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Effect of biotic and abiotic elicitors on *Salvadora persica* callus *in vitro*

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Abstract

The research was conducted to study the effect of different concentrations of nanoparticles (chitosan and titanium dioxide) of *Fusarium oxysporum* as elicitors to increase the production of active compounds from *Salvadora persica* callus. Determine the total flavonoids and alkaloids rustle that chitosan in 5 mg/L, titanium dioxide in 1 mg/L, and *Fusarium oxysporum* in 5 mg/L give 93.10 mg100mg, 128.7 mg/100mg, and 107.61 mg/100mg respectively whereas alkaloids give 2.39%, 3.91%, and 2.20 % respectively. The results showed superiority in the presence of flavonoids in the samples compared with alkaloids. HPLC (High-performance liquid chromatography) analysis shows significant differences in increasing flavonoid production (Rutin, Kaempferol, Quercetin, Catechin, Luteolin, and Apigenin) the addition of chitosan at 5 mg/L led to an increase in the production of Rutin, at 39.89mg/L. As for the induced callus treated with titanium dioxide at 1 mg/L, it increased Rutin to 35.89mg/L. While Rutin increased its production to 30.12 mg/L when treated with *Fusarium oxysporum* at 5 mg/l.

Keywords: Chitosan, Flavonoids, HPLC, *Salvadora persica*, Titanium dioxide.

Introduction

Depending on their metabolic routes and functions, plants can produce chemical molecules as primary or secondary metabolites. The primary metabolites guarantee the plant's essential functionality. However, the production of secondary metabolites does not directly contribute to the growth and development of plants. Nevertheless, they play a significant part in interactions with the environment as a form of defense and adaptation to environmental stresses[1]. Although secondary metabolites have a variety of biological characteristics[2]. Numerous secondary metabolites, including terpenes, phenolic acids, alkaloids, and flavonoids, have been discovered through phytochemical research[3]. Plant tissue culture techniques have been employed as a potent method for producing secondary metabolites due to their many benefits[4]. For millennia, scents, dyes, food additives, conventional medical ingredients, health advantages, pesticides, and industrial raw materials have all been extensively[5]. By inducing the stress response with the help of elicitors, precursors, and biotransformation, as well as by varying the environmental conditions and changing the composition of the medium, tissue culture techniques are used to increase the content of secondary metabolites[6]. Elicitation is one of the key methods used in biotechnology to increase the production of secondary compounds by introducing specific chemicals known as elicitors[7]. Elicitors are of two types: Biotic elicitors can be either unprocessed extracts or products that have undergone some level of purification. They can come from either pathogen (fungi, bacteria, or yeast) or the plant itself. They either have a specific composition, such as polysaccharides, glycoproteins, inactivated enzymes, pure chitosan, pectin, chitin, alginate,

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curdlan, xanthan, elicitin, etc., or a complicated composition, such as yeast extract and fungal homogenate[8]. while Abiotic elicitors include various chemical and physical factors including light, UV radiation, heavy metal salts ($AgNO_3$ $CuCl_2$ $CuSO_4$ $NiSO_4$) temperature change, and osmotic stress, as well as intracellular plant growth hormones like jasmonic acid (JA), methyl jasmonate (MJ), salicylic acid (SA), etc[9]. Chitin and its deacetylated counterpart (chitosan) are a family of linear polysaccharides made up of various proportions of N-acetyl-2 amino-2-deoxy-D-glucose (glucosamine, GlcN) and 2-amino-2-deoxy-D-glucose (N-acetylglucosamine, GlcNAc) residues[10]. Fungal elicitors (both free-living and entophytic) are the most significant and frequently used as biotic elicitors for the synthesis of chemicals[11] and secondary metabolites[12]. Different types of (NPs), have been utilized in several ways as elicitors in plant tissue culture mediums for increased secondary metabolite production[13]. Additionally, NPs are atomic or molecule assemblies with sizes ranging from 1 to 100 nm that exhibit a variety of physicochemical characteristics according to the elements that make up their composition[14] as organic materials include lipids and polymers of natural or synthetic origin and Inorganic materials include silica and metals such as gold, silver and iron oxide[15] the production of and use of nanoparticles (NPs), such as silver, silica, aluminum, zinc oxide, copper, carbon nanotubes, or titanium dioxide, is growing. NPs are manufactured on a massive scale from a wide range of bulk materials and have been used in a variety of fields, including agriculture and medicine[16,17]. For the long-term preservation and usage of major secondary metabolites in rare and endangered medicinal plants, particularly those with difficulties in conventional propagation, such as *Salvadora persica* L., the tissue culture technique is commonly used[18]. *Salvadora persica*, often known as Miswak (toothbrush), is a member of the Salvadoraceae family.

This plant has a long history of usage in traditional medicine for the treatment of scurvy, cough, asthma, piles, rheumatism, and ulcers. The *Salvadora persica* plant has a wide range of secondary substances, including volatile oils, flavonoids, alkaloids, terpenoids, and saponins in various parts of the plant. Numerous pharmacological actions, antimicrobial antioxidant, and anticancer properties are included.

Materials and Methods

Callus production Calls were obtained from *Salvadora persica* nodes sterilizer with 0.1% mercury chloride ($HgCl_2$), then culturing on MS media that contain 1 mg/L (KIN) and 2 mg/L (NAA) incubated under a temperature 25± 2 °C at (8:16) (dark: light) for 30 days[19]

Preparation of elicitors

Chitosan and titanium dioxide nanoelisitores were obtained as a ready solution from Phi center where they dissolved the nanopowder in distilled water and then sonicated for 10 seconds five times and examined with SEM Fig. 1, 2. As a biotic elicitor, an extract of the fungus *Fusarium oxysporum* was utilized, which was taken from the microbiology lab of the biology department at the College of Science for Women. The fungus was grown on potato dextrose agar (PDA) and produced by [20] with a few changes. The cultures were cultured for 5-7 days at 25°C, and then the mixture of cultures was filtered using milepore filter paper with a 0.22 m pore size. The solution was then kept at 4 °C to be used again.

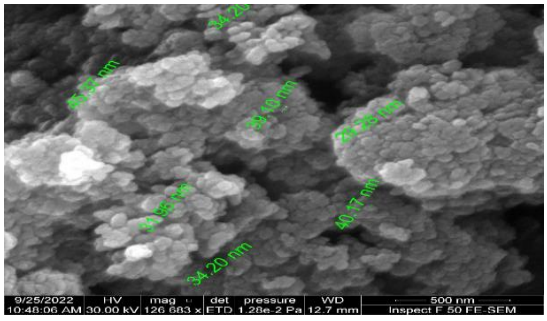

Figure 1. SEM of Chitosan.

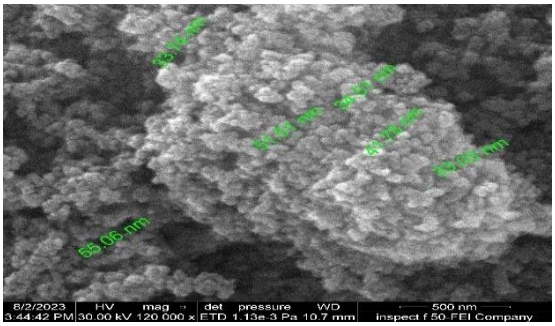

Figure 2. SEM of TiO_2.

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Elicitation

To enhance the biosynthesis pathway, subculture callus on MS media containing elicitors, Chitosan (CH), and *Fusarium oxysporum* (F) as biotic elicitors, titanium dioxide (TiO_2) as abiotic elicitors at (1,3,5) mg/L concentrations for each one of them in addition to 1 mg/L (KIN) and 2 mg/L (NAA), after that incubated at temperature (25±2°C) for 16/8 (light/ dark) for 21 days by ten replicates for each concentration[21].

Plant extraction

1 g of callus that resulted from the elicitation experiment was extracted by hot alcoholic (75%) in Soxhlet apparatus with a ratio (1:10 callus: Alcohol) for 6-8 h at 60 °C, then the extract was filtered and dried[22].

Qualitative estimation (Test for alkaloids)

Dragendroff's method was used to confirm the existence of alkaloids. A couple of drops of Dragon drops and an amount of the extract were dissolved in adjusted HCL. Alkaloids can be detected by a crystalline precipitate. The sample that contained alkaloids positively was subsequently submitted for further quantitative analysis [23].

Alkaloid separation:

In 2N HCl, a portion of the extract residue has been dissolved and then faltered.1 ml of the resulting solution was added to a separatory funnel and rinsed with 10 ml of chloroform. This solution's pH was neutral by adding 0.1 N NaOH. This solution was then mixed with 5 ml of Bromocresol Green (BCG) solution and 5 ml of phosphate buffer, and the result was measured at 470 nm[24].

Total flavonoid content (TFC)

The aluminum chloride colorimetric technique was used to estimate (TFC) of crude extract. In simple terms, 50 µL of crude extract (1 mg/mL ethanol) was brought up to 1 mL together with methanol, incorporated into 4 mL of distilled water, and added to 0.3 mL of 5% NaNO2 mixture before being incubated for 5 minutes. After that, the combination was allowed to stand for 6 minutes. The total volume of the combination was then raised to 10 mL using double-distilled water after 2 mL of a 1 mol/L NaOH solution had been added. After 15 minutes of standing time, the mixture was tested for absorbance at 510nm.A calibration curve was used to determine (TFC), which was then reported as mg of rutin equivalent per g of dry weight[25].

HPLC Conditions:

A SYKAMN HPLC system (Germany) with a C18-ODS column (250 4.6 mm, 5 m) was used to perform the high-performance liquid chromatography study.100 µL of samples was put into the system. At a flow rate of 1 mL/min, the mobile phase included 95% acetonitrile and 0.01% trifluoroacetic acid (solvent A) and 5% acetonitrile and 0.01% trifluoroacetic acid (solvent B). This was the gradient portion of the program: 10% A for the first 0–5 minutes; 25% A for the next 5-7 minutes; 40% A for the next 7–13 minutes; and then we return to the beginning situations. A phenolic UV-visible detector operating at 278 nm was used[26]. The concentrations of the active substances were determined quantitatively using the comparison between the standard and model under psychological conditions using the following equation Model the concentrations of active compounds: Concentration of compound= {area of sample / (area of standard) × (concentration of standard) × (dilution factor)}[27]

Experiment design statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of different factors on study parameters. The least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study[28]

Results and Discussion

Enhancing secondary metabolite production in plant tissue culture and cell culture through the application of biotic or abiotic elicitors has proven to be a successful method.

Total alkaloids and flavonoids

According to Table 1, 1 mg/L Titanium dioxide (TiO_2), 5 mg/L chitosan (CH), and 5 mg/L *Fusarium oxysporum* (F) had the highest concentration of flavonoids (TFC) with 93.10 (mg / 100gm),128.7 (mg / 100gm) and 107.61 (mg / 100gm) respectively,

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while the lowest content of flavonoids was for non-treated samples including leaf (L) and control (C) with 15.76 (mg / 100gm) and 23.61(mg / 100gm) respectively. The highest Total alkaloid content was T1, CH5, and F5 with 2.39%, 3.91%, and 2.20% respectively, whereas leaf and control had the lowest Total alkaloid content with 0.47% and 0.50% respectively.

Table 1. Total Flavonoids and alkaloid content

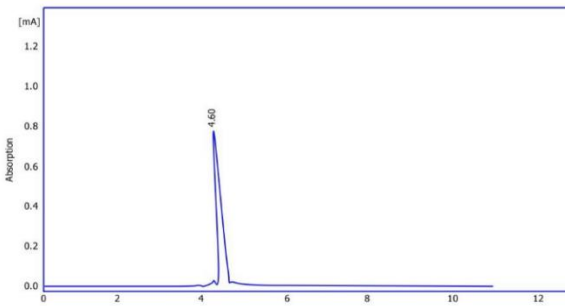
Treatment mg/L	TFC (mg / 100gm)	Total alkaloid content %
TiO_2 1	93.10	2.39
TiO_2 3	56.29	1.36
TiO_2 5	41.06	1.01
CH 1	33.06	2.00
CH 3	69.4	2.06
CH 5	128.7	3.91
F 1	61.0	1.20
F 3	85.10	1.86
F 5	107.61	2.20
L	15.76	0.47
C	23.61	0.50
LSD value	12.475 *	0.816 *

* (P≤0.05).

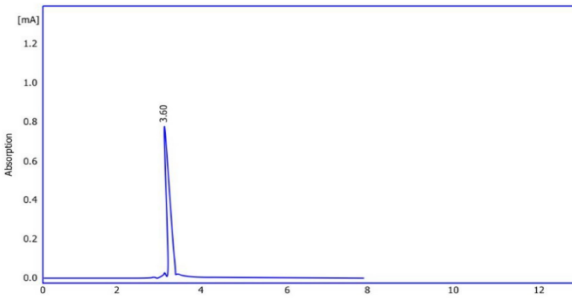
Qualitative and quantitative analysis of flavonoids

Chitosan in Table 2 shows the highest significant increase of flavonoids recorded for Rutin, Kaempferol, and Quercetin with 39.38 ppm, 28.69, and 26.44 ppm respectively Fig. 4 (A) Compared with leaf and control the results elucidate the increase of secondary compounds at a low level, for leaf Rutin 17.65 ppm, Kaempferol 17.08 ppm and Quercetin is 15.22 ppm, for control Rutin 21.00 ppm, Kaempferol 18.98 ppm and Quercetin 16.25 ppm Fig. 4 (D, E).

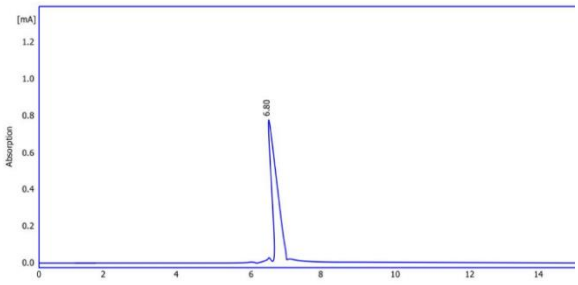
The best performance of titanium dioxide among increasing secondary compounds in Table 2 clarifies that Rutin, Kaempferol, and Quercetin with 35.89 ppm, 24.58 ppm, and 22.65 ppm respectively Fig. 4 (B). while leaf and control did not lead to the required level in increasing the production of the active compound for leaf Rutin 17.65 ppm, Kaempferol 17.08 ppm and Quercetin is 15.22 ppm and for control Rutin 21.00 ppm, Kaempferol 18.98 ppm and Quercetin 16.25 ppm Fig. 4 (D, E).
Fusarium oxysporum data in Table 2 illustrate there is a significant increase of Rutin, Kaempferol, and Quercetin when treated with F. at 30.12 ppm, 22.58 ppm, and 18.98 ppm respectively Fig. 4 (C). Leaf and control show the lowest increase of flavonoids compared with the other treatments for leaf Rutin 17.65 ppm, Kaempferol 17.08 ppm and Quercetin 15.22 ppm and for control Rutin 21.00 ppm, Kaempferol 18.98 ppm, and Quercetin 16.25 ppm Fig. 4 (D, E). Standard curves have been clarified in Fig. 3 (A, B, C, D, E, F).



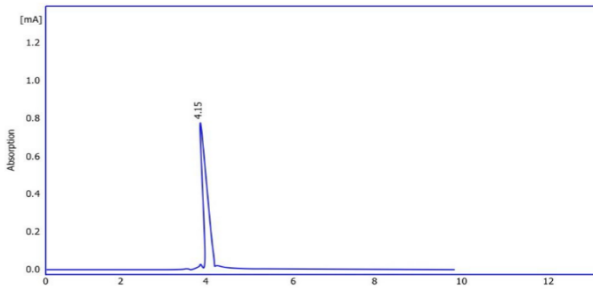
A



B



C



D

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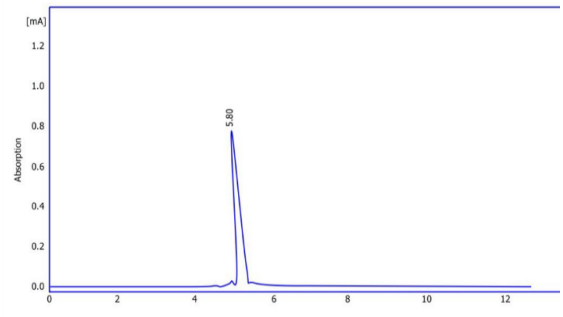
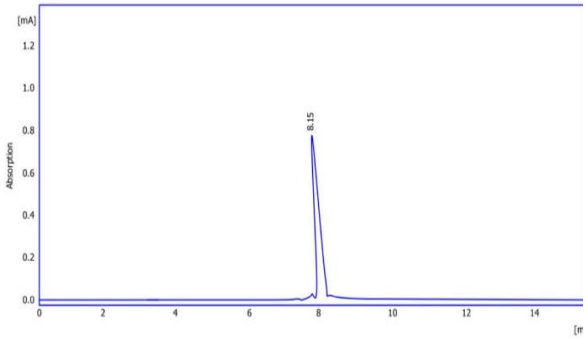


E



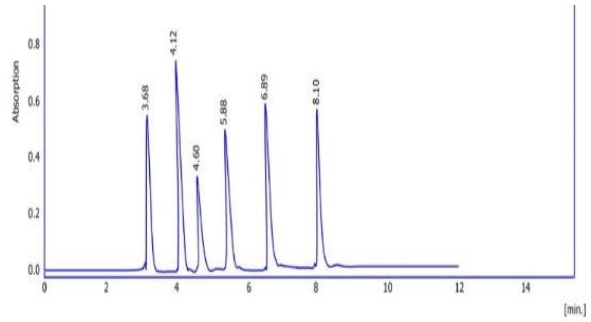
F

Figure 3. Standard curves of standers A) Rutin B) Kaempferol C) Qurcetine D) Catechin E) Luteoli F) Apigenin.

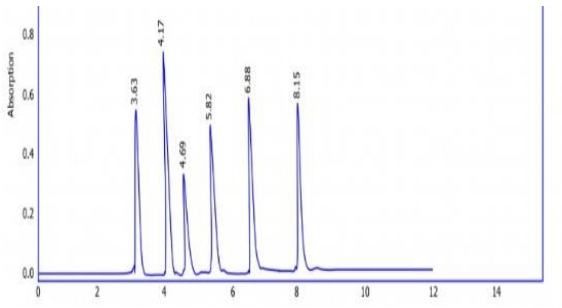
Table 2: HPLC analysis of flavonoids from *Salvadora persica* callus

Flavonoids (ppm)	CH 5mg/L	TiO_2 1mg/L	F 5mg/L	L	C	LSD value
Rutin	39.87	35.89	30.12	17.65	21.00	5.369 *
Kaempferol	28.69	24.58	22.58	17.08	18.98	4.911 *
Qurcetine	26.44	22.65	18.98	15.22	16.25	5.026 *
Catechin	23.96	20.11	17.98	9.32	11.65	4.702 *
Luteolin	18.98	15.98	13.69	8.56	10.05	4.188 *
Apigenin	14.55	11.47	8.98	5.33	6.89	3.027 *

* ($P \leq 0.05$).



A



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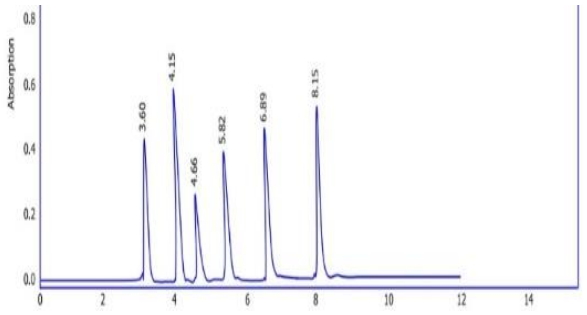

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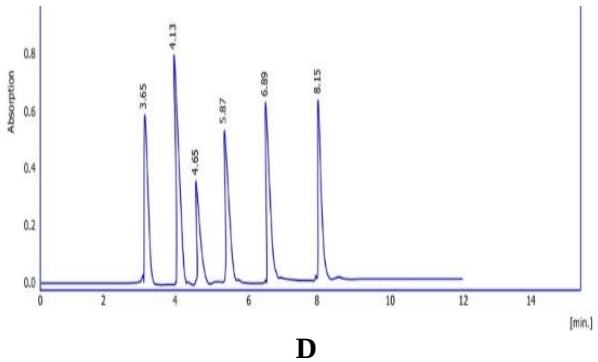

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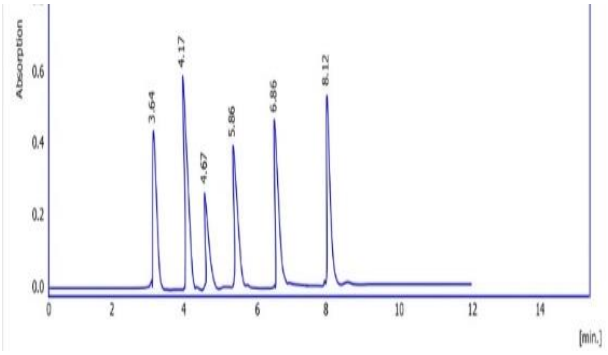

E

Figure 4. HPLC chromatogram of *Salvadora persica* extract of identified flavonoid compounds when treated with different elicitors. A) Chitosan, B) Tio2, C) *Fusarium*, D) Leaf, and E) Control

When chitosan nanoparticles (CNPs) are small (smaller than 100nm), have a high aspect ratio, and have a large surface area, they are more efficient[29]. They increase plant metabolism and facilitate the more effective translocation of chemically active substances across cell membranes[30]. Increased total phenolic content[31], and stimulation of defense enzyme activity[32].Chitosan is known to stimulate enzymes crucial to the formation of phenolic chemicals and phenylpropanoids. Numerous genes associated with flavonoid metabolism were dramatically upregulated by chitosan[33]. Also, CSNPs help in increasing alkaloid content according to[28] which confirms that CSNP's effect on alkaloid accumulation. The rustles show that Low concentrations of titanium led to an increase in the production of the active compound, which means an increase in cells[34]. While the higher concentration of titanium, led to lower production of the active compound, whether it is flavonoids or alkaloids, so there is an inverse relationship between the concentration of the inorganic nanomaterials (TiO_2) with the amount of production, due to the higher concentration there was an increase in the oxidative stress of the cell membrane, the rupture of the membrane and thus the death of the cell[35] as the lack of production of effective compounds that are considered defensive compounds for the plant led to a decrease in the defensive activity of the plant[36,37].

Elicitation of plants with a fungal elicitor molecule activates several defense mechanisms, including the deposition of lignin to strengthen the cell wall, the stimulation of a variety of defense enzyme activities, and the formation of phenolic compounds[31, 38]

The results in Table 2, indicate that the biotic and abiotic factors contribute significantly to increasing the production of the active compounds, in the untreated samples there is some increase in flavonoids but did not show a sufficient amount of flavonoids, when adding factors led to an increase in production, chitosan and *Fusarium oxysporum* as abiotic elicitors and titanium dioxide as abiotic elicitors have a significant effect in increasing the secondary metabolite flavonoids and alkaloids, and this is consistent with[39]. also, conclude that is one of the abiotic factors that have an effective role in increasing the active compound compared to control. Also, the control without elicitors (CH, TiO_2, and *Fusarium*) addition causes the lowest increased production of flavonoids This is what[40] confirmed callus without the addition of cobalt (abiotic factor) produced the lowest production of flavonoids. It is very important to highlight that growth regulators affect the increase of secondary metabolite production. Also, Plants respond differently to various types and concentrations of plant growth regulators in vitro, especially when auxins and cytokines are combined[41].

Conclusion

The work confirms the importance of biotic and abiotic factors in the production of flavonoids and alkaloids and how the amount of production varies according to the composition and type of the material stimulating the production, as the biotic factors (chitosan and mushroom extract) were superior to the abiotic factors (titanium dioxide) in terms of productivity.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

The study was done by H. Y. J. who did most of the work and wrote this research with this design, modified it, and corrected it. H. M. H. the creator of the study project

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تأثير المحفزات الحيوية والغير حيوية على كالس نبات المسواك خارج الجسم الحي

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الخلاصة

أجري البحث لغرض دراسة تأثير تراكيز مختلفة من الجسيمات النانوية (الكيتوزان وثاني أكسيد التيتانيوم) و *Fusarium oxysporum* كمحفزات لزيادة إنتاج المركبات الفعالة من كالس نبات المسواك .اظهرت نتائج المحتوى الكلي مركبات الفلافونويد والقلويدات التي ازاد انتاجها عند اضافة الكيتوسان بتركيز 5 ملغم / لتر، وثاني أكسيد التيتانيوم بتركيز 1 ملغم / لتر، و*Fusarium oxysporum* بتركيز 5 ملغم / لتر حيث اعطت 93.10 ملغم / 100 ملغم، 128.7 ملغم / 100 ملغم، و 107.61 ملغم / 100 ملغم على التوالي للفلافينودات بينما ساعدت المحفزات على انتاج القلويدات بمعدل 2.39 %، 3.91%، و2.20% على التوالي .وأوضحت النتائج التفوق في وجود مركبات الفلافونويد في العينات مقارنة مع القلويدات. بين تحليلHPLC (تحليل كروماتوغرافي سائل عالي الأداء) اختلافات كبيرة في زيادة إنتاج الفلافونويد (Rutin, Kaempferol, Quercetin, Catechin, Luteolin, and Apigenin) أدت إضافة الكيتوسان عند التركيز 5 ملغم / لتر إلى زيادة إنتاج Rutin ، بمقدار 39.89ملغم/لتر .أما الكالس المستحث المعالج بثاني أكسيد التيتانيوم بتركيز 1 ملغم/لتر فقد أدى إلى زيادة Rutin بمعدل 35.89 ملغم/لتر .بينما ارتفع إنتاج Rutin إلى 30.12 ملغم/لتر عند معاملته بـ *Fusarium oxysporum* بتركيز 5 ملغم/لتر.

الكلمات المفتاحية: الكيتوسان,الفلافينودات, HPLC ,المسواك ,تيتانيوم ديوكساید.