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The effect of cold plasma on gene expression of major genes in the biosynthesis of phenylpropanoids and essential oil contents in *Ocimum basilicum* L

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ABSTRACT

The current study was conducted for studying the impact of cold plasma on the expression level of three genes that participate in the biosynthesis of the phenylpropanoid pathway in *O. basilicum* . These studied genes were cinnamate 4-hydroxylase (C4H), 4-coumarate coA ligase (4CL), and chavicol O-methyl transferase (CVOMT). Also, the cold plasma impact studied on the essential oil components and their relation with the gene expression level .The results appeared that cold plasma increased the germination seeds of treated groups 2 (treated for 3 minutes and 3 minutes after 7 days) ,and group 3(treated for 5 minutes and 3 minutes after 7 days) was faster than the control group. Also, The height average of the mature plants of groups 2and 3 was between (50 to73), (50 to 100) cm, respectively comparing to control group .

Moreover, the results indicated significant differences (P value ≤0.01) in the level of gene expression. Which increased for the C4H, 4CL, and CVOMT genes in group 2 about (5.63 ±0.39), (3.42 ±040) and (24.53±0.35) folds respectively compared with untreated control. Additionally, the level of gene expression increased for the C4H, 4CL, and CVOMT genes in group 3 about (42.34 ±049), (4.13 ±0.38), and (34.05 ±0.97)folds compared with untreated control.

Concerning the contents of essential oil for the control group, group 2, and group 3 were 0.434%, 0.713, and 0.792% (v/w) respectively. Moreover, the general component of the essential oil in the examined sweet basil, phenylpropanoids was the chavicol compound and its derivatives for the control group, group 2, and group 3 which were 6.77%,17.3%, and14 (v/w) respectively. We concluded the atmospheric cold plasma has shown an effect on gene expression and essential oil content of phenylpropanoid compounds in *Ocimum basilicum* L. cultivated in Iraq, as the essential oil contents have important therapeutic properties.

Keyword: Cold plasma; Cinnamate 4-hydroxylase (C4H); 4-coumarate coA ligase (4CL); Chavicol O-methyl transferase (CVOMT); *Ocimum basilicum*

Introduction

The essential oils of plants included major constituents such as general terpenoids and phenylpropanoid, which are substantial sources of aromatic and food flavoring, industrial, and the products of pharmaceutical (Charles & Simon, 1990).The phenylpropanoids are phenolic small molecules, which are the important component in many herbs such as basil, cinnamon, cloves, and tarragon (Tahsili et al.,2012) . *Ocimum basilicum* has belonged to Lamiaceae family , is the famous genera for its medical features and aromatic oils which are important

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economic (Rastogi et al.,2014), and is mostly cultivated to produce essential oils , also which has the high component of phenylpropanoid derivatives, like eugenol, methyleugenol, chavicol ,metheylchavicol, linalool, a monoterpene, and sesquiterpenes (Tahsili et al., 2012). These components are synthesized and stored in peltate glands which are found on the surface of leaves, stems, and flowers (Maurya et al., 2019) . *Ocimum* genus is known with high of phenolic compounds and also has therapeutic potentials (Ramesh & Satakopan, 2010). *O. basilicum* has analgesic, anti-inflammatory, antimicrobial, and cardiac stimulating (Bilal et al., 2012) .The essential aroma components in the basil volatile extracts displayed anti-oxidative activity, inhibit oxidative damage which is related to cancer, atherosclerosis, premature aging, and diabetes (Lee et al.,2005) . Although the importance of sweet basil essential oils and are widely using while it has known little about the biosynthesis and organizing of the compounds responsible for the flavor quality of the herb (Wang & Pichersky, 1999).The biosynthesis of phenylpropanoid produced from the pathway of shikimate and organized by many groups of the reactions enzymatic via metabolic channels (Dixon et al., 1992) . The phenylpropanoid derivatives are obtained from cinnamic acid, which is created from phenylalanine via the deamination action of phenylalanine ammonia-lyase (PAL). PAL is an enzyme important which has a role in the organizing for the production of phenylpropanoid in plants (Achnine et al.,2004).Cinnamate4-hydroxylase is a major enzyme in the pathway of phenylpropanoid (Dong & Lin, 2021). The C4H has a role in catalyzing the hydroxylation of trans-cinnamic acid to p-coumaric acid (Kumar et al., 2016) ,which considered the second enzyme in the biosynthetic pathway of phenylpropanoid (Chen et al.,2007). 4-coumarate CoA ligase (4CL) considered the third enzyme for the phenylpropanoid pathway, which has a focal role in organizing the total flow of the hydroxycinnamic acids into pathways of subsequent biosynthetic (Rastogi et al., 2013) . Concerning the final biosynthetic step include the formation of methyl chavicol catalyzed via chavicol O-methyltransferase enzyme (CVOMT)(Gang et al., 2001) . So there are three genes responsible for the organizing of gene expression of these enzymes these are C4H, 4CL and CVOMT(Tahsili et al., 2012) . The sequence availability for these genes especially assists in the recognition of the conditions under which promoted expression levels (Nasorllahi et al.,2014).The cold plasmas have developed especially and purposefully rely on its non-equilibrium properties and their ability to cause physical and chemical reactions with the gas at relatively low temperatures (Song et al., 2020; Zainal et al.,2015). Atmospheric non-thermal plasma dielectric barrier discharge (DBD) is a plasma type that has a non-uniform

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distribution of energy (Niemira, 2012). It gains reactivity from the high-energy electrons, while the ions and neutral species remain cold(Zainal et al., 2015) . Also, DBD is considered typical to generate a large volume of non-equilibrium atmospheric pressure diffuse plasma. The utilization of cold plasma technology in many various fields of work include the effect of cold plasma on seed germination (Thirumdas et al.,2017). Furthermore, the promising properties for cold atmospheric pressure plasma (CAP) appeared, because it has a dual feature that it eliminates pathogenic microorganisms from the surface of seeds and it assists the germination of seed (Brust et al., 2021). Also, the atmospheric non-thermal plasma is important for the treatment of biological systems in its application at physiological temperatures. Moreover, the occurrence and constancy of plasma constituents(reactive oxygen , UV, nitrogen species, visible light, and electric magnetic fields) are restricted in time and space and do not drop out residues of synthetic chemical on the target (Brandenburg et al., 2019) . Therefore, this study aimed to the utilization of cold plasma feature in assists the germination of *Ocimum* seeds and increasing organizing of gene expression of these enzymes these are C4H, 4CL, and CVOMT that have a role in the biosynthesis of phenylpropanoid derivatives production, which consider the major compounds in the essential oil of *Ocimum* and has therapeutic potentials.

Material and methods

Plant material and germination conditions

5 Kg of *Ocimum basilicum* seeds (sweet basil) were cultivated in Iraq and have been obtained from the reliable company and belonging to family Lamiaceae which classified by the herbarium of Department Biology/College of Science/ Baghdad University .The seeds were divided to three groups and all these groups have been planted and grown in the green house of the Botanical Garden/College of Science / University of Baghdad.

Experimental Apparatus (DBD)

The experiment was carried out in cold plasma technique laboratory, Department of Physics, College of Science for women, University of Baghdad. An atmospheric dielectric barrier discharge (DBD) system was used to generate non-thermal plasma or cold plasma, the device was composed of two parallel electrodes made of copper material with a diameter of (90mm), surrounded by an insulating material of teflon with thickness of (10mm), the high power, as well as to be exposed to the plasma are placed on it, while the upper electrode is connected to a vertically moving base in order to change the distance between the two

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electrodes and this electrode is connected to the end of the high voltage outlet of the high power supply and the two electrodes are separated from each other with an insulating material of coloration or glass. It is placed above the lower pole of the system, and the dielectric system is fed with a variable high voltage by connecting it to a transformer whose output voltage is (12) kv, type (Ac) with specifications (220 volt input, frequency 50 Hz, and 15 Kvolt output voltage).

Seeds treatment via atmospheric pressure plasma system

The plant seeds were divided into three groups as following:

Group 1. Control group (1 Kg), seeds without Non thermal Plasma Dielectric Barrier Discharge (DBD) treatment.

Group 2. Seeds group (1 Kg), treated with Non thermal plasma Dielectric Barrier Discharge (DBD) for 3 minutes and repeat the treatment for 3 minutes after 7days.

Group 3. Seeds group (1 Kg), treated with plasma for 5 minutes and repeat the treatment for 3 minutes after 7 days.

Sequentially all seeds groups were subjected to direct treatment which are directly placed between the electrodes or placed under the plasma system and the treatment worked by placed inside glass of Petri dish. The seeds are uniformly distributed at the bottom dielectric plate were treated for regular time intervals in a DBD system operated at atmospheric pressure in atmosphere dielectric barrier discharge system (Baczek et al.,2019; Liu et al., 2019).

Conditions of growth and harvesting of *Ocimum basilicum*L. plant

The groups of seeds from sweet basil (*Ocimum basilicum*), have been planted and grown between August and October in 2020. The plants were watered every day and maintained at day/night temperature of 37-35^{o} C and 30-25 ºC, respectively .The first harvesting phase of control group and treatment group 2 and treatment group 3 sequentially, took place in the seedling stage, Once the seeds sprouted and there was visible evidence of seedlings, in the stage of two pairs of leaves, (two weeks), and all these samples were immediately frozen and preserved at -20 °C for genetic analysis. The other of the seedlings from all groups was left to grow and all plant height were monitored up to the time of second harvesting. Once a critical mass was reached, plants were harvested for the next step (Aldarkazali et al.,2019; Bączek et al., 2019).

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RNA extraction and cDNA synthesis

Total RNA extraction from the plant was done according to the protocol of (Heidery and Pahlevan 2014) by using TRIzol reagent (Thermo Scientific, USA). Quantus Fluorometer (Promega, USA) was used to detect the concentration of extracted RNA or cDNA to evaluate the goodness of samples by using Quantifluor RNA System .199µl of diluted QuantiFluor Dye was mixed with 1 µl of cDNA or RNA, after 5 minutes of incubation at room temperature, RNA concentration was measured.

PCR primer and the analysis of Real- Time PCR

Syber Green quantitative PCR was done according the manufacturer instruction of Go Taq one-Step RT- qPCR (Promega ,USA).The used primers (Macrogen, Korea) for quantitative real time polymerase chain reaction are chosen according to(Kim et al., 2014) for(C4H) forward 5`GGATCATTCTT GCCTTGCCTATACT3`and reverse 5`ATA ACAATGGTGGAGTGCTTCAAAA-3`. While according to (Tahsili et al., 2012) the primer of 4CL was chosen the forward 5`-TCGCAAAA CAGCCACTA CCGAC-3`and the reverse 5`-AGGTG CAGCAAGTTT GGC TCTC-3`. Whereas according to(Zarei et al., 2015) the forward of (CVOMT)was 5`-GATCCCACTT TCACAAA TC C-3`and the reverse5-GAGTACATGTGCCACAACCC-3`. While tubulin chosen according to (Zarei et al., 2015) the forward 5`-CTCCTTGAGCTA GTCGTCGC-3`and the reverse 5-AACAAGG CAAAA ACATTCCG 3`.

Ocimum basilicum C4H , 4CL ,and CVOMT genes was amplified from the synthesized cDNA with primers. Moreover, tubulin (Tub) was established as a house –keeping gene to normalize the dissimilar RNA concentrations during RNA extraction. The reverse transcriptase enzyme activation was performed in duplicate at 37 ˚C for 15 min in 1 cycle, to convert RNA to cDNA, and initial denaturation began at 95˚C for 5 min, followed by 40 cycles for the last three-step: denaturation at 95˚C for 30 second,annealing at 60˚C for 30 sec and extension at 72˚C for 30 second.

Plant Extraction by Supercritical Fluid Extraction Technique (SFE)

After the second harvested of the plants from each group (control group, group 2 and group 3), the collected plant material (aerial parts of plant) were dried and stored at room temperature .The samples of each group was ground .An essential oil (phenylpropanoids) compounds were extracted from aerial parts of the plants, the experimental runs were conducted in the SFT unit, containing extraction cell of approximately length of 30 cm and inside diameter of 5 cm) and 200 bar pressure, the fixed bed was formed inside a nylon basket

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. Commercial carbon dioxide was used for Super critical fluid SFT (99.99) purity. A total of 500 g of *Ocimum basilicum* L. of each groups was added to the extraction basket; the temperature was 50^{o}C . After reaching thermal equilibrium, the system was slowly pressurized by opening the valve at the extractor's inlet and allowed CO_2 to flow through the extraction basket at 200 bar pressure, when the system reached the operating pressure and stabilized, the valve from the extractor's outlet were opened and the extraction process began.

Qualitative and quantitative identification of active compounds (Phenylpropanoids)in *Ocimum basilicum* extracts groups by using GC- MASS technique.

A combination of plasma-treated groups (2, 3) and non-treated group oil extracts that was extracted from *Ocimum basilicum* L. (Sweet basil) groups grown in greenhouse that was super critical fluid extraction from the aerial parts of the plant were analyzed performed using GC-MS (model Shimadzu Qp 2010, Germany) equipped with a VF-5 fused silica capillary column (30 m ^{x} 0.25 i. d. mm film thickness 0.25µm, Germany). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml. Injection temperature was 280°C. Injector and mass transfer line temperature set at 250 $^{\circ}$C to 280° C. The oven temperature was 80 $^{\circ}$C . Diluted samples (which prepared in methanol) of 0.3 µl were manually injected in the split less mode .Identification of Compounds of the samples was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with standard .Start time was 2.98 min and end time was 33.00 min.

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study(SAS, 2012)**.**

Results

Effect of treated plasma on seed germination and growth

The groups of *Ocimum basilicum* L. plant seeds showed morphological changes in the external surface when exposed to non-thermal plasma by atmospheric dielectric barrier discharge DBD technique. The seed surface of groups 2 and 3 became post-treated more hydrophilic than the control group. Also, the germination of treated groups 2 and 3 was faster than the control group where the germination process of 2 and 3 treated groups sprouted after

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5 days after planted.The height average of the mature plants of groups 2and 3 was between (50 to73),(50 to 100) cm, respectively, whereas the height average of the control group was (40 to 70cm).

The effect of atmospheric non thermal plasma Dielectric barrier discharge (DBD) on gene expression

The plasma treatment for 3 minutes and 3 minutes after 7days by dielectric barrier discharge up-regulated the transcription of the C4H, 4CL, and CVOMT genes in group 2 about (5.63 ±0.39), (3.42 ±040) and (24.53±0.35) folds respectively compared with untreated control .Moreover, The plasma treatment for 3 minutes and 5 minutes after 7days up-regulated the transcription of the C4H, 4CL, and CVOMT genes in group 3 by about (42.34 ±049) ,(4.13 ±0.38), and(34.05 ±0.97) folds compared with untreated control.

So the results indicated significant differences with a p-value ≤ 0.01 as shown in the table (1).Strikingly that plasma treatment by dielectric barrier discharge demonstrated to alter the expression for genes at the transcriptional level. Additionally, the exposure times caused the raising of the induced expression of C4H, 4CL, and CVOMT genes .Moreover, the effects post-treatment appeared in seedlings of group 3 which had a maximum stimulatory effect than in seedlings of group 2 as shown in the figures (1,2, and 3).

Table (1) show the comparison between difference groups in fold change of C4H, 4CL and CVOMT genes in *Ocimum basilicum* L.

Groups	**Groups**			**LSD**
	Mean±SE			**(P-value)**
	Group1: Control	**Group2 :3+3**	**Group3 : 3+5**	
C4H	1.00 ±0.00 **c**	5.63 ±0.39 b	42.34 ±049 a	1.26** (0.0001)
4CL	1.00 ±0.00 **b**	3.42 ±040 a	4.13 ±0.38 a	1.112** (0.0012)
CVOMT	1.00 ±0.00 **c**	24.53 ±0.35 b	34.05 ±0.97 a	2.06** (0.0001)
Means having with the different letters in same row differed significantly. ** (P≤0.01).				

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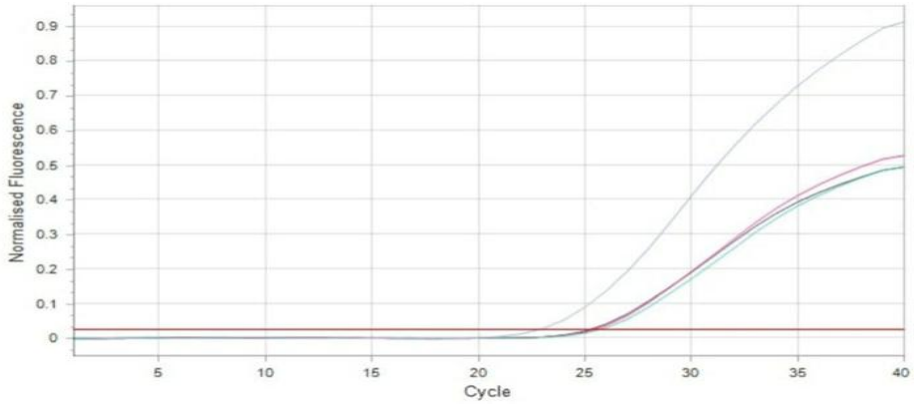
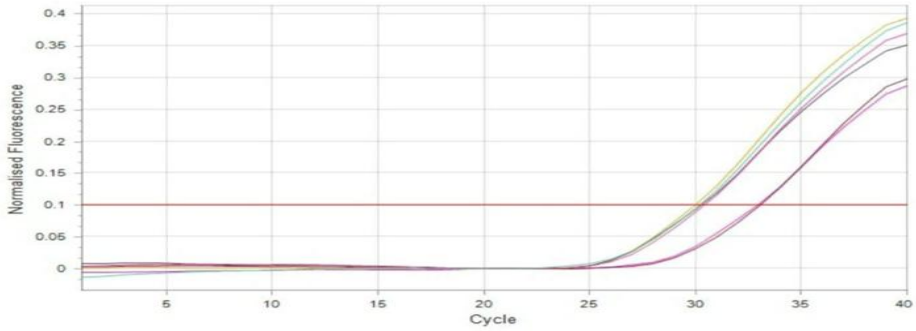


Figure (1) Quantitative real time PCR curve of C4H gene in *Ocimum basilicum* L. for three groups



Figure(2) Quantitative real time PCR curve of 4CL gene in *Ocimum basilicum* L. for three groups

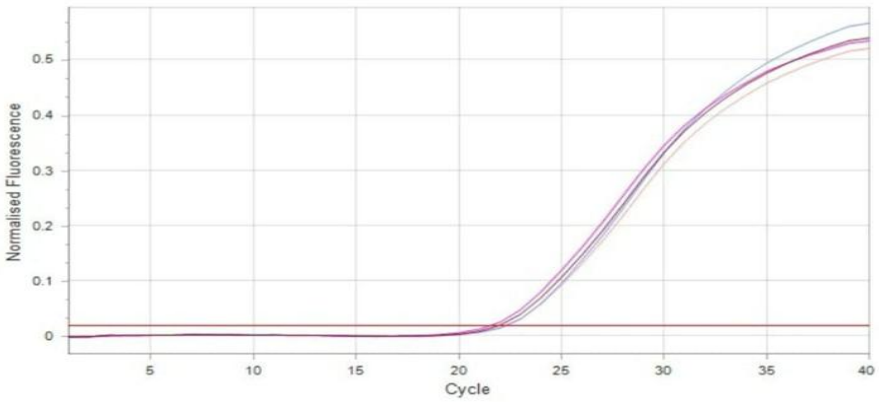


Figure (3) Quantitative real time PCR curve of CVOMT gene in *Ocimum basilicum* L. for three groups

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Qualitative and Quantitative Characterization of chavicol and its derivatives in *Ocimum basilicum* L. Plant Extracts using GC-MASS

The contents of essential oil ,which extracted using supercritical fluid extraction technique (SFT) for the control group, group 2, and group 3 were 0.434%, 0.713, and0.792 % (v/w) respectively. The samples were characterized as qualitative and quantitative by chromatographic method GC-MS . Moreover, the results were revealed that the general component of the essential oil in the examined sweet basil, phenylpropanoids was the chavicol compound and its derivatives for the control group, group 2, and group 3 which were 6.77%,17.3%, and 14(v/w) respectively.

Discussion

Through the results for our study observed agreement with another study that used an atmospheric pressure discharge and show that NTP can increase gene expression which is responsible for growth and development. Additionally, other researchers who applied cold plasma technology have confirmed which it is superior in the treatment of particulates to other technologies (Lee et al.,2021). Besides, the exposure time of cold plasma affects the germination of *Moringa oleifera* seeds by using the different exposure times (1, 5, 10, and 15 minutes) compared to untreated seeds (Dawood, 2020).The processing of cold plasma is a novel technique that increases the yield of plants that are known to be key producers of essential oils specially phenylpropanoids compounds (Buonopance et al.,2017; Song et al., 2020).

In other studies were observed an increase in the expression of genes in roots and shoots of NTP-treated wheat seedlings post-treatment by non-thermal plasma also the induction of expression being more faster in aerial parts and roots (Holubová et al., 2020; Iranbakhsh, Ardebili, Ardebili, Shafaati, & Ghoranneviss, 2018).Also, one of the studies proved that treatment by cold plasma improves the rice seed germination which was affected via heat stress through affecting the epigenetic regulation (Suriyasak et al., 2021) .

Strikingly, there is a relationship between atmospheric non thermal plasma dielectric barrier discharges (DBD), gene expression, enzyme activity, secondary products, and essential oil yield. As explained in the results, the extracts obtained using the supercritical fluid extraction technique had major compounds identified and detected in essential oils (phenylpropanoids) in the aerial parts of *Ocimum basilicum* L. plant at 50 ˚C and 200 bar of pressure. Supercritical fluid extraction (SFE) is interesting for processing natural products

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because it produces extracts without organic residues, also the temperature of the process can be reduced, therefore it can preserving thermo sensitive compounds (Capuzzo et al.,2013). Total essential oil contents in this study according to GC-MASS results indicated that the effect time exposure of atmospheric non-thermal dielectric barrier discharge (DBD) in group 3 influenced strongly in the expression of essential oil phenylpropanoids compounds in group 3 and more effective than control and group 2.

According to the results of this study, the maximum quantity of essential oils was chavicol and its derivatives for the aerial parts of the plant during the vegetative growth it was observed the maximum value in group 2.

Although the gene expression of the CVOMT gene in group 3 was higher than in group 2, which may be due to alter the proportion of methyl chavicol and its derivatives by drying(Lachowicz et al., 1996) . In addition, the changes in the environmental factors affect essential oils composition (Dzida, 2010) .

It has been concluded that the C4H, 4CL, CVOMT represent regulatory enzymes in the phenylpropanoid pathway play a key role in the regulation of chavicol and it is derivatives level in the *Ocimum basilicum* L. plant where the level of gene transcription and activity for C4H, 4CL, and CVOMT increased during the vegetative growth. The results in this study were in accordance with other researcher's observations, the gene expression of several genes for seeds of diverse plant species was affected by cold plasma (Moghanloo et al., 2019) . Also, atmospheric non-thermal plasma dielectric barrier discharge (DBD) influenced the priming of sunflower it was related to transcriptional responses and variation in phytohormones (Mildažienė et al., 2019).Taking together, the results along with these reports underline this hypothesis that atmospheric non-thermal plasma dielectric barrier discharge (DBD) can modify transcription of genes, that way improving plant growth and metabolism. Consequently, more genetic studies are required to elucidate the potentially involved mechanisms in plant responses by using atmospheric non thermal plasma Dielectric barrier discharge (DBD).

Conclusion

Through this study conclude the critical importance of the atmospheric non-thermal plasma dielectric barrier discharge (DBD) effect on gene expression and essential oil content of phenylpropanoid compounds in *Ocimum basilicum* L. cultivated in Iraq, and this study is considered the first of its type as it has evaluated the effect of atmospheric non-thermal

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plasma dielectric barrier discharge (DBD) on gene expression and the essential oil content which has therapeutic characteristics.

Conflicts of interest

The authors announce, there is no discrepancy of interest related to this article.

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