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RESEARCH ARTICLE

Nano Zinc as a Source to Stimulate *Calendula Officinalis* Callus to Produce the Active Triterpenoid Glycoside

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ABSTRACT

Plants can synthesize a large number of different active compounds and ingredients, which can be influenced under stress conditions. The plant *in vitro* culture technique is considered a suitable and useful method to propagate and protect the medicinal and herbal plants, and to extract active natural compounds. The main target of this work was to obtain callus of *Calendula officinalis* explants stimulated by Nano zinc, and then to extract the natural glycosides from callus *in vitro* culture and apply them against certain bacterial and fungal isolates. The explant of *C. officinalis* was grown on media containing a mix of hormones, at concentrations of 0.5 mg·L⁻¹ of benzylaminopurine (BA) and 1-naphthaleneacetic acid (NAA). The dry callus was extracted with methanol, and the extracted glycoside were hydrolysed to release the aglycone. Six selected species of bacteria were isolated from wound infections, identified and verified using the Vitek2 technique. Three species of yeasts were used to investigate the influence of oleanolic acid (OA) on the viable counts of colonies. Current work revealed that *Proteus mirabilis* and *Candida krusei* were isolated the most affected by OA. The application of elicitors, such as nano zinc, can be considered as a useful elicitation technique to enhance OA biosynthesis and accumulation in callus culture, and it seems to exert a promising effect against certain bacteria and yeasts.

Keywords: Antimicrobial activity, *Calendula officinalis*, Callus, Nano zinc, Oleanolic acid

Introduction

The callus is often used in various experiments regarded as plant tissue culture technique. Plant *in vitro* culture techniques have been treated as a source for large-scale production of important and valuable natural ingredients, which belong principally to plant secondary metabolic products. Under stress and in non-normal environmental conditions, plants can synthesize a large number of different bioactive compounds and ingredients that the plant produces as a part of the chemical defense. One of these important groups of compounds are oleanane-type triterpenoids, including the most characteristic

oleanolic acid. *Calendula officinalis* L. callus has been successfully induced and stimulated for the production of saponins of the triterpene oleanolic acid.¹ There were several studies on callus *in vitro* cultures representing an alternative way for the production of naturally derived antimicrobial and antiviral compounds. The tissue plant culture technique produces a diversity of secondary active plant metabolites, occasionally in a high concentration compared to the intact original plant source.²

Oleanolic acid Fig. 1 is considered an active compound that occurs throughout the plant kingdom and is found in many plants as glycoside derivatives. It is a pentacyclic triterpenoid that has been found in

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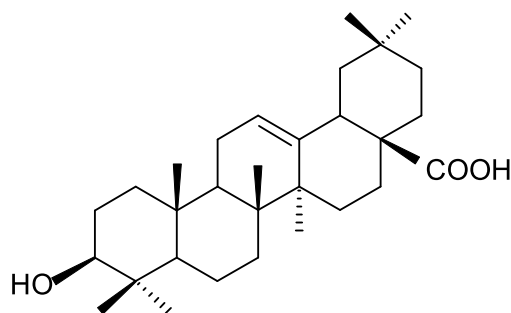


Fig. 1. Oleanolic acid (chemical structure), the aglycone of triterpenoid glycosides occurring in *Calendula Callus*.

about 1600 herbal plants, edible, and pharmaceutical plant species.³ Oleanolic acid glycosides, along with other derivatives, possesses many promising pharmacological activities, such as anti-inflammatory, hepatoprotective, antioxidant, and anticancer properties. With the recent elucidations of oleanolic acid biosynthetic pathways and the imminent commercialization of the first oleanolic acid-derived drugs or supplements, this compound remains an important and promising active ingredient for diverse pharmacological and biochemical studies.⁴ Oleanolic acid is distributed widely among the family (Oleaceae) and the name originated from the olives (*Olea europaea*).⁵ Oleanolic acid as an active plant constituent has many pharmacological medicinal properties including immunostimulatory, anti-HIV, anti-tumor, hepatoprotective, anti-inflammatory, antiparasitic and antibacterial properties,⁶ and some of the functions of OA might be effective in the defense against the fungal attack.¹

The first step of the present study was to obtain the callus culture from *Calendula officinalis* explants. In previous studies on tissue cultures of this plant, it was revealed that the MS medium with half salt concentration usually can give high averages of explants.¹ In the present work, the obtained callus culture was elicited by Nano zinc to stimulate the biosynthesis of bioactive oleanolic acid glycosides.

There are focused studies on alternative antimicrobial agents, such as antibacterial peptides and secondary metabolites produced by microorganisms, bacteriophages, and plants. The plant-derived oleanolic acid (OA) has a high antimicrobial potential; it is worth applying because it is an ingredient naturally found in plants, and almost no microbial resistance has been found till now.⁷ The spectra of causative infection microbes and their sensitivity patterns have been found to vary from one setting to another. Microbial resistance develops naturally over time, but the misuse of antibiotics in humans and animals could accelerate this phenomenon. When there are no strong regulations on antimicrobials, it is a

major factor in the access and misuse of antimicrobials. Antimicrobial drugs could be basing with any medical prescriptions in many different countries in their culture and education.⁸ Microbial resistance to antimicrobial factors is a big challenge in healing infections.⁹ Therefore, this work aimed to test the active material of plant callus extract against certain pathogenic fungi and bacteria.

Material and methods

Plant material (elicitation by Nano zinc, initiation and maintenance)

C. officinalis seeds were washed three times with distilled water, then soaked and sterilized by a hypochlorite (commercial) diluted in sterilized distilled water (1:4, v/v). Nano zinc was prepared at a concentration of 1mg/L. Seeds of *C. officinalis*, after being washed in sterilized distilled water, were then soaked with Nano zinc for 4 min, and washed with distilled water for one minute. Nano zinc was used in this experiment as a potential stimulant of biosynthesis of bioactive plant secondary compounds. To keep the seeds of marigolds sterile and to prevent contamination, the treatments were applied to all plant parts (seeds, explants) and the next steps of in vitro culture initiation techniques, and to maintaining explants, were made in a spatial laminar (cabinet) air-flow. The marigold seeds were obtained and grown on MS (solid agar) during the first 5–7 days in a special growth chamber with darkness. On the eighth day of cultivation, the obtained seedlings were checked and moved to an incubator (a growth chamber) for the installed plant.

Callus initiation

Explants of marigold were growing in room 17h (Light)/.7 h of darkness photoperiod with 200 $\mu\text{m m}^{-2} \text{ s}^{-1}$ Photosynthetically Active Radiation, the temperature between 24/22°C and 71% relative humidity. The explants were cut into 1.0 cm pieces and subjected to the method of growing on MS media,¹⁰ supplemented with plant hormones at concentrations of 0.5 mg L⁻¹ of benzylaminopurine (BA) and 1-naphthaleneacetic acid (NAA). The callus was obtained after four weeks. Then a subculture of callus was made to maintain and obtain a large mass of callus.

Extraction of callus

Callus of marigold was weighed and dried at room temperature for 3 days, powdered by grinding special mortar, and extracted with methanol for 8 hours in a Soxhlet Extractor. The obtained plant extract

was removed to special round-bottom flasks and then evaporated by rotary evaporator under pressure reflux to dryness at 45°C.

Hydrolysis and oleanolic acid extraction

The dried plant extract was boiled under reflux using a concentration of 10% hydrochloric acid in methanol for about 2h. Then, distilled water was added and evaporated in a vacuum. The solution was removed to extract 3 times with 25 ml of diethyl ether. Solutions were washed with distilled water. The extracts were evaporated, and subjected to the thin-layer chromatography technique. The plates were developed in solvent system of 95:5 (v/v) chloroform-methanol. The location of oleanolic acid on the plate was compared with the standard. The scrubbed area gel was eluted in the elution columns in diethyl ether, the eluted fraction contained oleanolic acid Fig. 1.

Bacterial and yeast culture conditions

Traditional bacterial diagnosis was performed using micro and macroscopic examinations, cultural characterization, and biochemical tests, including IMVC tests, urease test to indicate urea formation, oxidase test to investigate cytochrome production, as well as performing catalase test, sugars fermentation test, motility test, and coagulase detecting enzyme. Isolates were further diagnosed at the species level using the Vitek2 compact technique, while the pathogenic yeasts used in this study, *C. albicana*, *C. tropicalis*, and *C. krusei*, were also verified by Vitek2. All the bacteria and yeasts were purified on Nutrient Broth and Sabouraud Dextrose Agar (SDA), respectively, at 37 °C for 24 hours and preserved on slants until used for evaluating antibacterial and antifungal activity.

Microbial viability assay

Counting viable colonies was conducted using the procedure presented by:¹¹ about 0.1ml from bacterial inoculum with a density check of 0.5 McFarland's standard added to an equal volume of OA prepared, incubated for 30 min. at 37 °C, then poured onto (sterile) Petri dishes with media and spread with a glass spreader. All the dishes were incubated at 37°C for 24 h.

Antimicrobial activity test

Antimicrobial activity was conducted using the Kirby-Bauer agar diffusion method (Mueller-Hinton

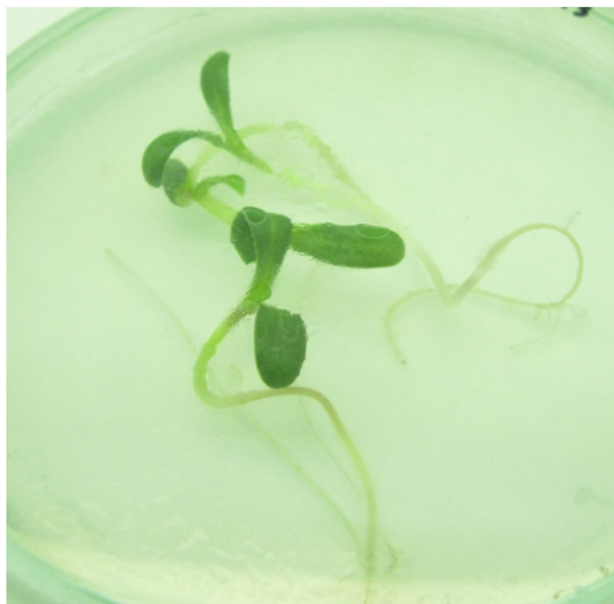


Fig. 2. Calendula seedlings after 11 days of growth in the growth chamber.

agar (Himedia). The inoculum suspension of each tested bacterium was adjusted to a turbidity of 0.5 McFarland standards to sustain 1×10^8 colony-forming units. 0.1 ml of bacterial suspension grown in nutrient broth (Himedia) was poured onto petri dishes using a glass rod spreader. The inoculum was dried at room temperature for a couple of minutes, then wells were made. Those holes were filled with 100 μ L of plant-derived oleanolic acid in concentration of 120mg/ml. Standards of antibacterial agents (Azithromycin/Bioanalyse) and antifungal (Nystatin 1000UI) were used as positive controls. Then, microbial isolate culture was incubated at 38°C, and the diameters of the inhibition zone were evaluated in millimetres.¹²

Results

The plant *in vitro* culture technique enables the production of plant tissues in a sterile environment, and controlled conditions, leading to the growth and development of the tissues Fig. 2. These methods are considered suitable methods to obtain the active compounds from medicinal and herbal plants.

Nano zinc caused significant changes in the accumulation of active compounds in *C. officinalis* callus. The content of oleanolic acid was enhanced in the marigold explant after being stimulated by nano zinc. Such stimulation was also¹³ demonstrated previously by using a Laser Diode and UV as agents to increase flavonoid production in *C. officinalis*.

Table 1. The influence of BA and NAA (0.5 mg L^{-1}) on regeneration of *C. officinalis* shoots from sterilized explants cultured on MS solid media.

BA&NAA	Shoots (number)	Shoots (length, cm)	Leaves (number)
0.0	1.4	3.1	5.2
1.0	1.9	2.7	3.9
2.0	6.1	5.0	6.2
3.0	5.5	4.0	6.0

Explant of marigold (*C. officinalis*) was grown on a mix of cytokinin and auxin on MS containing hormones BA and NAA Table 1. Callus was obtained at the best concentrations with a large mass. Fig. 3 illustrated the interaction with the concentration of BA and NAA at 0.5 mg.L^{-1} concentrations (variant 2 according to Table 1). Oleanolic acid obtained from the callus showed significant inhibitory activity against bacteria. Viability assay results showed significant differences in CFU number comparing the control non-treated isolates with exposed isolates related to same species that decreased dramatically, although the exposure time were 30 minutes and serial dilution were not conducted. Since the data were distributed across independent groups (each isolate is a group), and each group had a single numerical variable (inhibition diameter in mm), the current study used one-way ANOVA (one-way analysis of variance Fig. 4, and revealed differences between colonies before and after exposure to OA.

Discussion

The obtained result concerning the callus growth is similar to the previously observed effect of the

effective cytokines (BA) used in the micropropagation of *Atropa belladonna*, which enhanced all explants, shoot number, and the length of the shoots, as well as the number of leaves.

The inhibitory activity of oleanolic acid against bacteria in this work is presented in Table 2 using the ANOVA test, where $a > b$. Different letters in a column mean high differences of mean values according to the bacterial isolate. Results revealed that Gram-negative bacterial isolates compared with the two Gram-positive isolates showed no effect or the least inhibition zone. However, *Proteus mirabilis* and *Candida krusei* (14mm,24mm, respectively) seem to be the most affected by oleanolic acid Fig. 5. Wolska et al.¹⁴ postulated that both carboxy and hydroxy groups existing in triterpenes and their derivatives are important for antibacterial activity.

It was reported¹⁵ that OA can inhibit the efflux pumping, which could directly inhibit bacterial viability, or interfere with the stress response. Additionally, it can alter DNA synthesis, eventually inducing a heat shock response. Also, OA can inhibit peptidoglycan by inhibiting expression of peptidoglycan biosynthesis-related genes or affecting the amount of mucopeptides, otherwise interacting with undecaprenyl pyrophosphate and, ultimately, leading to blocking bacterial cell walls, suggesting that these biochemical pathways could be a target for OA triterpenoids. Changes in the metabolic pathways usually lead to mediate the uptake of carbohydrates, as well as phosphorylation of carbohydrates, in addition to controlling the metabolism in response to energy deficiency. Furthermore, Wolska¹⁴ had posted that OA might affect proteins involved in translation processing, which leads to the accumulation of mRNA and

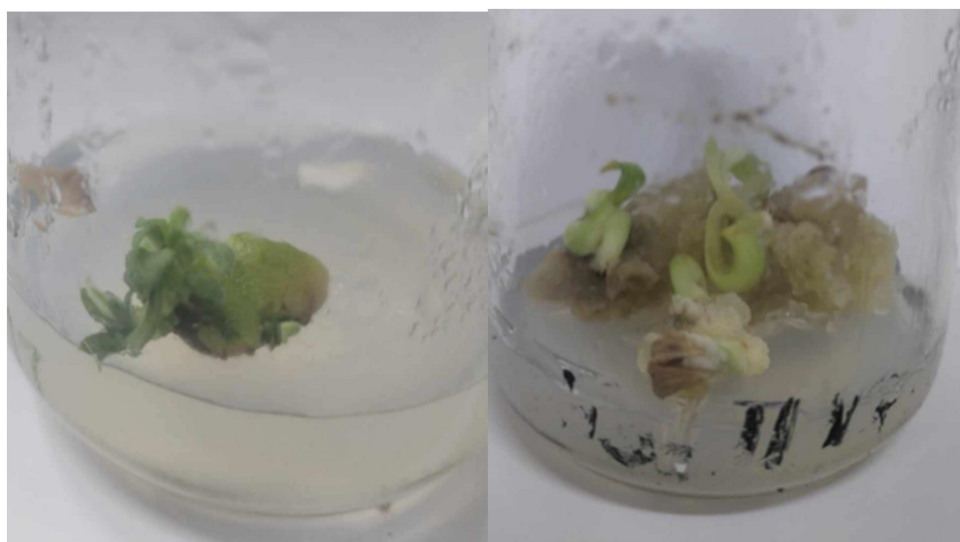


Fig. 3. Calendula callus after 6 weeks of growth in a growth chamber in interaction with the concentration of BA and NAA 0.5 mg L^{-1} .

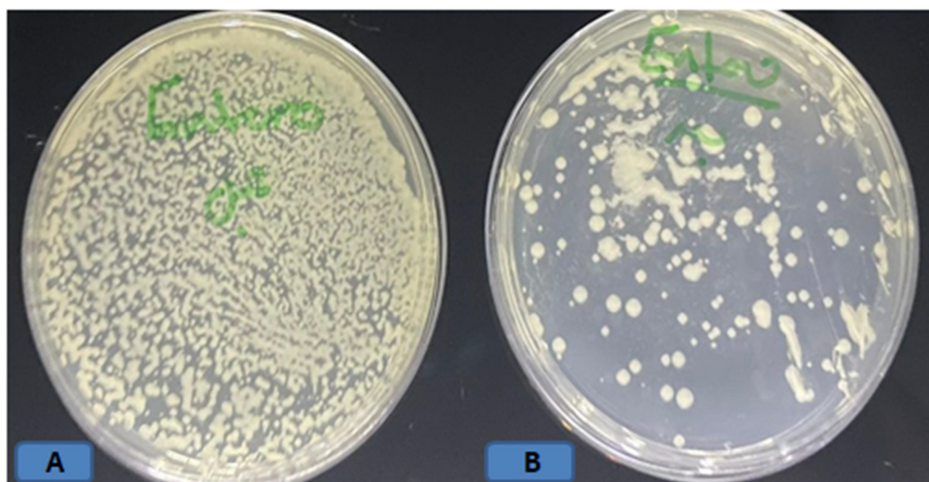


Fig. 4. Viability Assay. A: colonies of non treated bacteria; B: number of colonies after being exposed to oleanolic acid.

Table 2. Inhibition diameters of oleanolic in mm (0.01mg/ml).

Bacterial Isolates	Inhibition diameter	Yeast Isolates	Inhibition diameter
<i>Proteus mirabilis</i>	18 a	<i>C.albicans</i>	16 b
<i>Escherichia coli</i>	15 b		
<i>Klebsiella oxytuca</i>	14 b	<i>C.tropicalis</i>	18 b
<i>Pseudomonas aeruginosa</i>	17 a		
<i>Enterococcus faecalis</i>	10 c	<i>C.krusei</i>	24 a

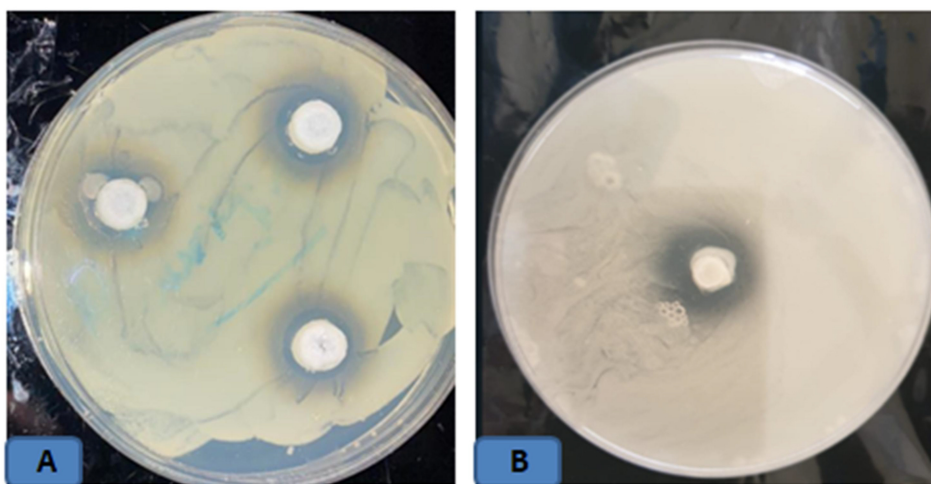


Fig. 5. Inhibition zone of OA of A: *P. mirabilis*; B: *C. albicans* cultured on Muller-Hinton agar.

misfolded proteins with the induction of chaperone and ribonuclease subunits.

Callus extract showed in this study was also similar to several studies that indicated the antifungal activity of oleanolic acid glycosides against fungi such as *Rhizoctonia solani* Kuhn, *Sclerotinia sclerotiorum* Lib, and *Phytophthora parasitica* Dast.^{16,17} *C. tropicalis*, *C. guilemondi*, and *Microsporum cain*.¹⁸ Extended studies are required in order to characterize the antifungal activity against fungi such as *C. albicans* since the

mode of actions is not yet well established.¹⁹ In some modern experiments, synthesized active secondary metabolite compounds can exhibit strong inhibitory effects on bacterial growth, with some compounds performing comparably or even better than the reference antibiotics, highlighting their potential as effective compounds and antibacterial agents, that could be applied as promising substances instead of the antibiotics.²⁰ Different chemical, biological, and physiological plant processes are connected to basic

processes such as photosynthesis in plants, as well as, for example, stimulating cell divisions and elongations in different types of plants.²¹

Conclusion

Nano zinc could cause significant changes in the accumulation of active compounds in *Calendula calus*. The oleanolic acid, as an active plant secondary compound, was elevated in explants after being stimulated by Nano zinc. Oleanolic acid, as a plant secondary compound, can affect bacteria and fungi, which could directly inhibit bacterial viability. The current study suggests the potential use of the plant-derived chemicals as antimicrobial drugs. However, further studies are needed to observe the cellular target and molecular mechanism of oleanolic acid as well as fungal and bacterial gene expression. The other important future research trend is the synergy with ursolic acid (UA), the triterpenoid often occurring in the mixture with OA in numerous plants, as well as the synergy with the antibiotics.

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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 republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Tikrit.

Authors' contribution statement

A. S.M.A., S.M.A, S.Q.S, and A.S designed the study. A.S.M.A, S.M.A., S.Q.S and A.S performed the stimulate *Calendula officinalis* Callus experiments. A.S.M.A and A.S. performed extraction *Calendula officinalis* Callus simulations. A.S.M.A, S.M.A and S.Q.S expressed and purified by applying active triterpenoid glycoside on microorganisms. A.S.M.A, S.M.A and A.S

analyzed the data. A.S.M.A, S.M.A, S.Q.S and A.S wrote the paper with input from all authors.

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الزنك النانوي كمصدر لتحفيز كالوس نبات الأذريون الطبي (*Calendula officinalis*) لإنتاج الغليكوسيد التريتربينويدي الفعال

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المخلص

تستطيع النباتات تخليق عدد كبير من المركبات الفعالة وتركيز مختلفة من المكونات التي تنتجها، والتي يمكن أن تتأثر بأنواع ظروف الإجهاد. وتعد تقنية زراعة الأنسجة النباتية في المختبر (in vitro) طريقة مناسبة ومفيدة لإكثار النباتات وحمايتها واستخلاص المركبات الطبيعية الفعالة من النباتات الطبية والعطرية. إن إنتاج مركب فعال من خلال تحفيز النبات على إنتاج مركبات ثانوية نباتية يمكن أن يكون وسيلة فعالة لحماية النبات تحت ظروف الإجهاد، ولا سيما عندما يُنتج هذا المركب في مزارع الأنسجة المخبرية. كان الهدف الرئيس هو الحصول على كالوس من أجزاء نبات الأذريون الطبي (*Calendula officinalis*) المحفزة بالزنك النانوي، واستخلاص الغليكوسيدات الطبيعية من الكالوس المزروع مخبرياً، ثم اختبار فعاليتها ضد بعض العزلات البكتيرية والفطرية. زُرعت الأجزاء النباتية على أوساط غذائية تحتوي على خليط من الهرمونات بتركيز 1 و 2 و 3 ملغم/لتر من BA مع 0.5 ملغم/لتر من NAA واستخلص الكالوس الجاف باستخدام مذيب الميثانول، ثم أجريت عملية التحلل المائي الحمضي لفصل الجزء السكري عن الأجليكون. عُزلت ستة أنواع مختارة من البكتيريا من إصابات الجروح، وتم تشخيصها يدوياً والتأكد منها باستخدام تقنية Vitek2. كما استُخدمت ثلاثة أنواع من الخمائر لدراسة تأثير حمض الأوليانوليك (OA) في أعداد المستعمرات الحية. أظهرت النتائج أن *Proteus mirabilis* و *Candida krusei* كانتا الأكثر تأثراً بحمض الأوليانوليك. وتشير النتائج إلى أن التحفيز باستخدام المحفزات (elicitors) يمكن أن يؤثر في إنتاج بعض المركبات النباتية الفعالة ويقلل من أخرى. ويُعد استخدام محفزات مثل الزنك النانوي لتعزيز تراكم حمض الأوليانوليك، بوصفه مركباً ثانوياً نباتياً فعالاً في زراعة الكالوس المخبرية، تقنية واعدة ذات تأثير ملحوظ ضد بعض أنواع البكتيريا والخمائر.

الكلمات المفتاحية: مضاد ميكروبي، *Calendula officinalis*، الكالوس، الزنك النانوي، حمض الأوليانوليك.