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Detection of Bioactive Compounds Produced by *in Vitro* Culture of Jojoba Plants (*Simmondsia chinesi* (Link) Schn.) Using GCMS and FTIR

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Abstract: The jojoba tree is economically important plant due to its high contains of oil and its various industrial, commercial and medical applications. A practical experiment was carried out to determine the optimal combination of plant growth regulators affecting the response of transplanted buds to unfolding, growth and callus induction on the formed explants. The study was conducted in the Plant Tissue Culture laboratory at the College of Agriculture-University of Basrah. Apical shoots were used to produce the initial callus when cultured on MS nutrient medium with combinations of plant growth regulators (0.5, 1 and 2 mg L^{-1} NAA and 1, 2.5 and 5 mg L^{-1} TDZ). The quality of the formed callus and the most important secondary compounds were analyzed using Gas chromatography–mass spectrometry (GC–MS)and Fourier-transform infrared spectroscopy (FTIR).The MS media(murashige and skoog) supplemented with 1 mg L^{-1} NAA + 2.5 mg L^{-1} TDZ and 2 mg L^{-1} NAA + 5 mg L^{-1} TDZ was most effective for callus formation, with the short period of time and the highest percentages of callus formation and fresh weight.GC-MS analysis identified various active compounds in the jojoba plant callus. The callus tissue contained a wide range of secondary metabolic compounds, including ethylene diol, sitosterol, vaccenic acid and ethyl ester methyl mannose, which all exhibit antioxidant activity.FTIR method was integrated to the spectrophotometric system to detect characteristic peak values and functional groups. Chemical compounds included the main functional groups such as phenols, alkanes, amine salts, benzenoid and sulfoxide compounds, primary amine groups and a class of halocarbon compounds. The jojoba plant can be propagated by ex vivo under the influence of plant growth regulators, producing secondary and chemical compounds significant in industrial and medical applications.

Keywords: Jojoba, Plant tissue culture, Secondary compounds.

Introduction

The jojoba plant, *Simmondsia chinesi* (Link) Schneider, known as goat nut and wild hazelnut, belongs to the family Simmondsiaceae (Al-Ghamdi *et al.*, 2017). It originates from southwestern Arizona and northern Mexico, an area shared by the United States and Mexico (Al-Obaidi *et al.*, 2017). This plant is significant due to its high contents of proteins, glycosides, acids, and fatty alcohols. The seeds contain a high percentage of oil, free fatty, alcoholic acids, and antioxidants (Singh *et al.*, 2008).

Micropropagation of jojoba is a promising method for commercial mass production of

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superior clones, single explant source can produce thousands of true plants per year, meaning plants that are genetically similar to the parental stock, thereby maintaining the genetic line (El Sherif *et al*., 2023).

The plant was propagated in the laboratory, and callus formation was stimulated by growing the shoots and single nodes on MS medium supplemented with 1.5 mg.L^{-1} BA with 1 mg.L^{-1} IAA to increase the percentage of callus (Ibrahim *et al*., 2012). The levels of TDZ used particularly promoted callus formation (Alrazn et al., 2023; Guo et al., 2011). Additionally, plants produced in the laboratory grow more vigorously than those grown from seed cuttings because they have a stronger root system (Gonçalves & Romano, 2013). Several studies have reported on the in vitro propagation of jojoba by using various excised explants, such as buds and stem nodes (Singh *et al*., 2008). Understanding compounds in cultivated jojoba plants is crucial to improve their commercial applications, Gas chromatography–mass spectrometry (GC-MS)(Raji *et al*., 2019); and Fourier transform infrared spectroscopy (FTIR) are widely used techniques to identify and analyze compounds in plants, such as jojoba (Murali *et al*., 2021).

GC-MS is a powerful analytical method that combines the features of gas chromatography and mass spectrometry to identify different substances in a test sample (Léon *et al*., 2004). This technology plays a vital role in the separation and identification of compounds, including fatty acids, esters and alcohols in cultivated jojoba plants (Badawy *et al*., 2013). Specific compounds can be identified and quantified by analyzing peaks in chromatograms, providing valuable insights into the chemical composition of the plant (Fancello *et al*., 2017; Tripathi *et al*., 2020). Several techniques can be used to identify phytochemical compounds in plant extracts, (FTIR) is used to identify functional groups in gases, liquids and solids through infrared beams (Hayat *et al*., 2020). It has been used to identify biomolecules in plant extracts and analyze bioactive compounds in medicinal plants, plant dyes and food additives (Pharmawati & Wrasiati, 2020).

Cultivation of medicinal plants in the laboratory has become a valuable technique for producing large quantities of plant materials and is considered an effective method for stimulating callus tissue (Khateeb *et al*., 2017). Regenerating plants and producing natural plant compounds using biotic or abiotic stimulants (Khoddami *et al*., 2013). Many techniques have been tried to increase and improve plant cell response and production of secondary compounds (Khan *et al*., 2021). Therefore, the present study aims to evaluate growth regulators in callus production from the cultivation of jojoba plant and estimate the effective compounds using GC-MS and FTIR.

Materials & Methods

This study was conducted in the Plant Tissue Culture Laboratory of the College of Agriculture at University of Basrah from October 2023 to June 2024. Plant parts (explants, Fig. 1) were collected from 3-month-old cultivated jojoba seedlings, which were obtained from one of the private nursery in Babylon Governorate.

Fig. (1): Explants used in culture.

The sterilised explants were grown in nutrient medium consisting of MS salts obtained from Phyto Technology Lab, USA. The explants were cultivated on a sterilized laminar flow table. After planting, the explants were incubated in a growth chamber at 27 ± 1 °C under 16 hours of light and 8 hours of darkness(fig.2)(Ibrahim *et al*., 2012).

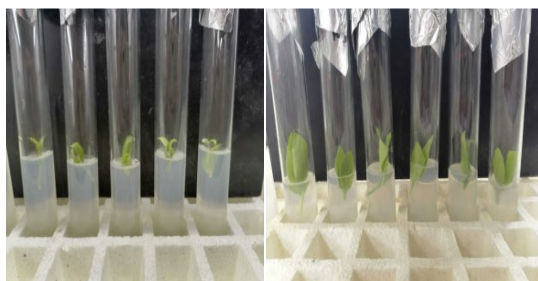

Fig. (2): Establishing a tissue culture and growing explants on MS media.

Induction of primary callus

Effect of the growth regulator auxin NAA and cytokinin TDZ

The effect of plant growth regulators (auxin NAA, 0.5, 1 and 2 mg.L^{-1}) and cytokinin TDZ (1, 2.5 and 5 mg.L^{-1}) on callus formation was studied. Explants were grown in the nutrient medium MS salts at a rate of 10 replicates. After planting, the explants were incubated in the growth chamber at 27 ± 1 °C under 16 hours of light and 8 hours of darkness.

Extraction, quantitative and qualitative estimation of the active substances of the jojoba plant using GC-MS technology

Samples of the callus were dried at 40 °C for 24 hours and ground. Approximately 1.5 g of the powder was mixed with 7.5 mL of 96% pure ethyl alcohol. The samples were placed on a vibrating device for 24 hours. The solution was filtered using filter paper and placed in an electric oven at 40°C for 24 hours to turn it into a powder. The powder was dissolved in 3 mL of pure ethyl alcohol, and the active ingredients were estimated using GC-MS device. The gas chromatographic separation was used according to the method of (Hamedi *et al*., 2013). Active compounds were estimated using a gas chromatograph integrated with an Agilent 5977A MSD mass spectrometer, Mass Hunter GC/MS Acquisition Software and Hunter qualifying program.

Detection of active aggregates using FTIR Fourier transform infrared spectrophotometer (FTIR)

Samples were dried in an oven for 48 hours at 60°C. Approximately 2 mg of the powder was mixed with 200 mg of potassium bromide (KBr) at a mixing ratio (100:1 part of the sample.part^{-1} of KBr) and crushed in a ceramic bowl to mix uniformly. The sample was homogeny zed and pressed into tablets for FTIR spectroscopy and to obtain absorption spectra between 400 and 4000 cm-1. The spectra of the samples was analyzed using an American-made Jasco FTIR 4200 FTIR spectrometer (Raji *et al*., 2019).

Statistical analysis

The laboratory experiment was designed using a completely randomized design (C.R.D.). Data were analyzed using one-way ANOVA. The Least Significant Difference (L.S.D.) test was adopted to compare the means at the 0.01 probability level. The statistical program Statistical Package for the Social Science (SPSS) version 21 was used.

Results & Discussion

Time Period for Primary Callus Induction

Table (1) shows the influence of growth regulators NAA and TDZ on the period of callus formation in the MS nutrient medium. Auxin NAA at a concentration of 2 mg.L^{-1} outperformed the other concentrations resulting in a minimum period of 20.77 days. The concentration of 0.5 mg.L^{-1} had the longest period of 34.77 days. Cytokine TDZ at a concentration of 5 mg.L^{-1} led to the

formation of callus in a minimum time period of 20.44 days and was significantly different from the other concentrations. Regarding the interaction between growth regulators, the treatment with 2NAA and 2.5 TDZ resulting in the shortest time period for callus formation at 18.33 days, showing no significant difference from the treatments with 1NAA and 5 TDZ and 2 NAA and 5 TDZ, which amounted to 20.33 and 20.66 days, respectively, while it differed significantly from the other treatments (Fig. 3).

Table (1). Effect of NAA and TDZ on callus induction period in vitro (Mg L^{-1}).

Concentration NAA (mg. L^{-1})	Concentration TDZ (mg.L^{-1})			Averages of NAA
	1	2.5	5	
0.5	$39.66^{g}\pm1.52$	$33.66^{f}\pm0.57$	$31.00^{e}\pm1.00$	$34.77^{C*}\pm3.96$
1	$25.66^{d}\pm1.52$	21.33 ± 0.57	$20.33^{ab}\pm0.57$	$22.44^{B}\pm2.60$
2	$23.33^{c}\pm0.57$	$18.33^{a}\pm0.57$	$20.66^{b}\pm0.57$	$20.77^{A}\pm2.22$
Averages of TDZ	$29.55^{B}\pm7.73$	$24.44^{A}\pm7.05$	$24.00^{A}\pm5.29$	
LSD	NAA= 1.24	TDZ= 1.24	Interaction = 2.16	

*Similar letters indicate that there are no significant differences between samples at a confidence level of $p \geq 0.01$.

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Percentage of primary callus

Table (2) shows the effect of growth regulators auxin (NAA) and cytokine (TDZ) on the percentage of callus formed. Treatment with 1 and 2 mg L^{-1} of NAA resulted in the highest percentage (82.22% and 80.00%, respectively). A significant difference was observed compared to the 0.5 mg.L^{-1} concentration, which resulted in 52.22%. Cytokine TDZ at a concentration of 5 mg.L^{-1} produced the highest percentage of 78.88% with an insignificant difference from the concentration of 2.5 mg.L^{-1}. The interaction between auxin and cytokines led to high percentages of callus formation. The treatment with 1 NAA and 2.5 TDZ in mg.L^{-1} achived the highest percentage of 93.33%, which was not significantly different from the treatment with 2 NAA and 2.5 TDZ mg.L^{-1} (90.00%) but differed significantly from the other treatments.

Fresh weight of primary callus

Table (3) shows the effect of the growth regulators auxin NAA and Cytokinin TDZ and their interaction the fresh weight of callus

(G). Auxin NAA at 1 mg.L^{-1} resulted in the highest fresh weight of 3.17 g, significant difference from the other concentrations. Cytokinin TDZ at a concentration of 5 mg.L^{-1} produced the highest fresh weight of 2.75 g, also significantly different from the other concentrations. Regarding the interaction effect, treatments with 1NAA and 5TDZ as well as 1 NAAA and 2.5 TDZ resulted in the highest fresh weight rates of 3.40 g and 3.30 g, respectively, with a significant difference from the other treatments.

Plant growth regulators, such as auxins and cytokines, are key components of the nutritiont medium that influence tissue culture performance (Schaller *et al.*, 2015).

Auxins play a fundamental role in the callus formation and its development into vegetative embryos and germination (Al-Asadi *et al.*, 2019). The most important plant growth regulators are 2,4 dichlorophenoxy acetic acid (2,4-D) and 1-naphthalene acetic acid (NAA)(AL-Alwani & Mohammed, 2023). Cytokines are important factors in the formation of vegetative branches and the induction of somatic embryos, including benzyl adenine (BA) and kinetin (Kn), isopentenyl adenine (2ip) and Thiodiazoron (TDZ) (Ibrahim *et al.*, 2012; Khateeb *et al.*, 2017).

Table (3) Effect of NAA and TDZ concentrations on the fresh weight of callus (g).

Concentration NAA (mg.L^{-1})	Concentration TDZ (mg L^{-1})			Averages of NAA
	1	2.5	5	
0.5	0.83^{g}±0.05	1.20^{f}±0.10	1.76^{e}±0.15	1.26^{C}±0.41
1	2.83^{d}±0.05	3.30^{ab}±0.10	3.40^{a}±0.20	3.17^{A}±0.28
2	2.70^{d}±0.10	2.90^{cd}±0.10	3.10^{bc}±0.10	2.90^{B}±0.19
Averages of TDZ	2.12^{C}±0.97	2.46^{B}±0.96	2.75^{A}±0.76	
LSD	NAA= 0.15	TDZ= 0.15	Interaction = 0.26	

*Similar letters indicate that there are no significant differences between samples at a confidence level of $p \geq 0.01$.

The presence of growth regulators in the nutrient medium stimulates the formation of DNA (RNA) and provides energy through its activity, which is associated with the oxidation of nutrients and the formation of growth-related enzymes, including respiratory enzymes (Solliman *et al.*, 2017). These enzymes lead to a large amount of energy production, which is exploited by tissues for division and growth (AL-Alwani & Mohammed, 2023). Cytokines greatly influence on the growth and division of cells, particularly the division of the cytoplasm of cells (Corredoira *et al.*, 2008). Auxins also affect cell elongation and division through their effect on nucleus division (Ikeuchi *et al.*, 2013). The addition of TDZ cytokinins in the nutrient medium leads to higher rates than the other types of cytokinin by stimulating plant tissues for growth and division as well as stimulating the accumulation of hormones (da Silva *et al.*, 2013 ; Al-Drisi *et al.*, 2022). Cytokinin play an important role in stimulating vegetative multiplication by breaking apical dominance, thereby increasing the area of vegetative multiplication in the meristematic region (Guo *et al.*, 2011).

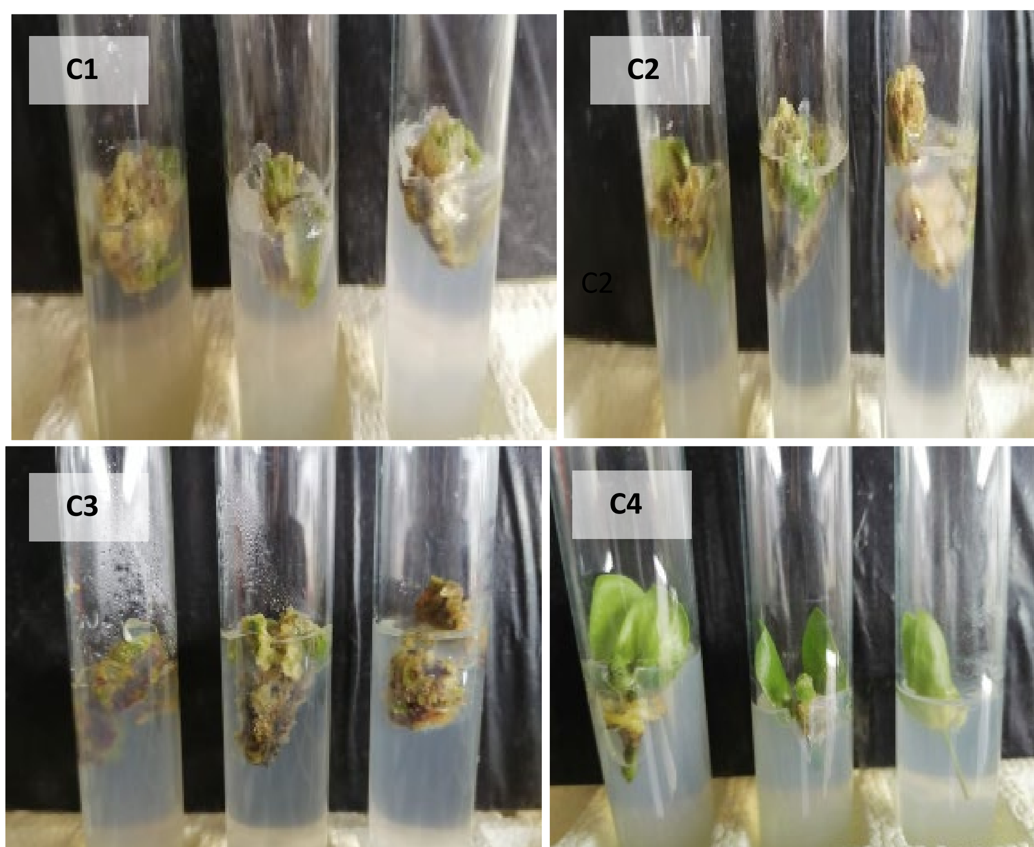

Fig. (3): Callus formed 25 days after being culturing on the MS medium: C1 (1 NAA, 2.5 TDZ); C2 (1 NAA, 5 TDZ); C3 (2NAA, 2.5 TDZ); and C4 (2NAA, 5TDZ).

Analysis of compounds in callus by GC-MS

Gas chromatography- mass spectrometry (GC-MS) was used to analyse the chemical components in the callus extract of the jojoba plant, with data compared to the chemical characteristics of the spectrum database. The study adapted findings for growing the jojoba plant on the MS nutritional medium under the influence of plant growth regulators NAA and TDZ (Table 4 and Figs. 4, 5, 6 and 7). Key chemical components identified include ethylene diol, sitosterol, vaccenic acid and ethyl ester methyl mannose, which were present in the highest proportions. These compounds are crucial due to their roles in secondary metabolism, which contributes to cell regeneration and resistance as antioxidants. The chemical content in the alcoholic extract, represented by long system chains, showing significant proportions 0.506–0.0255 %, 3.258–7.565%, 3.011–10.241%, 16.923–20.042%, and 2.939–20.663%, sequentially. According to (Tripathi et al., 2013), plant growth regulators like auxin impact the synthesis of secondary plant chemicals and lipid metabolism, altering fatty acid content and composition. Wild plant environments face numerous risks and instances of environmental instability. Thus, extracting natural substances and metabolites from plants in a pure form are essential. For these plants, tissue culture techniques provide a vianle alternative to traditional agriculture (Léon *et al.*, 2004). These compounds are more biologically effective and stable than those produced industrially. Research has focused on callus induction and its application in the synthesizing secondary metabolic compounds from medicinally significant plants, comparing them with those from plants grown in the field (Zambari *et al.*, 2021).

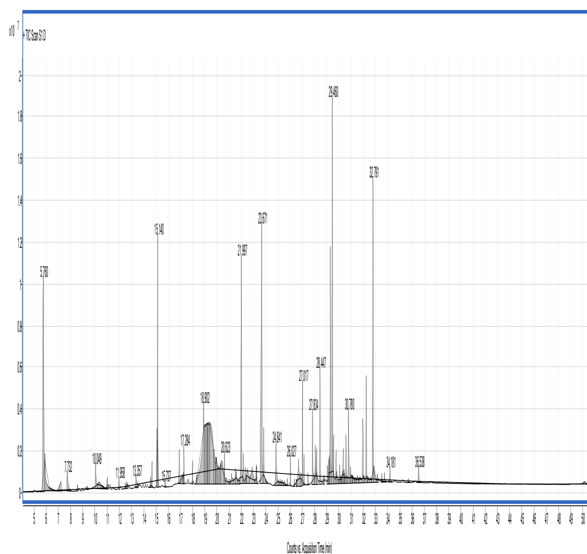

Fig. (4): GC-MS chromatographic separation of treatment C1 (1 NAA, 2.5 TDZ).

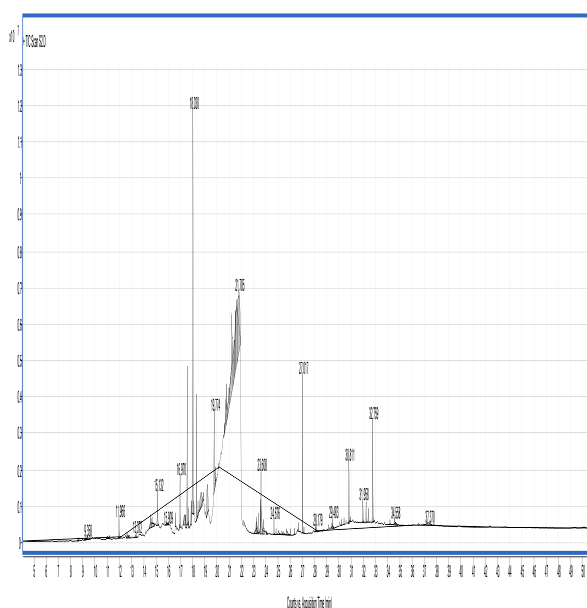

Fig. (5): GC-MS chromatographic separation of treatment C2 (1 NAA, 5 TDZ).

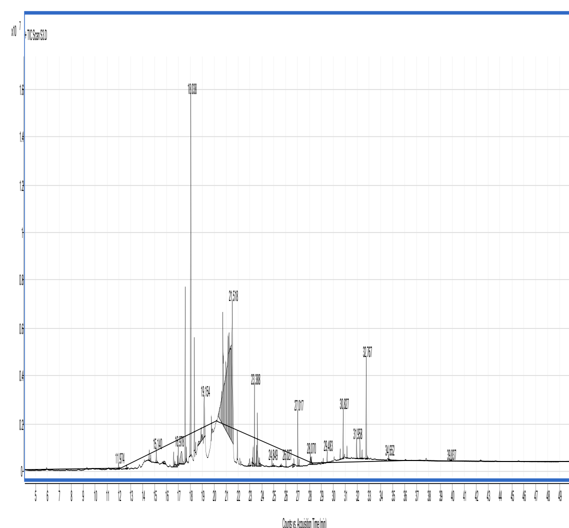

Fig. (6): GC-MS chromatographic separation of treatment C3 (2 NAA, 2.5 TDZ).

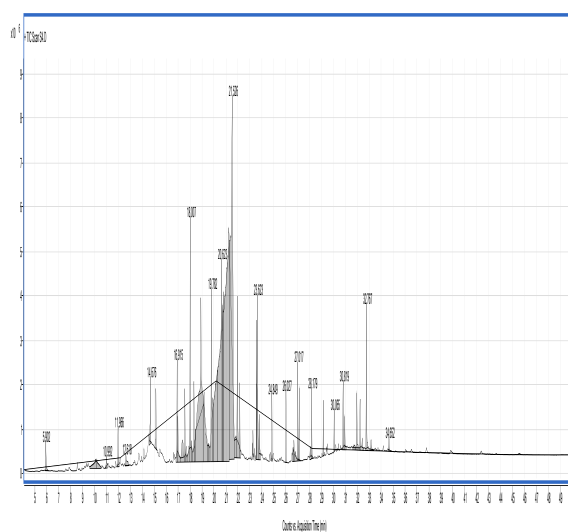

Fig. (7): GC-MS chromatographic separation of treatment C4 (2 NAA, 5 TDZ)

Table (4): GC-MS Analysis of secondary compounds in jojoba callus.

Compounds	C1 1NAA,2.5 TDZ	C2 1 NAA, 5TDZ	C3 2NAA, 2.5 TDZ	C4 2 NAA, 5 TDZ
Eugenol	5.025	1.495	0.506	1.150
gamma.-Sitosterol	7.565	4.730	3.935	3.258
Stigmasterol	2.144	0.909	0.710	1.020
Campesterol	1.453	1.198	0.842	--
D-Glucopyranoside, methyl	5.493	4.028	--	--
n-Hexadecanoic acid	5.544	--	1.278	3.259
Vaccenic acid	10.241	--	3.011	--
6-Hydroxy-5-methoxyflavone	8.315	--	--	--
Octadecanoic acid	1.647	0.334	0.336	6.719
Acetic acid, (ethylthio)-, ethyl ester		20.042	16.923	
Benzeneacetic acid, .alpha.-cyano-, ethyl ester	1.331	9.160		
4-O-Methylmannose		2.939	3.515	20.663

C1(1 NAA, 2.5 TDZ) ; C2(1 NAA,5 TDZ); C3(2NAA , 2.5 TDZ);C4(2NAA, 5TDZ) treatments of callus induction

Analysis of compounds in callus by FTIR

Table (6) displays the application of FTIR on the spectrophotometric system to detect characteristic peak values and their functional groups of the most important chemical compounds of jojoba callus under the influence of various plant growth regulators (Fig 8, 9, 10 and 11). In treatment C4, the group at 3290 cm^{-1} was of medium intensity, while the group did not show in the 500–600 cm-1. These findings provide insights into the chemical bonds and functional groups present in the dry powder of the callus tissue of jojoba. Specific transactions (C1, C2 and C3) showed the same functional groups, ranging from 3390 to 500 cm^{-1} and did not differ significantly between them, being clear and strong indicative. The major functional groups found in the jojoba plant callus extract included phenols, alkanes, amine salts, benzenoid compounds, sulfoxide, primary amine groups, and halo compounds

Table (6): FTIR analysis of compounds in cultivated jojoba plants.

Peak values	Functional groups		Treatment of plant growth regulators			
			C1	C2	C3	C4
3300-3400	N-H stretching	aliphatic primary amine	++	++	++	++
3200-3290	O-H stretching	alcohol	+	+	+	-
2800-3000	N-H stretching	amine salt	++	++	++	++
1600-1650	C=C stretching	conjugated alkene	++	++	++	++
1085-1150	C-O stretching	aliphatic ether	++	++	++	++
1030-1070	S=O stretching	sulfoxide	++	++	++	+
1050-1025	C-O stretching	primary alcohol	++	++	++	+
500-600	C-I stretching	halo compound	++	++	++	-

++ powerful + mediocre - subpar

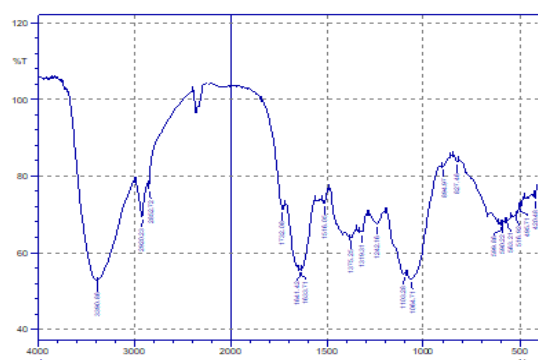

Fig. (8) FTIR spectroscopic analysis of treatment compounds C1 (1 NAA, 2.5 TDZ)

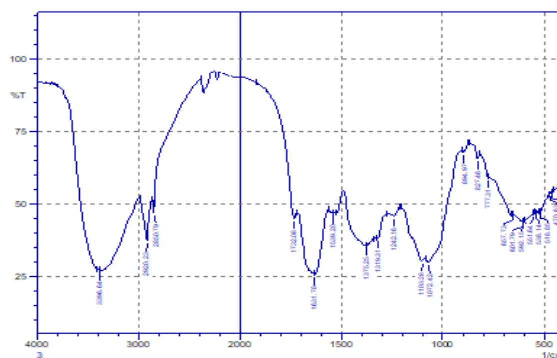

Fig. (9) FTIR spectroscopic analysis of treatment compounds C42 (1 NAA,5 TDZ)

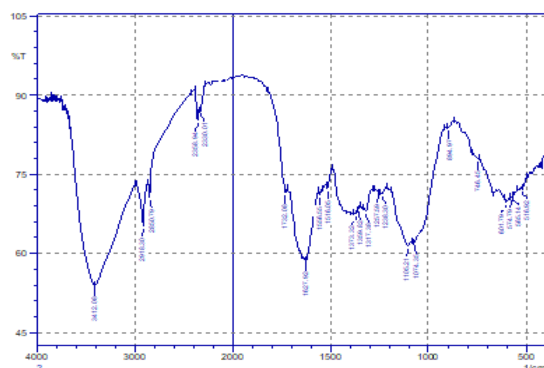

Fig. (10) FTIR spectroscopic analysis of treatment compounds C3 (2 NAA, 2.5 TDZ)

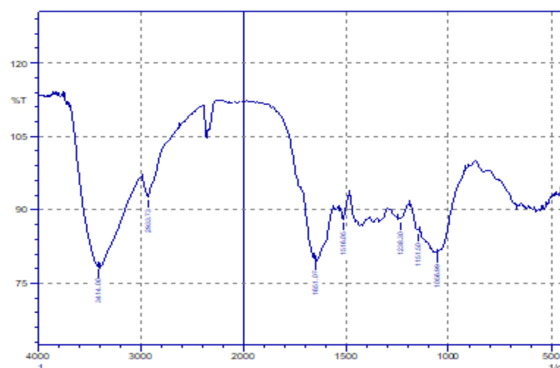

Fig. (11): FTIR spectroscopic analysis of treatment compounds C4 (2 NAA,5 TDZ).

According to this study, alkaloids cultivated in plants are a good source of antioxidants and therapeutic substances (Singh *et al*., 2022). Amines are metabolized in tandem with the cell cycle and are crucial for development and proliferation. Conversely, plants collect a variety of related specialized chemicals in free or conjugated forms, known as binary and polyamines (Bouchereau *et al.*, 2000). The current study discusses the most recent advancements in the extraction, measurement and estimation of

amines in plant tissues, with a particular emphasis on mass spectrometry and chromatographic separation (Wang *et al.*, 2019)

Amino salts are compounds of great importance in the pharmaceutical industry because many active pharmaceutical ingredients are amino salts (Smith, 2019). Amino salts are important because they are used to make pharmaceutical substances soluble in water and therefore more bioavailable (Pawar & Kamble, 2017).

Numerous metabolites with varying structural and functional diversity are produced by plants, and these metabolites serve a variety of purposes in the growth and development of the plant as well as in the plant's adaptation to its ever-changing environment (El Sherif *et al.*, 2023). Many medications are organic halides or two-halocarbon compounds, in which one or more carbon atoms are joined to a single halogen atom by covalent bonds (Cheynier *et al.*, 2013).

Conclusion

The results showed the possibility of using plant tissue culture technique for the propagation of the Chinese jojoba plant. The study found that the use of auxin NAA with cytokinin TDZ led to the highest results in the duration of callus formation, proportion and wet weigh.t Analyses using GC-MS and FTIR, revealed the presence of many secondary metabolic compounds and chemical compounds with antioxidant properties. This technique is one of the important methods in the production of important secondary and chemical compounds in the pharmaceutical industry to reduce complex methods of production

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Contributions of authors

A.H. A., Suggesting the research and writing the manuscript.

A.A.S.; Practical experiments and analysing the data.

M.S.A., Designing the experimental approach.

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Conflicts of Interest

The authors declare no conflicts of interest.

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الكشف عن المركبات الفعالة الناتجة من زراعة نباتات الجوجوبا (*Simmondsia chinesi* (Link) Schn.) خارج الجسم الحي باستخدام تقنيات GCMS وFTIR

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المستخلص: تعتبر شجرة الجوجوبا من النباتات المهمة اقتصاديا لاحتوائها على نسبة عالية من الزيت ولها استخدامات صناعية وتجارية وطبية. تم إجراء الدراسة لتحديد أفضل تركيبة من منظمات النمو النباتية على استجابة البراعم المزروعة للتكشف والنمو وتكوين الكالس.أجريت الدراسة في مختبر زراعة الأنسجة النباتية بكلية الزراعة–جامعة البصرة. تم استخدام البراعم القمية لإنتاج الكالس الأولي استخدم الوسط الغذائي(MS) (موراشيج وسكوج) المجهز بمجموعتين من منظمات نمو النبات الاوكسين NAA بالتراكيز 0.5، 1 و 2 ملغم لتر$^{-1}$ والسايتوكاينين TDZ بالتراكيز 1 و 2.5 و 5 ملغم.لتر$^{-1}$. تمت دراسة صفات الكالس الاولي وأهم المركبات الثانوية باستخدام تقنية GCMS و FTIR .وجد ان الوسط الغذائي المجهز 1 NAA مع TDZ 2.5 ملغم. لتر$^{-1}$ والمعاملة 2 NAA مع TDZ5 ملغم.لتر$^{-1}$ كانت الأفضل في تكون الكالس بأقل فترة من الزمن وسجلت أعلى نسبة مئوية لتكون الكالس وأعلى معدل الوزن الرطب. تم استخدام تقنية GCMS للكشف عن المركبات الثانوية النشطة من الكالس لنبات الجوجوبا. يحتوي نسيج الكالس على مجموعة واسعة من المركبات الأيضية الثانوية ذات النشاط المضاد للأكسدة ، مثلethylene diol و sitosterol و vaccenic acidو ethyl ester و methyl mannose. بينت تقنية FTIR لنظام الطيف الضوئي للكشف عن بعض المركبات الكيميائية و قيم الذروة المميزة ومجموعاتها الوظيفية. احتواء مستخلص الجوجوبا للعديد من المركبات الفعالة مثل الفينولات والألكانات وأملاح الأمين ومركبات البنزينويد والسلفوكسيد ومجموعات الأمين الأولية وفئة من مركبات الهالوكربون.خلصت الدراسة بامكانية اكثار نبات الجوجوبا عن طريق الزراعة خارج الجسم الحي تحت تأثير منظمات النمو النباتية وانتاج المركبات الثانوية وتشخيصها كيميائيا لما لها من دور مهم في المجالات الصناعية والطبية.

الكلمات المفتاحية: الجوجوبا ، زراعة الأنسجة النباتية، المركبات الثانوية