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Increase Production Secondary Substances in *Hedera helix* L. Callus by Abiotic Elicitor

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Abstract

Ivy (*Hedera helix* L.) leaves contain variety of secondary substances that have therapeutic effects on many diseases. Naturally these substances are low concentrations and high cost, researchers have turned to a new method aimed to increase production in callus culture from leaf as explant by using a safe elicitor polyethylene glycol (PEG) at concentrations (0, 20, 30, 40, and 50)%. This experiment was carried out in tissue culture laboratory at College of Science, University of Mustansiriyah in a completely randomized design (CRD) with three replicates to each concentration. The results showed that there was a significant effect of PEG on the fresh weight and highest value was achieved at 20%. Based on the high-performance liquid chromatography (HPLC) analysis of the alcoholic callus extract shows that significant increase in flavonoids (Nicotiflorin, Quercetin, and Kaempferol) and terpeniods (Hederacoside C, α-Hederin, Hederacoside B, and Hederacoside D). The highest values of flavonoids and terpeniods were achieved the highest average at 50% of PEG. In general, the conclusion of the study indicates that the PEG elicited the callus to medicinal compounds production.

Keywords: *Hedera helix* L., Polyethylene glycol, Callus .

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1. Introduction

Plant tissue culture (PTC) refers to a group of laboratory methods used to cultivate explants in a sterile environment using various types of medium. Also, PTC is technique a proven to be a useful and effective method for producing many secondary substances (Mohammed *et al*., 2025 p.107-117). Secondary substances produced by plants have active properties, defensive role against microorganisms, environmental stresses tolerances and active importance to pathogens (Kumar & Parasurama, 2024p.1034-1044). Ivy (*Hedera helix* L.) is known as "Habl Almasakeen" in Arabic, which belongs to Araliaceae family and characterize by evergreen perennial plant, stem creeping, leaves are alternate and have palmate lobed, flowers are tiny and yellowish-green color and fruit, a drupe contains 2-6 seeds (El-Sohafy *et al*., 2020 p.68-77). *H. helix* at long time ago used as a medicinal herb because it contains more active substances so it is anti-parasitic, anti-microbial, anti-tumor, anti-spasmodic, and anthelmintic (Ragab, Otay & Diab, 2023 p.99-111). The active substances in *H. helix* leaves: tannins, flavonoids, terpeniods, glycosides, saponins, alkaloids, coumarins, phenolic acids and volatile oils (Sen *et al*., 2023 p.1394). Flavonoids compounds are found in high concentrations in ivy leaves and play importance role as antimicrobial and antioxidant activities (Fadaei *et al*., 2025 p.1-15). Moreover, flavonoids have many roles in plants such as: allelopathy, UV protection, signaling molecules, antioxidants, detoxification and tolerance to various stresses (Shomali *et al*., 2022 p. 3158). Terpeniods are one of secondary substances and that perform a wide range of physiological functions including: stress protection, improving pollination, herbicidal activity and membrane permeability (Al-Haidari & Abdulhalem, 2025 p.121-127). α-Hederin is one of terpeniods isolated from *H. helix* leaves to treatment symptoms of bronchitis and COVID-19 because has bronchospasmolytic properties (Osama *et al*., 2023 p. 203-245). Elicitation is one of biotechnology methods in PTC to stimulated plant to increase medicinal substances production by factors that called elicitors and that can be divided into two groups according to origin: endogenous and exogenous

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or nature: biotic or abiotic (Jasim & Habeeb, 2024 p. 2829-2837). Biotic elicitor is extracts of microbes such as: bacteria, fungi, and yeast, while abiotic elicitor is physical factors (ultrasound, ultraviolet irradiation UV, temperature, and light) or chemical compounds (phytohormone, heavy metal, and nanoparticles) (Longkumer *et al*., 2025 p. 919-945). Polyethylene glycol (PEG) is abiotic elicitor, hydrophilic polymer, high solubility, low toxicity, low polydispersity and least cost; moreover have multiple applications in agriculture, food industry, pharmaceutical compounds and cosmetics (Gaballa *et al*., 2024 p. 721-730). In PTC PEG improved rooting of *Spathoglottis plicata* (Manokari *et al*., 2022 p. 435-450), induced somatic embryogenesis of *Vigna radiata* (Gnanaraja *et al*., 2022 p.721-730) and use as elicitor to increase medicinal compounds production in various plants such as: *Nigella sativa* (Neamah, 2018 p. 435-450), *Mentha pulegium* (El-Naggar & Osma, 2024 p. 1-16), *Cathranthus roseus* (Abdul Wahid, *et al*., 2024 p.119-129) and *Stevia rebaudiana* (Ghazal *et al*., 2024 p.14714).This study aimed to establish a callus from *H. helix* and enhances secondary substances production using PEG, offering a novel approach.

2. Materials and Methods

2.1. Site experiment and explant source

This experiment was carried out in tissue culture laboratory, biology department, College of Science, University of Mustansiriyah. *Hedera helix* L. leaves were collected as explants from mother plant at gardens in Baghdad, Iraq on 14/11/2024.

2.2. Preparation of nutrient media

Murashige and Skoog (MS) medium was used in this experiment and supplement with chemical compounds. The pH of medium was adjusted to 5.8 using sodium hydroxide or hydrochloric acid at 1N concentration and sterilized using an autoclave at 121C° for 20 minutes (Murashige & Skoog, 1962 p. 473-497).

2.3. Sterilization and callus induction

The explants were cut into segments and washed with tap water for 30 minutes. After which they were moved to laminar air flow cabinet and

immersed in 99% ethyl alcohol (CH_3CH_2OH) for one minute then washed with sterile distilled water (SDW) for three times followed by immersed in 1% of NaOCl (sterilizer) for five minutes and washed for three times to sterilizer removing (Alaakel, Al-Ammouri, & Al-Ouda 2024 p. 61-69). Finally, culturing on universal vials contains MS medium for ten replicates and incubated in incubator under a temperature 25±2C° at 8/16 (dark/light) for 21 days to callus induction.

2.4. Elicitation

After callus induction take a certain weight of callus and put in vials contain MS, in addition PEG at different concentrations (0,20,30,40, and 50%) by three replicates for each concentration. The vials incubated in incubator under same conditions mentioned above to for 30 days. The fresh weight (F.W.) (mg) and dry weight (D.W.) (mg) (dried under 65 C°) of callus was calculated using a digital balance.

2.5. Callus extraction site

The extraction was carried out in the laboratory back to scientific research commission, Baghdad, Iraq.

2.6. Extraction of flavonoids compounds

One gram of elicited callus was dissolved in 20ml hexan to remove fat layer, then the organic layer was dissolved in 100ml (90:10 methanol : water). The extract was subjected to ultra-sonication at 60% duty cycles for 25min at 25C° followed by centrifugation at 7.500rpm for 15min. the extract was evaporated under stream of liquid nitrogen to reach approximately 1ml and then complete the volume to 1ml using HPLC grade methanol by overtaxing, the mixture was passed through 2.5µm disposable filter, and stored at 4C° for further analysis, then 20µl of the sample was injected in HPLC system according the optimum condition (Suárez *et al*., 2025 p.105-110).

2.7. Extraction of terpeniods compounds

One gram of elicited callus was maceration in 100 ml hot water for 70C° for 60 min. The extract was subjected to ultra-sonication at 60% duty cycles for 25 min. at 25C° followed by centrifugation at 7.500 rpm for 15min. The clear supernatant of each sample was subjected to charcoal

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treatment to remove pigments prior to evaporation under in vacuum. Dried samples were re-suspended in 1ml HPLC grade methanol by vortexing, the mixture were passed through 2.5µm disposable filters and stored at 4C° for further analysis, then 20µl of the sample was injected in HPLC system according the optimum condition (Havlíková *et al*., 2015 p. 1529-1531).

2.8. Separation conditions of HPLC

The separation was occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu, the eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer. The separation conditions of HPLC explained to flavonoids in Table 1 and terpeniods in Table 2.

Table 1- Separation conditions chromatographic of flavonoids.

Column length	**50×2.0 mm I.D**
Mobile phase	Acetonitrile : 0.1% phosphoric acid
Flow rate	1.0 ml.min^{-1}
Detection	UV 280 nm
Temperature	30 C°
Sample volume	20 µl

Table 2- Separation conditions chromatographic of terpeniods.

Column length	**10.0×4.5 mm I.D**
Mobile phase	Acetonitrile : ammonium acetate
Flow rate	1.2 ml.min^{-1}
Detection	UV 220 nm
Temperature	30 C°
Sample volume	20 µl

The concentration of each constituent was calculated by comparison retention time and the peak area of authentic standard with that of the sample under the peak area of authentic standard (Tables 3 and 4) (Figures 1 and 2) with that of the sample under the same optimum separation conditions.

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Table 3- Retention times and areas of standard flavonoids.

Seq.	Flavonoids	Retention time (minute)	Area (µ volt)	Conc. ($mg.ml^{-1}$)
1	Rutin	2.070	314522	2
2	Nicotiflorin	3.625	311299	
3	Quercetin	4.693	337989	
4	Kaempferol	5.788	305984	

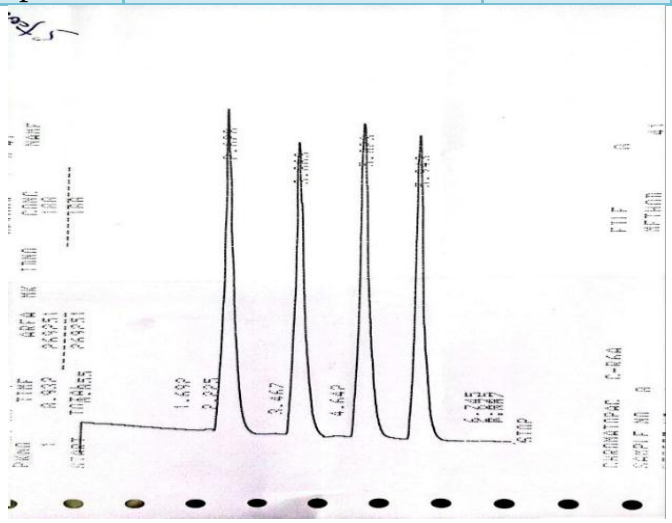

Figure -1 HPLC of standard flavonoids.

Table 4- Retention time and area of standard terpeniods.

Seq.	Terpeniods	Retention time (minute)	Area (µ volt)	Conc. ($mg.ml^{-1}$)
1	Hederacoside C	2.628	410600	2
2	α-Hederin	3.863	344115	
3	Hederacoside B	5.023	340372	
4	Hederacoside D	5.943	313750	

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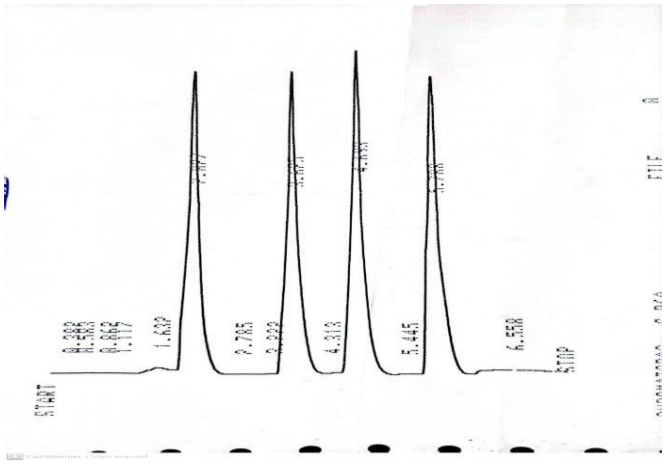

Figure -2 HPLC of standard terpeniods.

Calculation:

The concentrations of the secondary substances were calculated according to the following formula (Al-Hadithi, Al-Saady & Faleh, 2023 p. 1-8).

$$\text{Conc. of compound } (mg.ml^{-1}) = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{conc. of standard} \times \text{dilution factor}$$

2.9. Statistical analysis

The results were presented as means. The Completely Randomized Design (CRD) was using. Following one-way analysis of variance (ANOVA), the data was subjected to multiple comparisons using Fisher's LSD test at a probability level of 0.05 (SAS, 2018).

3. Results and Discussion

Results, shown in figures (3 and 4) illustrate the significant decrease in fresh weight of ivy (*H. helix*) callus affected with high concentrations of PEG in culture media. The highest fresh weight 629.70 mg of callus was observed in 20% of PEG, while the least 326.70 mg in 40%. Moreover, there was no significant difference in the dry weight in all concentrations of PEG (Figure 5). Our results were in agreement with Khashan & Athary (2016 p.75-90). who found that the fresh weight of *C. roseus* callus significantly decrease as a result of increasing PEG concentrations (5,10 and 15%) in MS medium.

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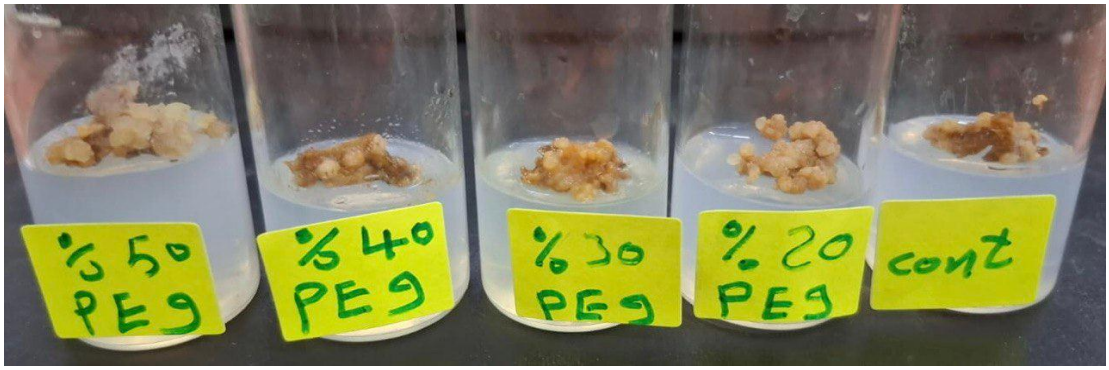

Figure -3 Callus of ivy under various concentrations of PEG (%).

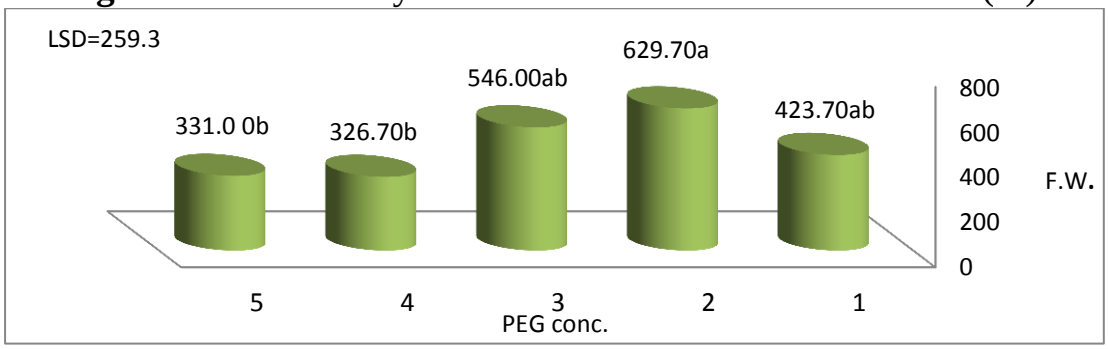

Figure- 4 Fresh weight (mg) of ivy callus under various concentrations of PEG (%).

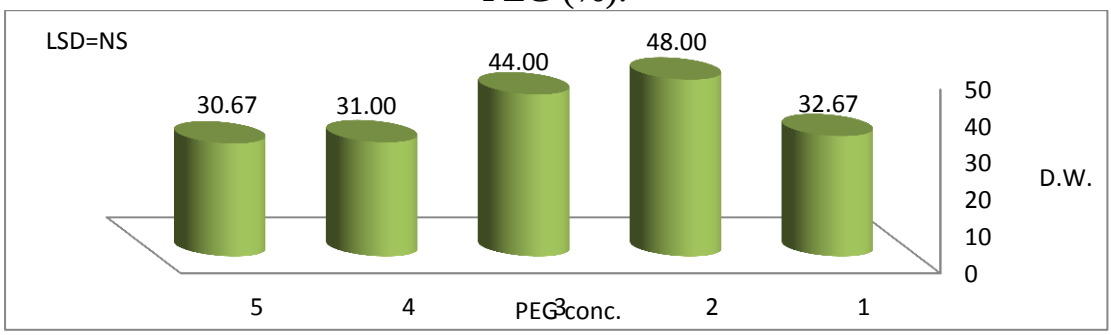

Figure -5 Dry weight (mg) of ivy callus under various concentrations of PEG (%).

PEG is no regulate in cells because it a macromolecule and cannot pass through plasma membrane but prevent water from entering the cell therefore decrease content of water (Almukhtar, 2018 p. 282-287). According to Hellal *et al*. (2017 p. 203-221) when the PEG concentration is increased in culture media a caused decrease in the ability of cells to

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absorb water, rises the osmotic pressure and reduce division and enlargement of cells. Similarly, study Ghassemi-Golezani *et al*. (2018 p. 237-248) who reported that PEG increase in MS media caused reactive oxygen species (ROS) accumulation in cells and distribute the metabolism (photosynthesis and respiration) and plasma membrane breakdown and then negative effect on biomass of callus. Under water deficit result from PEG the cytokinin decrease and abscisic acid increase which leads to increase the activity of Chlorophylase to break down the photosynthetic pigments and adversely affecting fresh weight of callus (Abdelgawad *et al*., 2024 p.1979-1998).

In general, PEG positively affects in secondary substances (flavonoids and terpeniods) concentrations of *H. helix* callus culture by increasing values. However according to Table (5), results showed the concentration of 50% PEG gave height value of Nicotiflorin 6.16 $mg.l^{-1}$ and significant different with other concentrations. Also, height value of Quercetin in concentration 50% of PEG that gave 5.05$mg.l^{-1}$ and significant different with other concentrations expect concentration 40% of PEG that gave 4.79$mg.l^{-1}$. The height value of Kaempferol occur in concentration 40% of PEG that gave 5.18$mg.l^{-1}$ and significant different with other concentrations expect concentration 50% of PEG that gave 5.15$mg.l^{-1}$. Also, results in table (5) indicate no significant effects on Rutin values in *H. helix* callus between PEG concentrations. The findings align with Abdul Wahid *et al*. (2024 p. 119-129) and Abdelgawad *et al*. (2024 p.1979-1998) who reported that PEG could enhance to alkaloids and flavonoids production in callus of *Cathranthus roseus* and *Maesa indica* respectively.

Table 5- Effect of PEG concentrations (%) in flavonoids of ivy callus.

Flavonoids ($mg.l^{-1}$)	**Concentrations of PEG (%)**					**LSD 0.05**
	Control	20	30	40	50	
Rutin	4.59 a	5.29 a	5.43 a	5.98 a	4.14 a	N.S
Nicotiflorin	3.59 d	4.14 c	4.62 c	5.18 b	6.16 a	0.54
Quercetin	2.84 c	2.74 c	3.27 b	4.79 a	5.05 a	0.41
Kaempferol	3.96 b	3.48 bc	3.68 c	5.18 a	5.15 a	1.03

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The current experiment showed significant differences among PEG concentrations in terpeniods concentration Table (6), the concentration 50% PEG caused a high value in Hederacoside C production in ivy callus that gave 8.59mg.l^{-1} and significant different with other concentrations expect control that gave 8.00mg.l^{-1}. The height value of α-Hederin occur in concentration 50% of PEG that gave 14.91mg.l^{-1} and significant different with other concentrations. Furthermore, the highest value of Hederacoside B occur in concentration 40% of PEG that gave 5.86mg.l^{-1} and no significant different with concentrations 30 and 50% of PEG that gave 4.84 and 5.73mg.l^{-1} respectively. While, the highest value of Hederacoside D occur in concentration 50% of PEG that gave 4.81mg.l^{-1} and no significantly different with 0, 30% and 40 % concentrations of PEG that gave (4.06, 3.73 and 4.07) mg.l^{-1} respectively. Finally, the table 7 showed that the callus treatment with 20% of PEG gave least terpeniods studied production. Similar to current study, El-Naggar & Osma (2024 p.1-16) showed that the application of PEG results in a significant increase secondary compounds of *Mentha pulegium* callus.

Table 6- Effect of PEG concentrations (%) in terpeniods of ivy callus.

Terpeniods (mg.l^{-1})	Concentrations of PEG (%)					LSD 0.05
	Control	20	30	40	50	
Hederacoside C	8.00 a	6.40 c	7.17 b	7.42 b	8.59a	0.56
α-Hederin	11.46 b	9.55 c	11.19b	12.42 b	14.91a	1.25
Hederacoside B	4.27 b	3.10 c	4.84 ab	5.86 a	5.73a	1.05
Hederacoside D	4.06 ab	3.42 b	3.73 ab	4.07 ab	4.81a	1.27

The accumulation of secondary compounds in plant tissues, it is one of the strategies to confront environmental stresses, enhance plant immunity and ROS scavenging (Mehmandar *et al*., 2023 p.870-882). Increase PEG concentration in media significantly enhanced flavonoids and terpeniods accumulation in *H. helix* callus. PEG is good elicitor to induced drought

stress and increases the negative osmotic pressure of the cells, therefore levels of abscisic acid (ABA) hormone rises, which help to production of several secondary metabolites (Zhou *et al*., 2015 p.7035-7045). Also, added PEG in MS medium of *Stevia* simulated water stress and increased the production of several active compounds as mechanism to defend to against water stress (Ahmad *et al*., 2020 p. 1552).

Numerous studies have demonstrated that PEG can reduce CO_2 fixation indirectly and increase ROS in cell followed by induce cells to induce some genes to synthesis of secondary metabolites (Iskandar & Iriawati, 2016 p.7-16; Nestorovi´c Živkovi´c *et al*. 2023 p.1277). Results were in accordance with that presented by Neamah (2018 p. 435-450) and Ghazal *et al*. (2024 p.14714) who reported that add PEG in MS medium caused increasing of terpeniods in *Nigella* and flavonoids in *Stevia* respectively.

4. Conclusion

Through this work, it was concluded that PEG concentrations induced dehydration in *H. helix* callus and decreased water content and biomass, while increased the flavonoids and terpeniods accumulation especially in 50% concentration.

5. Recommended

It is recommended to use the concentration 50% of PEG to produce secondary substances from the callus of other plants.

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زيادة انتاج المواد الثانوية في كالس نبات الهايدرا بواسطة محفز لاحيوي

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مستخلص البحث:
تحتوي اوراق نبات الهايدرا (*Hedera helix* L.) على مجموعة متنوعة من المواد الثانوية ولها تأثيرات علاجية للعديد من الامراض، ولقلة كمية هذه المواد طبيعيا وارتفاع كلفة، لجأ الباحثون في طريقة جديدة تهدف الى زيادة الانتاج في مزارع الكالس المنتج من الورقة كجزء نباتي باستخدام محفز آمن وهو بولي اثيلين كلايكول بتراكيز(0و20و30و40و50)%. نفذت التجربة في مختبر زراعة الانسجة في كلية العلوم/الجامعة المستنصرية وبثلاث مكررات وضمن التصميم العشوائي الكامل. كشفت نتائج التجربة ان بولي اثيلين كلايكول له تأثير معنوي في الوزن الطري واعلى قيمة تحققت عند التركيز 20%، وبالاعتماد على تحليل الكروماتو غرافي السائل ذو الأداء العالي للمستخلص الكحولي للكالس بين هناك زيادة معنوية في تركيز الفلافونويدات (Nicotiflorin و Quercetin و Kaempferol) والتربينات (Hederacoside C و α-Hederin و Hederacoside B و Hederacoside D)، حيث سجلت اعلى القيم في تركيز 50%، وخلاصة الدراسة تشير الى ان بولي اثيلين كلايكول حث الكالس على انتاج المواد الثانوية.

الكلمات المفتاحية: هايدرا، بولي اثيلين كلايكول، الكالس
ملاحظة : هل البحث مستل من رسالة ماجستير او اطروحة دكتوراه ؟ نعم : كلا :√