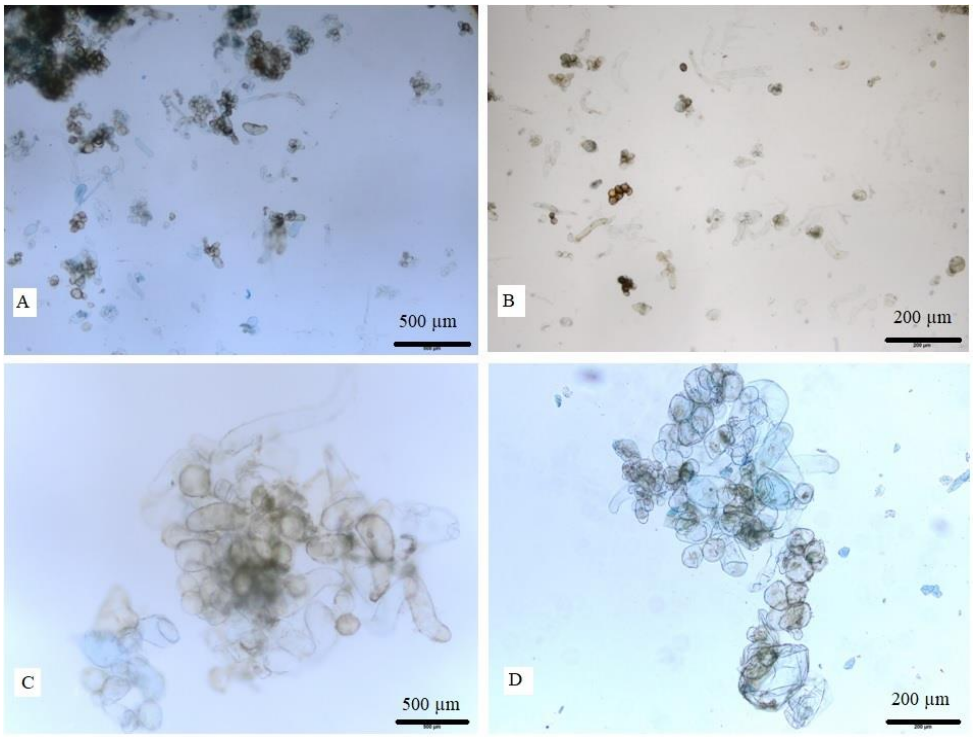


Table 4. DPPH free radical scavenging activity results (%) (p<0.05).

Extract	DPPH scavenging activity (%)
100 mL	41.63±3.1
250 mL	45.22±1.1
500 mL	47.26±1.3
1000 mL	51.36±1.2
May herb sample	80.51±2.5
September herb sample	85.69±2.1
Ascorbic acid	97.83±0.9
BHT	95.24±0.9
BHA	95.29±1.1

Antioxidants are radical scavenging compounds used in the treatment of various diseases. High antioxidant capacity in plants is an indication that the plant has medicinal importance. Bora and Sharma (2010), reported that *M. sativa* Linn. has 71.05% DPPH scavenging activity. Karimi et al. (2013), analyzed dry leaf samples of *M. sativa* (provided from a Taghavi farm in Iran) and quantified the total phenolic content as 45.2 mg/g and DPPH scavenging activity as 54%. Zinca and Vizireanu (2013), evaluated 2, 3, 4, 5, 6 and 7-day-old *M. sativa* L. seedlings (provided from a health store in Canada) for their total phenolic content and antioxidant activity. The highest total phenolic content (0.9 mg/100 g) was measured in 4-day-old seedling and the highest antioxidant activity (64%) was shown by 6-day-old seedling. Silva et al. (2013), analyzed *M. sativa* L. (provided from market) herb samples and reported 56% DPPH scavenging activity. Different from these researchers, in this study, *M. sativa* L. herb samples were collected separately in May and September. These samples were compared according to their total phenolic content and DPPH scavenging activity. The highest total phenolic content

(44.2 mg/g) and the highest DPPH scavenging activity (85.69 %) was observed in the September herb sample.

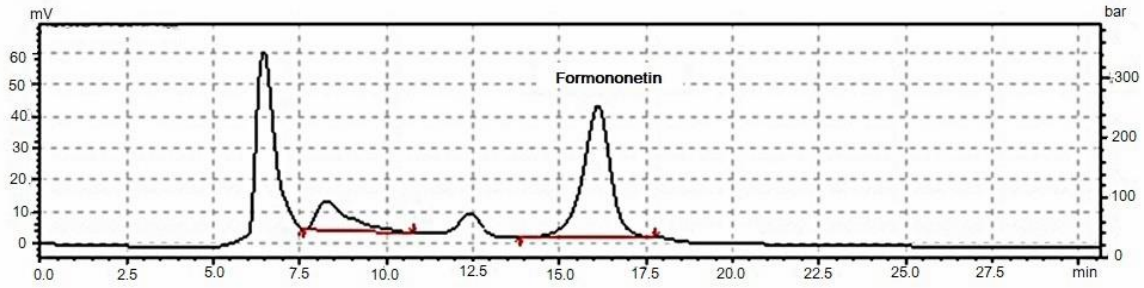
3.5. HPLC-UV Measurement Results

The calli derived from large-scale cell suspension cultures of *M. sativa* L. cv. Elçi (alfalfa Elçi) and the herb samples collected in May and September were analyzed by HPLC-UV method. The retention time for formononetin was 16 mins (Figure 3). Of the herb samples, May sample had the highest formononetin content with 2.07 mg/g, while of the cell suspension cultures, 1000 mL has shown the highest formononetin concentration of 4.99 mg/g. Additionally, formononetin content increased in cell suspension cultures compared to naturally occurring *M. sativa* L. cv. Elçi (Alfalfa Elçi) herbs (Table 5).

Table 5. HPLC-UV analysis results (Formononetin) (mg/g) (p<0.05).

Extract	Quantity (mg/g)
100 mL	3.72±0.04
250 mL	3.46±0.06
500 mL	3.36±0.08
1000 mL	4.99±0.09
May herb sample	2.07±0.05
September herb sample	1.84±0.09

Figure 3. Chromatogram of 1000 mL cell suspension culture.



M. sativa L. is a valuable medicinal plant which is rich in flavonoid compounds. The levels of these compounds increase in response to various factors. For example, elicitor trials may increase the amount of these compounds. *M. sativa* L. cv. Moapa 69 had been exposed to *Rhizobium meliloti* bacteria (Dakora et al., 1993). No formononetin was detected in the control group yet it was detected in the treatment group subjected to *Rhizobium meliloti* bacteria, but it could not be quantified. *M. sativa* cv. Nagyszenas was subjected to to different concentrations of potassium nitrate (KNO_3) and its formononetin content was determined by HPLC analysis (Coronado et al., 1995). Formononetin content was lower than 1 mg. The data suggested that low concentrations of KNO_3 increased formononetin content compared to high concentrations. Although no elicitor trials were conducted in the present study, formononetin was determined in herb sample and the quantity was 2,5 times higher.

Culture media are also important to increase flavonoid production. SH (Shenk and Hildebrandt) callus and suspension culture media was prepared for *M. sativa* cv. Apollo (He et al., 1998). The scale of cell suspension culture media was 50 mL. The researchers revealed formononetin content during HPLC analyses of calli, but the amount of formononetin was not measured. Tetrahydrofuran and distilled pure water were used as solvent for HPLC analysis. No formononetin content could be observed in *M. sativa* L. cv. Elçi (alfalfa Elçi) herb samples,

yet they revealed formononetin production in cell suspension cultures (Çölgeçen et al., 2014). Formononetin content was measured as 0.32 mg/100 mg in 100 mL suspension cultures. In this study, MS medium was used both for callus and suspension culture. Herb samples were found to contain formononetin. The highest formononetin content of 4.99 mg/g was found in 1000 mL cell suspension cultures while the lowest of 0.37 mg/g was found in 100 mL cell suspension cultures.

The use of different solvents or different chromatographic methods may result in varying flavonoid contents. A quantitative analysis had been conducted on some flavonoids in *M. sativa* cv. Lucerne (Martin et al., 2006). 260 nm wavelength was used in LC-MS analysis for this quantification of flavonoids in herb samples. Formononetin content was measured as 40 mg/kg. The herb samples of *M. sativa* cv. Azurara and 6 different *Medicago* species were evaluated for their flavonoid content (Rodrigues et al., 2014). HPLC analysis was performed with the columns C18 150 mm x 4.60 mm. *M. sativa* extracts prepared with aqueous and ethanol solutions were compared. No formononetine and daidzein could be detected in the *M. sativa* extract that was prepared with aqueous solution, while 2.40 mg/kg formononetine was quantified in the *M. sativa* extract that was prepared with ethanol extract. In this study, analyses were performed by HPLC at a 254 nm wavelength with C18 4,6 x 250 mm columns. 70% MeOH-distilled pure water was used as solvent. The highest formononetin content was measured as 4.99 mg/g. As a remarkable finding, this study indicated that herp samples of *M. sativa* L. cv. Elçi collected in May (2.07 mg/g) and those collected in September (1.84 mg/g) contain different amounts of formononetin. It was clearly revealed that seasonal changes have significant effect on formononetin content in plants.

4. CONCLUSION

Clinical trials have shown that formononetin reduces the symptoms of menopause by increasing estrogen levels. Thus, the extracts of *M. sativa* with high content of formononetin can be a starting point for the development of new pharmacotherapies. Hence, a method has been tested to increase the production of this compound with such high medicinal value. In this study, formononetin production was increased in large-scale cell suspension cultures of *M. sativa* L. cv. Elçi (Alfalfa Elçi). Future studies should focus on the development and application of modern sample preparation techniques, and better cell suspension culture methods should be developed for the production of formononetin in *M. sativa.* Meanwhile, the development of advanced methods for purifying special and biologically active compounds will enable our future understanding of their actions on organisms. Also, this study provides a basis for achieving high-efficiency production with bioreactors and can be investigated in further studies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Tayfun Aktaş: Investigation, Resources, Visualization and Writing -original draft. **Hatice Çölgeçen:** Methodology, Supervision, and Validation. **Havva Karahan:** Investigation, Resources, Visualization and Corresponding.

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