JFBE 5(1): 86-92, 2022

Journal of Food and Bioprocess Engineering

Journal homepage: https://jfabe.ut.ac.ir

Original research

Comparison of active ingredients properties in callus culture of *Zataria multiflora* with native plant

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A B S T R A C T

Recent studies show that the extraction of active ingredients from medicinal plants is subject to various parameters includes plant type, cultivation conditions and the extraction method. This research aimed to compare the callus cultivated of *Zataria multiflora* with its native plant. Thus, Murashige and Skoog (MS) medium were used to grow the plant *in vitro* and then the callus was obtained. Then a combined ultrasound-microwave method was applied to extract the chemical compounds of the native plant as well as its callus. Herein, extraction conditions were 20 min for ultrasound time, 350 watts for ultrasonic power, and 800 watts for microwave power. According to the results, the cultivated callus obtained from Zataria multiflora could not produce measurable amounts of oily compounds found in the native plant. Therefore, callus extracts were studied in terms of antioxidant and antimicrobial activity. The results of gas chromatography-mass spectrometry analysis showed that the highest compositions for essential oil of the native plant were carvacrol (86.1%) and thymol (2.01%). Whereas, the major chemical compounds for callus extract were also carvacrol (71.65%) and thymol (11.25%). In addition, the study of antioxidant activity by DPPH method revealed that the callus has a weaker antioxidant activity than the native plant. Whereas, the results were opposite in the case of antimicrobial characteristics. To put it another way, callus did better in antimicrobial properties.

Keywords: Callus culture; Medicinal plants; Antioxidant; Lamiaceae; Antimicrobial

Received 13 May 2022; Revised 13 June 2022; Accepted 30 June 2022

1. Introduction

In the past, humans used herbal products as medicinal and food sources, and now, nearly all of world's population relies on plant products (Efferth, 2019). According to the literature in the field of chemical and effective components of various plants, researchers around the world are working to replace traditional extraction methods in modern pharmaceutical enterprises for chemical compositions (Anwar et al., 2022; Picot-Allain et al., 2021; Wyk & Wink, 2017). Many chemical compounds, including phenols, flavonoids, terpenes, amines, and other secondary metabolites, can be extracted from plant parts such leaves, stems, roots, and flowers (Chiocchio et al., 2021). These compounds have been identified as the primary source of therapeutic qualities, because of their antioxidant and antibacterial capabilities. On the other hand, since the increasing number of natural habitats is rapidly being destroyed, biotechnological *in vitro* techniques may help to counteract the extinction of endangered species. Therefore, *in-vitro* culture has been introduced as a biological system for the production of these metabolites. Due to the lack of environmental constraints, this technique is expected to produce better metabolites than farm cultivation (Babich et al., 2020; Nazir et al., 2020). To acquire the active elements of medicinal plants, *in-vitro* culture has been considered since the early twentieth century. In this procedure, cells, tissues, organs, or the entire plant are cultured in the laboratory under nutritional and environmental conditions to produce specific plant clones. Controlled conditions can be nutrients, pH, temperature, and appropriate gas and liquid environment, which provides a suitable environment for growth and proliferation (Patil et al., 2021). As mentioned earlier, many secondary plant metabolites have medicinal value to humans. As a

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https://doi.org/10.22059/jfabe.2022.344430.1119

result, plant cell culture offers valuable resources for producing secondary metabolites in a simple and scalable manner (Efferth, 2019).

Zataria multiflora, a member of Lamiaceae family, is one of these plants which are used for medicinal and condimental purposes in the world. *Zataria multiflora* usually grows in the central and southern regions of Iran. The most reported compounds for this plant are flavonoids and essential oils (Nakhaee Moghadam et al., 2020). This plant has been mentioned in almost all the medical resources as one of the most important medicinal plants and has been written for many properties in the treatment of diseases, especially infectious diseases. Today, its compounds are widely used in various food and pharmaceutical industries (Makkizadeh Tafti et al., 2010). So far, the current literature available focuses on chemical properties and compounds of essential oil of *Zataria multiflora*.

According to the study on *Zataria multiflora Boiss*, chemical compounds of essential oil were identified, and the compounds identified indicated that thymol (41.81%), carvacrol (28.85%), and p-cymene (8.36%) are the main compounds (Dezaki et al., 2016). Ebrahimzadeh et al. also reported a study on essential oil extracting from *Zataria multiflora* with supercritical carbon dioxide under different conditions and also by steam distillation to compare the extraction methods. The results showed that the major components of *Z. multiflora* Boiss were thymol (44.6%), λ-terpinene (21.5%) and ρ-cymene (13.7%), based on steam distillation, while under different supercritical fluid extraction the percentages of constituents were widely different [thymol (14.2-67.6%), λ-terpinene (0.1-19.5%) and ρ-cymene (3.6-12.0%)] (Ebrahimzadeh et al., 2003). Moreover, in our previous study, sequential ultrasound-microwave associated extraction, which is a combination of ultrasound and microwave energy, was introduced as a green and safe method for extracting *Zataria multiflora* essential oil, and the results showed that the use of this method could be a more beneficial extraction method (Karimi et al., 2020).

On the other hand, due to the development and importance of in-vitro cultivation, there has been a lot of research on the study of the properties and chemicals of the callus cultivation. Shams Ardakani et al. reported the analysis of the chemical composition of essential oil from the whole plant and calli of *Foeniculum vulgare* Miller. The results showed that its callus had potential to produce volatile constituents (Shams Ardekani et al., 2005). According to Shams Ardakani and Pourshafiei research, callus production from *Nepeta Persia Boiss* and its ability to produce secondary metabolites compared to field plant were investigated. The results of this study revealed that tannins and flavonoids are formed in callus to a detectable level, but with the culture conditions of this study, callus cells are not able to produce essential oil (Shams Ardakani & Pourshafiei, 2003). Zaman et al. examined the callus of the plant *Polyantia bulata*, an endangered medicinal plant species. The results of their antioxidant research showed that the plant callus has the ability to accumulate antioxidants (Zaman et al., 2020).

According to these literatures, although it is expected that callus production could have the benefits of increasing secondary metabolites, the ability to produce essential oil has not been proven for all the produced callus, and it must be examined in various cases. To the best of our knowledge, a study has not been conducted to compare the compounds produced in *Zataria multiflora* callus cultivation with the native plant and its ability to produce essential oils. On the other hand, because of the importance of this medicinal plant in the pharmaceutical, food and cosmetics industries, it was decided to compare their antioxidant properties and antimicrobial potential.

2. Material and Methods

2.1. Chemical materials

1,1-diphenyl-2-picryl-hydrazyl (DPPH.) and 3-(2-Pyridyl)-5, butylated hydroxy toluene (BHT) were purchased from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Methanol was purchased from Merck Chemical Co. (Darmstadt, Germany).

2.2. Native plant sample

The native plant used in this study was purchased from Drug Pajouhan Spadan (Isfahan, Iran) and dried in the shadow. The dried leaves were placed in dark glass packaging until the tests were conducted and kept in a cool, dry space.

2.3. In-vitro cultivation

Callus of *Zataria multiflora* was prepared using tissue culture techniques. One half of the Murashige and Skoog (MS) medium were used for seed cultivation and *Zataria multiflora* separation. To sterilization, the seeds were placed in 30 ml of distilled water containing 2 drops of Tween (20%) and then in 70% alcohol solution (v/v) for 2 min. The seeds were then rinsed with sterile distilled water for 5 min, and after that, were immersed in 1% sodium hypochlorite solution. Finally, they were washed three times in the distilled water for 5, 10, and 15 min. The seeds were brought into the laminar flow hood and the media was autoclaved (for 15 min at 121°C). For two months, the cultures were incubated at a 16-hour photoperiod with a light intensity of 2200 lux and a temperature of 23 ± 3°C using fluorescent tube lights (Fig.1).

2.4. Preparation and extraction

In the present study, sequential ultrasound-microwave assisted extraction (SUMAE) was used to perform the process of extracting from the native plant and callus (Karimi et al., 2020; Sharifzadeh et al., 2021). Its schematic is shown in Fig. 2. The ratio of 1 to 20 was considered for the weight of the plant and the volume of solvent (distilled water). The process was also carried out in the optimal conditions obtained in our previous study (Karimi et al., 2020). For this purpose, a certain amount of plant or callus was added to the specified proportions of deionized water. The solution was kept 30 *min* at room temperature, then the resulting solution was placed in a 400-watt laboratory ultrasonic homogenizer with a frequency of 20 kHz (Topsonic, UHP 400, Iran). To prevent the rising temperature and evaporation of active materials, the beaker containing solution were placed in the cold-water bath during operation. In addition, the temperature was regularly controlled to be stable at 85 °C. Due to the optimal conditions in the previous research, the power of ultrasound was 350 *watts* for 20 *min* (Karimi et al., 2020). Then the mixture transferred to a 500 *ml* beaker and was placed into microwave oven (Samsung, ME341, 2450 MHz, South Korea) and heated by microwave irradiation at 900 *watts* (Fig .3). The final compounds obtained from the native plant and its callus was kept in separate microtubes in a refrigerator.

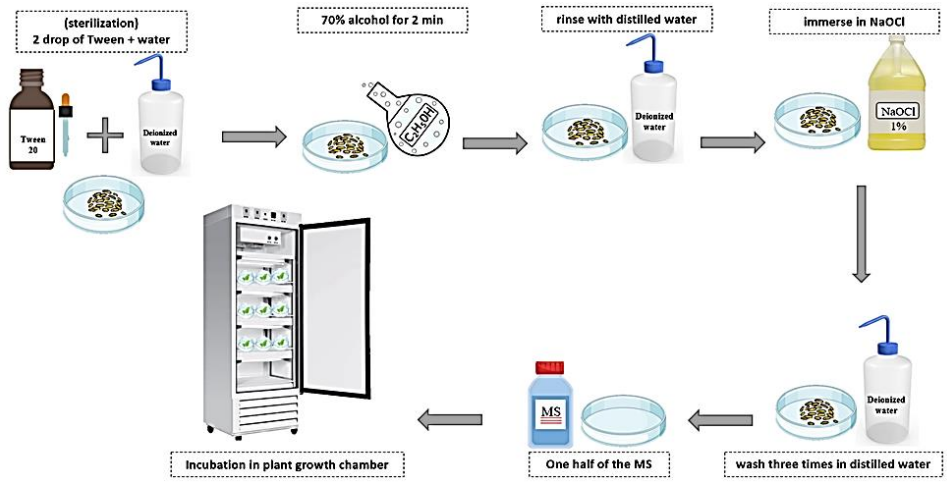

Fig. 1. Preparation of callus culture.

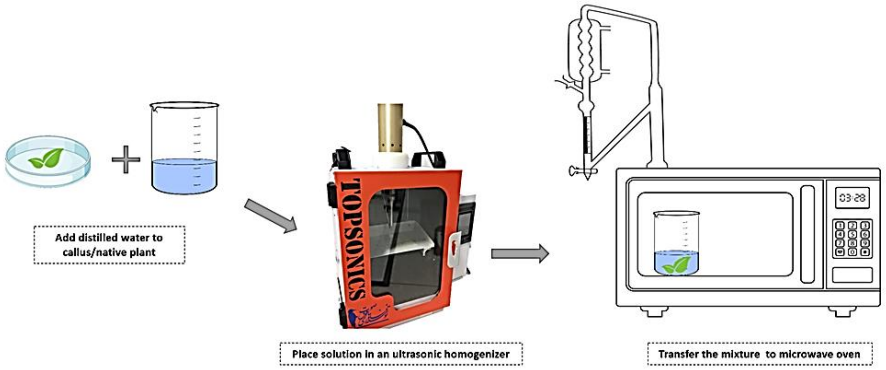

Fig. 2. Schematic diagram of extraction process.

Fig. 3. Dried callus culture obtained by in vitro method (a) and Native plant (b).

2.5. Antioxidant assay

One of the purposes of the present study is to compare the antioxidant activity of the native plant to callus extracts. Antioxidant activity was performed according to the method initially described by Espín et al. in the presence of DPPH (Espín et al., 2000). According to this method, to prepare 90 µM of DPPH stoke solution, weight 14.2 mg of DPPH and dissolve in the methanol up to 500 ml. It was then made by mixing 1 ml of DPPH Stoke solution and 4 ml of methanol as a control sample after homogeneity, and absorbed by the UV-Vis spectrophotometer (UNICO, UV/Vis 2100) at 517 nm. To measure the absorption of essential oils and extracts, 1 microliter from the DPPH Stoke solution was added to each concentration (0.15, 0.45, 0.75 and 0.99 mg/ml) and then add methanol up to 4 ml. The absorption of the solutions obtained in the same wave was read after 30 min of darkness. The percentage of DPPH inhibition is actually the concentration of essential oil or extract that inhibits 50% of the DPPH radical. The proportion of scavenging activity, I (%), was calculated using the formula below:

$$\mathrm{I}\ (\%) = \frac{(\mathrm{A_{control}} - \mathrm{A_{sample}})}{\mathrm{A_{control}}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample reaction.

2.6. Gas chromatography-Mass Spectrometry (GC-MS) analyses

To identify and determine the chemical compositions, the GC MS (AGILENT 7890 GC Series equipped with a massive detector (MSD), 5975 Series) was used. The length of column was 30 meters, the inner diameter of 0.25 mm and the thickness of the layer was 0.25 µm of HP 5MS. Helium was used as a carrier gas with a constant current of 0.8 ml/min, 1mm injectable volume and injection temperature 290°C. The column temperature program was adjusted as the temperature of the ion source 280°C, initial temperature of oven started at 50ºC and hold for 5min, with an increase of 3 °C/min, to 240°C and an increase of 15°C. In the minute, it was planned to 280°C for 10 min. The total time of the GC was 75 min. The Jeremy spectrum used was the Agilent 5975 model with 70 electron ionization voltage, EI ionization method, and ionization temperature of 220°C. Identification of essential oil compounds was based on the comparison of inhibition time and their mass spectra with published information and compliance with the Wiley 7N National Institute of Standards and Technology (NIST5.0). The mass spectra of unknown compounds were also compared with the known spectra in the library, and the names of all compounds were identified. Identification of compounds was confirmed by the analysis of the mass spectra and compared to the standard spectrum of standard compounds and the use of information available in the GC-MS library.

2.7. Antimicrobial activity in Zataria multiflora and its callus

2.7.1. Microorganisms

The antimicrobial properties of the samples were performed on two gram-positive bacteria (*Staphylococcus aureus* ATCC 1652) and the Gram-negative (*Escherichia coli* ATCC 1885). The standard strains of the tested bacteria were prepared by the Razi Karaj Serum Microbiology Group.

2.7.2. Preparation of microbial suspension

The microbial suspension was prepared in the Muller-Hinton broth culture Suspension, for each 24-hour fresh cultivation test. The incubator was at 37°C. The microbial suspension was compared and adjusted by adding a normal saline of 0.9%, it was compared and adjusted with 0.5 MacFarland solution, then the microbial suspension was used to inoculate in the Muller Hinton Agar medium.

2.7.3. Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of extract samples were evaluated using a broth microdilution technique in a 96-well microplate, as reported by Wikler et al. (Cockerill et al., 2012). Extract samples with a concentration of 20 mg/mL were dissolved in 5% dimethyl sulfoxide (DMSO) and Muller-Hinton broth culture, and then diluted to different quantities using the same combination. 75 µL of bacterial suspension (105-106 CFU/mL), and 75 µL of extract at different concentrations were placed in sterile 96-well microplates. Each well had a total capacity of 150 ml. The wells, which contained both culture and bacterial suspension, were employed as a positive growth controls. The target organisms were inoculated into the medium without extracts as a negative control. Then, the plates were sealed with sterile plastic lids and incubated for 24 h at 37°C. The MIC of an antibiotic was defined as the lowest concentration at which observable growth was suppressed (absence of turbidity). Experiments were repeated three times for each bacterium, and finally the average results were reported.

Table 1. Antioxidant activity of compounds extracted from callus, native plant and BHT.

Sample	IC_{50}*
compounds extracted native plant	107.2625
compounds extracted from callus	169.3460
BHT	88.8175

*Concentration (µg/ml) for a 50% inhibition.

3. Results

3.1. Extraction from callus and compares it with native plant

Fig. 1 shows the image of the in-vitro cultivated callus. In order to compare callus and native plant, the extraction was applied in the optimal experimental efficiency of the SUMAE method reported for the native plant (20 min of ultrasound, ultrasound 350 watts and microwave 900 watts) for both native plant and callus. Results

showed that the cultivated callus could not produce the measurable amount of essential oil. However, in similar conditions, a measurable quantity of essential oil was extracted from the native plant.

Although obtaining the essential oil from callus was not successful, the chemical compounds of the extract obtained from callus were identified as well as its antioxidant properties in order to compare the extracted compounds from the native plant and callus.

3.2. *Evaluation of antioxidant activities*

In order to investigate the antioxidant properties, IC50 values of native plant essential oil, extract of callus, and BHT (as a standard antioxidant) are shown in Table 1. Low IC50 expresses higher antioxidant activity. The results indicate the antioxidant activity of the essential oil from the native plant is higher than the callus extract.

3.3. *Antimicrobial activity*

Antimicrobial activity of essential oil of native plant and extract of callus culture are very different due to their chemical compounds. According to much research so far, some groups in these materials have more activity and some have minimal activity (Thormar & Hilmarsson, 2010).

The antimicrobial activity in this study was evaluated for the essential oil of *Zataria multiflora* and extract of callus against a gram-positive bacterium and a gram-negative by comparing their MIC. Antimicrobial activity stated in the minimum concentration of MIC bacterial growth insertion showed that the highest growth in insertion activity was observed by *Escherichia Coli* bacteria. According to Table 2, the extract from callus has a higher antimicrobial activity than the essential oil extracted from the native plant.

Table 2. MIC (µg/µl) values of *Zataria multiflora* and its callus.

	microorganism	
	S. aureus	*E. coli*
Essential oil	7.81	15.625
Extract of *Zataria* Callus	15.625	31.25

3.4. *Gas chromatography – Mass Spectrum analysis*

Table 3 shows the compounds in the essential oil from the native plant and the extract of the callus, along with the percentage of each and inhibitory coefficient (RT). The compounds identified in the extract of callus were 20 compounds, while for essential oil of native plant the 35 compounds were found. According to Table 3, carvacrol (86.1%) and thymol (2.01%) are the highest among the compounds identified from the essential oil of the native plant. These compounds also have the highest levels in the callus extract (carvacrol (71.65%) and thymol (11.25%)). In other words, carvacrol is an isomer of thymol and is commonly found in mint essential oils. Among the terpenoids, thymol and carvacrol have high anti-fungal and microbial properties. Thymol is the next compound in terms of high levels. It has the effect of hypertension in terms of medicinal properties and is used in the treatment of skin diseases with phenolic compounds. It also has antiviral properties against hepatitis A (Koufan et al., 2020; Memar et al., 2017).

Table 3. Chemical composition of callus and *Zataria multiflora* extract.

No.	Compound	RT	Area (%)	
			essential oil of native plant	extract of callus
1	ρ-Cymene	13.894	0.36	5.21
2	1,8-Cineole	14.212	0.51	-
3	γ-Terpinene	15.617	-	0.23
4	cis-sabinene hydrate	16.069	0.14	0.32
5	Pyridine	17.628	0.12	-
6	Linalool	17.761	0.22	5.54
7	Camphor	19.823	0.14	-
8	Borneol	20.939	1.17	0.46
9	Terpinen-4-ol	21.484	0.64	-
10	α-Terpinol	22.182	0.30	-
11	Glutaconic anhydride	22.469	0.20	-
12	Piperazine	22.807	0.19	-
13	Catechol	24.223	0.28	-
14	Carvacrol methyl ether	24.674	0.40	0.56
15	Thymoquinone	24.992	0.37	0.21
16	Nerol	25.320	0.54	-
17	Thymol	27.228	2.01	11.25
18	Carvacrol	27.710	86.1	71.65
19	2,5-Diethylphenol	28.140	0.31	-
20	Piperitone	29.135	0.13	-
21	Phenol, 2- methoxy-3- (2- propenyl)-	29.812	0.34	-
22	Geranyl acetate	30.951	0.11	0.21
23	β-caryophyllene	32.366	0.38	-
24	Norephedrine	34.766	0.12	0.11
25	β-Acoradiene	35.515	0.11	0.36
26	Phenylephrine	36.079	0.32	0.48
27	γ-Cadinene	36.294	0.23	-
28	δ-Cadinene	36.664	0.42	-
29	Chloracetamide	37.433	0.14	0.21
30	Phenylephrine	38.981	0.23	0.11
31	β-Cubebene	41.207	1.14	-
32	2- Bromoethanol	41.720	0.14	0.62
33	Methamphetamine acetylated	42.879	0.10	0.11
34	Adamantane	51.135	0.26	0.14
35	Allantoicacid	52.263	0.30	0.21
36	Benzenemethanol, alpha.- (1-aminoethyl)-	57.648	0.13	0.11
	Identification (%)		97.8	98.1

4. Discussion

According to recent research, the use of *in-vitro* cultivation is expanding due to its benefits to farm cultivation. In this study, callus was produced using the Tissue Culture technique in the medium of the Murashige and Skoog (MS) medium. Callus cultivated from *Zataria multiflora* does not produce essential compounds, while essential oils were obtained from the native plant. A similar result on the callus of the Bunium persicum plant was reported. The researchers only succeeded in obtaining extracts from these plants. Furthermore, in studies on calluses of plants such as Lemongrass, as well as the plant *Satureja hortensis L.* and *Origanum acutidens*, the calluses of these plants also did not have

the ability to produce essential oil (Fathi Rezaei & Rakee, 2018; Güllüce et al., 2003; Khosravinia et al., 2015; Sökmen et al., 2004).

The results of callus antioxidant properties were also compared to the native plant, and since the results showed that the callus was unable to produce essential oil, research was conducted on the extract. The antioxidant properties of the DPPH method and IC_{50} values were 107.2625 and 169.3460, respectively, for the native plant and callus. Results showed that callus had lower antioxidant properties than the native plant. In a study of callus from Argan Plant, the results showed that the antioxidant properties of the extract of the leaf of the native plant were high. Callus extract of this plant has moderate antioxidant properties compared to the native plant (Koufan et al., 2020). In another study, calluses from the lavender were investigated. The results of this study showed that in some cases, callus cultivation can produce higher or lower levels of antioxidant properties than the native plant. Differences in the results of callus's antioxidant properties depend on the culture and plant species (Mosafa, 2016).

In addition, the results of the antimicrobial properties against Gram-positive bacteria of *Staphylococcus aureus* and Gram-negative *Escherichia coli* showed that this property is better in the cultivated callus than a native plant. Also in a study on the extract of the *Pratensis* L. Callus, which is from the mint family, showed better results against gram-positive bacteria of *Staphylococcus aureus* and Gram -negative *Escherichia coli* than the native plant (Maslova et al., 2019). In another study, the antimicrobial activity of the *Bunium persicum* and the resulting callus was investigated, the results showed that callus could be a good alternative to chemicals by producing metabolites with antimicrobial properties in the pharmaceutical industry (Khosravinia et al., 2015).

In the study of the results of the analysis of chemical compounds for the full plant and the plant, 35 and 20 chemical compounds were identified for the native plant and callus culture, respectively. The two main compounds of carvacrol and thymol are the highest in the analysis of the chemical compounds of the plant and callus. The chemical compounds obtained in this study have also been identified by other researchers (Ebrahimzadeh et al., 2003; Gavahian et al., 2011; Golmakani & Rezaei, 2008; Khalili et al., 2018).

Moreover, many compounds were found in the composition extracted from the fresh plant but these compounds were not found in the callus. It indicates that the cultivation method and environmental conditions can greatly affect the composition of the plant. In some studies, the combination of linalool has been identified as the main substance. However, the amount in the present study compounds was not very significant. It should be noted that the efficiency and chemical compounds of the extracted from medicinal plants are severely influenced by seasonal changes, and this difference can also relate to the difference between geographical and climate conditions (Morales, 2002). Due to the preservation of the desired chemicals and the harmful properties, this callus can be used in the pharmaceutical industry.

5. Conclusion

The offered extraction technique (a combined ultrasound-microwave method) was applied to extract the chemical compounds of the native plant also as its callus. The results revealed that the callus of *Zataria multiflora* is not able to produce essential oil. Therefore, callus extracts were studied in terms of antioxidant and antimicrobial activity. The results of gas chromatography-mass qualitative analysis showed the same major compositions for essential oil and extract. In addition, the study of antioxidant activity by DPPH methodology unconcealed that the callus encompasses a weaker antioxidant activity than the native plant. Whereas, the results were opposite within the case of antimicrobial characteristics. This means, callus did higher in antimicrobial properties. In general, it can be argued that due to the issues with field cultivation, using the callus of this plant is a highly acceptable choice and may be preferable to a field plant in situations where the use of thyme's antibacterial characteristics is considered.

Acknowledgment

Not applicable.

Conflict of interest

The authors declare that there are no conflicts of interest.

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