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Artemisia Annua Response to Different Applications of Banana Peel Nanoparticles

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Abstract

This study was conducted as a randomized experiment to investigate the effects of banana peel Nanoparticles on the growth and biovital activity of *Artemisia annua*. The seeds of *Artemisia annua* were divided into four groups. Each group was soaked in different solutions: distilled water, Nanoparticles dissolved in distilled water, Nanoparticles dissolved in a free bacterial medium, and Nanoparticles combined with the endophytic Kocuria artinae bacterium dissolved in water. Furthermore, one group was treated with banana peel Nanoparticles and the endophytic bacterium grown in a bacterial medium. Each group was further subdivided into three subgroups, which were soaked for 4, 8, and 16 hours, respectively. The prepared banana peel Nanoparticles derived from banana peel were characterized using various techniques such as Fourier-transform infrared spectroscopy (FT-IR), potential analysis (ZETA), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray fluorescence spectroscopy (XRF). Plant growth parameters including plant height, plant fresh weight, plant dry weight, and the concentration of artemisinin, as an indicator of plant bioactivity were measured. The results indicated that all growth criteria and parameters exhibited significant differences among the groups. The expression of artemisinin biosynthetic pathway genes, including ADS, FDS, DBR, CYP, CPR, and HMG, was also investigated. The findings demonstrated that the expression of these genes played a role in the biosynthesis of artemisinin through specific pathways, and all genes collectively contributed to its production.

Keywords Artemisia *annua* Artemisinin Gene expression banana peels endophyte

1- Introduction

Artemisia annua, a member of the Asteraceae family, is a medicinal herb renowned for its diverse array of active compounds. Among these, artemisinin stands out as a powerful bioactive metabolite with therapeutic potential in treating malaria, and Covid-19 (1), hepatitis B, and schistosomiasis (2). Additionally, artemisinin has demonstrated effectiveness against various types of cancer, including breast, colon, lung, and leukemia (3). Extensive research has been conducted to unravel the genetic and enzymatic mechanisms underlying artemisinin biosynthesis. Notably, Graham et al. (4) identified five key genes (FDS, ADS, CPR, HMGR, CYP71AVI, and DBR) responsible for the biosynthesis of artemisinin

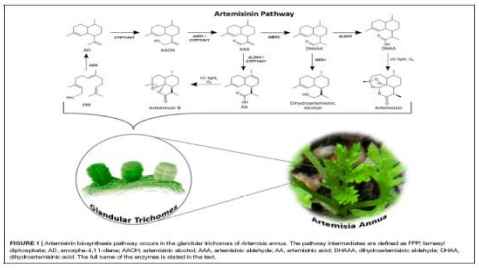

Fig (1) The biosynthesis of Artemisinin pathway. (5)

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However, the concentration of artemisinin within the plant is relatively low, resulting in high production costs. This limitation hinders its widespread utilization (6).

As the population grows and human activities expand, the consumption of food increases, leading to a rise in agricultural waste. Traditional disposal methods of agricultural waste contribute to climate-related issues, such as increased methane (CH4), nitrous oxide (N2O), and carbon dioxide (CO2) emissions, which are major contributors to greenhouse gas concentration and subsequent climate changes (7).Addressing these concerns, the development of sustainable and environmental-friendly practices for managing agricultural waste is crucial (8). These practices aim to improve soil quality, reduce the need for pesticides and irrigation water, and minimize the carbon footprint, presenting significant challenges in the face of climate change and sustainable agriculture (9).

Banana peels have shown promising antioxidant and antibacterial properties due to their higher content of tannins and phenolic compounds compared to banana pulps (9). They contain essential components such as cellulose (60-65%), hemicellulose (6-8%), lignin (5-10%), starch (3%), crude protein (6%-9%), crude fat (3.8-11%), total dietary fiber (43.2-49.7%), pectin, essential amino acids, polyunsaturated fatty acids, micronutrients, and minerals like potassium, calcium, sodium, iron, and manganese (11, 12). Banana peels are also rich in natural bioactive substances, including dietary fiber, polyphenols, and carotenoids, which offer health benefits such as protection against cancer, cardiovascular disease, and degenerative disorders (13). Moreover, banana peels exhibit antibacterial activity against various bacteria, fungi, and yeast (14). Consequently, banana peels can serve as a valuable resource for producing antibiotics (14), activated carbon, and a source of minerals (15).

The synthesis of Nanoparticles using plant and fruit extracts is an environmental-friendly and cost-effective approach, particularly with non-recycled food waste. Extensive research has been conducted on Nanoparticles synthesis using various plant and fruit sources, including apple, banana, pumpkin, kiwi, lemon, mango, mangosteen, potato, starfruit, avocado, carrot, watermelon, dragon fruit, lychee, rambutan, passion fruit, beetroot, pomegranate, pineapple, and orange (16).

Endophytes represent a unique group of nonpathogenic microorganisms that reside within the internal tissues of plants (17). They can be classified into endophytic fungi and endophytic bacteria (18). Endophytes play a crucial role in enhancing plant growth and resistance by participating in essential processes such as nutrient mobilization and phytohormone production, thereby improving plant tolerance and survival under stressful conditions (19).

The objective of this study is to investigate the response of *Artemisia annua*, particularly in terms of artemisinin production, to the application of banana peel Nanoparticles with or without specific endophytic bacteria.

2- Materials and Methods

2.1 Plant material

*Artemisia annua*seeds were kindly obtained from faculty of pharmacy, Cairo university while, endophytic *Kocuria artinae* bacterium isolate was kindly provided by Prof. Raslan who was previously identified (20). It belongs to the phylum Actinobacteria, class Actinobacteria, order Actinomycetales, suborder Micrococcinae, and family Micrococcaceae.

2.2 Preparation of banana peels Nanoparticles(BPNP)

The process involved in this study, as depicted in Fig(1), entailed the collection of banana peels, which were subsequently subjected to a thorough washing using distilled water, followed by the cutting of these peels into smaller fragments. These fragments were then subjected to drying in an oven at a temperature of 70°C for a duration of 48 hours. Upon completion of the drying process, the dried peels were subjected to grinding using a micro grinder ball mill, as per the specifications outlined in Table 1, for a period of 48 hours. It is worth noting that the grinding of the peels was conducted for a duration of 48 hours, as indicated in references (21) To obtain a desirable particle size, the ground peels were subsequently sieved, ensuring that the resulting particles were smaller than 150 µm. Finally, the sieved peels were stored at room temperature Fig (2).

Fig (2): Preparation of banana peels Nanoparticles(BPNP).

Condition	Description	Condition	Description
Time	48h	Ball/precipitate mass ratio	8.1 mass ratio
Vessel size	7.5 cm diameter	Speed	250 rpm
Balls diameters	ranged from 1.11 to 1.75 cm diameter	Materials of balls	Porcelain
Materials of vessels	St. steel	Temp	60°C

Table (1): Ball milling specification conditions.

2.3. Characterizationof banana peelsNanoparticles

Banana peel Nano particles (BPNP) were thoroughly and comprehensively analysed and examined through the utilization of Fourier-transform infrared spectroscopy (FT-IR), ZETA potential analysis, scanning electron microscopy (SEM), as well as energy-dispersive X-ray spectroscopy (EDS) and X-ray fluorescence spectroscopy (XRF) techniques in order to achieve a comprehensive understanding and characterization of their properties and attributes. (22)

2.3.1 Fourier transform infrared spectroscopy (FTIR)

By means of employing the Bruker Tensor 27 FTIR spectroscope, equipped with the Spectrometer Power 85 – 265VAC, 45 – 67 Hz, 70 W, as well as the DigiTectTM detector system, which possesses a High sensitivity DLATGS and an optional LN2cooled MCT detector, thereby allowing for convenient exchange, as illustrated in the detection of active compounds in banana peel, both before and after milling, was successfully carried out.

2.3.2 ZETA Potential

The zeta potential of banana peel Nanoparticleswas measured using a Zetasizer Nano-ZS (Malvern Instruments, UK).

2.3.3 XRF

The examination was conducted utilizing the X-ray fluorescence (XRF) method, employing the s6 JAGUA Bruker AXS-WD model. The instrument possessed High Sense Power with Helium purge, a Radium tube, and was operated at 400 w /50 kV and 17 mA. The light was analyzed using four crystals analyze light, a Zenon detector, and the High Sense feature. The quantity of the sample was 7gm, which was placed in sample cups with a diameter of 36 mm. The residue remaining after heating at 600ºC was subjected to X-ray fluorescence analysis to identify the inorganic materials.

2.3.4 Scanning Electron Microscopy (SEM /EDX).

The evaluation of the morphologies, microstructure, and Element composition of the prepared sample was conducted through the utilization of a JSM-IT639 scanning electron microscope (FESEM, JEOL JSM-6390), whereby the recorded data pertaining to these characteristics were meticulously recorded and analyzed.

2.4 Bacterial growth condition

Endophytic *Kocuria artinae* bacterium isolate was grown in a tryptic soy broth at 30°C to be used. It is an aerobic, gram-positive, pale yellow in color, round, smooth, and opaque bacteria.

2.5 Experimental Design

Seeds of *Artemisia annua* were divided into four groups and each group was prepared to be soaked in, distilled water, in Nanoparticles dissolved in distilled water,in nanoparticles dissolved in the free bacterial medium, Nanoparticles with endophytic bacteria dissolved in water, and Nanoparticles and endophytic bacterium grown in bacterial medium, respectively. Each group was sub divided into three subgroups to be soaked for 4 - 8- and 16- hours, respectively. Each subgroup consisted of 5 replicas thenThe cultivation took place for 6 months.

2.6 Plant vigour measurement

To estimate plant growth and to identify its vigour, as a preparatory and simple indicator, plant height, fresh and dry weights were measured after 3-monthes.

2.7 Metabolomics profiling of *Artemisia annua*

2.7.1 Sample Preparation

The air-dried powder of *Artemisiaannua*aerial parts (5 gm) were extracted with 70% ethanol (3 x 50 ml) and the ethanolic extract was filtered and concentrated under reduced pressure at 45 ºC to yield (1.2 g) dry residue. The dried ethanolic extract of *Artemisiaannua* was then suspended in 30 ml distilled water and successively fractionated with *n*-Hexane (3 x 30 ml), which was further filtered and evaporated using rotary evaporator at 45 ºC, then the extract was collected and stored at a low temperature.

2.7.2 LC-MS Analysis of *Artemisia annua*n-hexane extract:

The metabolomics profiling was performed on n-hexane extract according to Abdelmohsen et al on the QTrap sciex 6500 plus system. Chromatographic separation was carried out on C18 column (50 ×2.1 mm, 1.7 μm, Phenomenex Inc., Torrance, CA, USA) and a gradient binary solvent system water (0.1%formic acid) and acetonitrile (mobile phase B) at flow rate 1.2 mL/min. The injection volume was 2 µL, and the post running time was 5 min. The gases

for the MS system were supplied by a Genius 1024 gas gen- erator (Peak Sci., Billerica, MA, USA). Data were processed with Analyst 1.6.3 (Sciex Inc, Redwood City, CA, USA).

The electrospray mass spectrometer (ESI-MS) was operated under positive ion mode and an optimized collision energy level of 80%, scanned from m/z 100 to 600 to get the total ion chromatogram and the MS spectrum of artemisinin for qualitative identification. High-purity nitrogen (99.999%) was used as dry gas, the flow rate was 12 mL/min, the capillary temperature was 350 °C, and helium was used as nebulizer at 60 psi. For quantitative analysis of artemisinin, SIM was used to record the abundance of the [M - H2O + H]+ molecular ion peaks at m/z 265.3 for artemisinin (the scan range was between m/z 250 and 270 instead of between 100 and 600). Quantification was based on the LC-MS peak area of artemisinin, and standard curve was used for calculation.

2.8 Gene expression of artemisinin biosynthesis pathway

For detection of the gene expression of artemisinin biosynthesis pathway, 6 related genes in this pathway and their primer sequences were selected for this step (Table 2).

2.8.1 Total RNA was isolated with TRIzol RNA lysis buffer (Qiagen, Hilden, Germany). RNA purity and concentration were determined by using a NanoDrop™2000/2000c spectrophotometer (Thermo Fisher, Waltham, MA, USA).

2.8.2 The cDNA synthesis was obtained by reverse transcription of1μg RNA using an RT-qPCRkit (Takara Biotechnology Co. Ltd., Dalian, China) according to the manufacturer' s instructions.

2.8.3 Real-time PCR was adjusted based on the following conditions:10 μL2XSYBRGreenmix (Qiagen GmbH, Hilden, Germany),1μLprimers (F and R),and 8μL double distilled water. After denaturation at 95°C, 45 PCR cycles were performed using Step one real time PCR machine (ABI Corporation, Lee's Summit, MO, USA) at 95°C for 10 sec, 55°C for 40 sec, and 72°C for 25 sec. The samples were set in triplicates and the genes were normalized to 18s as a housekeeping gene Table (2) and the results were calculated using $2^{\Delta\Delta}$CT equation.

($^{\Delta\Delta}$CT = ΔCT test sample – ΔCT calibrator sample)
where ΔCT = CT target – CT reference.

Sequence	Primer name
TGCTGGTTCTCTTGGTGGAT	HMGR forward
CTCCAACTGTGCCAACCTCT	HMGR reverse
GAACTCGCCAATGAGGAACA	FDS forward
TTTCAGCACCGCTTGGACT	FDS reverse
TGTCAATGAGGAGTATGCCC	ADS forward
GTCTCCCATACGTGTGAAGT	ADS reverse
CTCCACTACCCTTGGTTCTG	CYP71AV1 forward
TCGTATTCTGCACCCATGAC	CYP71AV1 reverse
GCTCGGAACAGCCATCTTAT	CPR forward
CCGAAGCCTTCTGAGTCATC	CPR reverse
CCAATGGAAGTGAGGAGGAA	DBR2 forward
CAAGGTCAGGATTCGAGACA	DBR2 reverse
GCAACAAACCCCGACTTCTG	18s r RNA forward
TGCGATCCGTCGAGTTATCA	18s r RNA reverse

Table (2): Primer sequences designed for real-time amplification of artemisinin biosynthetic genes of *Artemisia annua* (23).

2.9 Statistical analysis

The data were analyzed using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 26; Inc., Chicago. IL). Descriptive data were expressed as mean μ and standard deviation (SD). One way analysis of Variance (ANOVA) test used for comparison between three or more groups having quantitative normally distributed data with Duncan's new multiple range tests at $p \leq 0.05$ level of significance. Two-way ANOVA test conducted for growth criteria as dependent factors estimated for type and time of treatment.

3- Results

3.1 Nanoparticles impact

3.1.1 FT IR (Fourier transform infrared)

The FT-IR spectra of banana peels (fresh peels without draying and ball milling "Before"), and "after" drying and 48 milling time are shown in Fig (3). FT-IR spectra of Nanoparticles derived from the peel of a banana were acquired to authenticate the presence of specific functional groups within Nanoparticles samples of banana peel. This was done

in comparison to the functional groups discovered in the banana peel powder of the control group, which can be observed in Fig(3). The absorbance spectra were assessed within the infrared range, spanning from 4000 to 600 cm^{-1}. This range exhibited numerous peaks, signifying the intricate composition of the substance being absorbed. Notably, there were peaks detected at 3327, 2937, 2852, 1626, 1559, 1373, 1369, 1019, and 789 cm^{-1}, as illustrated in Figure (3).

The bands at 3327-2937 cm-1 are due to O-H, while the C-H group is identified at 2937-2852 cm^{-1}. The band at 1626 cm-1 is attributed to the C=O bond of carboxylic acid, The band at 789 cm-1 is attributed to an amine (24).

The wavelength of O-H band becomes lower as the milling progresses suggesting that this phase might be sensitive to mechanical stress and the water release through drying processes. Also disappearing in C-H group and elongation in C=O and amine groups (25).

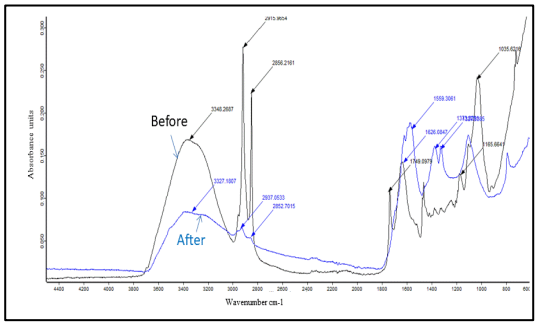


Fig (3): Fourier transform infrared spectroscopy (FTIR) wavenumber vs. absorbance units of functional groups present in banana peel. FTIR spectra was in wavelength region 4000-500 cm^{-1}.

3.1.2 Zeta potential values

The Zeta potential values have provided valuable insights into the surface charge and stability of the Nanoparticles derived from synthesized banana peels.

The zeta potential value of the Nanoparticles obtained from Banana peel, as depicted in Fig (4), stands at -39.5mV, thus indicating the inherent stability of the synthesized Nano particles. the synthesized Nanoparticles ".

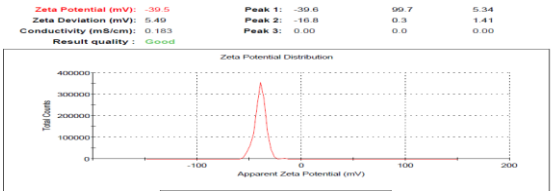


Fig (4): Zeta potential values for Banana Nano particles

3.1.3 XRF

The X-Ray Fluorescence (XRF) method exhibits remarkable precision and accuracy, enabling the identification of virtually any mineral content present in biological materials, thereby providing immediate results. The mineral composition of banana peel, as determined through the utilization of XRF, is presented in Table 3. Notably, the potassium content within the banana was found to be higher, while the sulfur content was comparatively lower. Specifically, the mineral content of potassium (K), chlorine (Cl), calcium (Ca), silicon (Si), phosphorus (P), iron (Fe), magnesium (Mg), and sulfur (S) in dry banana peels were measured as 69.86 mg/100g, 8.73 mg/100g, 7.98 mg/100g, 4.10 mg/100g, 2.11 mg/100g, 1.97 mg/100g, 1.45 mg/100g, and 0.66 mg/100g, respectively. These micro minerals serve the purpose of facilitating metabolic processes within the plant. (26).Table (3).

Element	K	Cl	Ca	P	Si	Mg	S	Fe
%	69.86	8.73	7.98	2.11	4.10	1.45	0.66	1.97

Table (3): X-Ray Fluorescence Analysis of the Main Chemical Elements Detected in Nanoparticles of banana peel

3.1.4 EDX SPECTRA of banana peel Nano particles

The elemental compositions of banana peel were determined using EDX analysis (Figure 4, 5 and Table 4). Banana peel Nano particles contained the primary elements C and O, as well as trace elements, K, and Si, Ca. Fig (5 & 6).

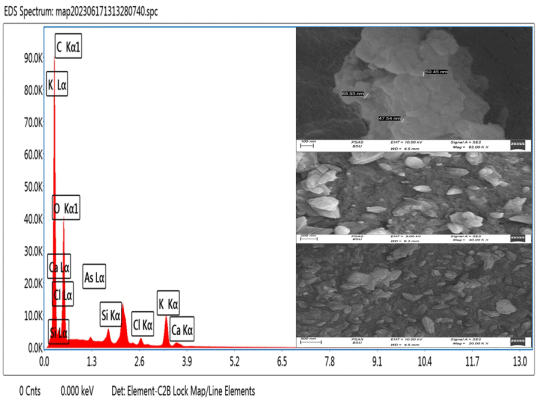


Fig (5)**:** EDX & SEM analysis of banana peel Nano particles.

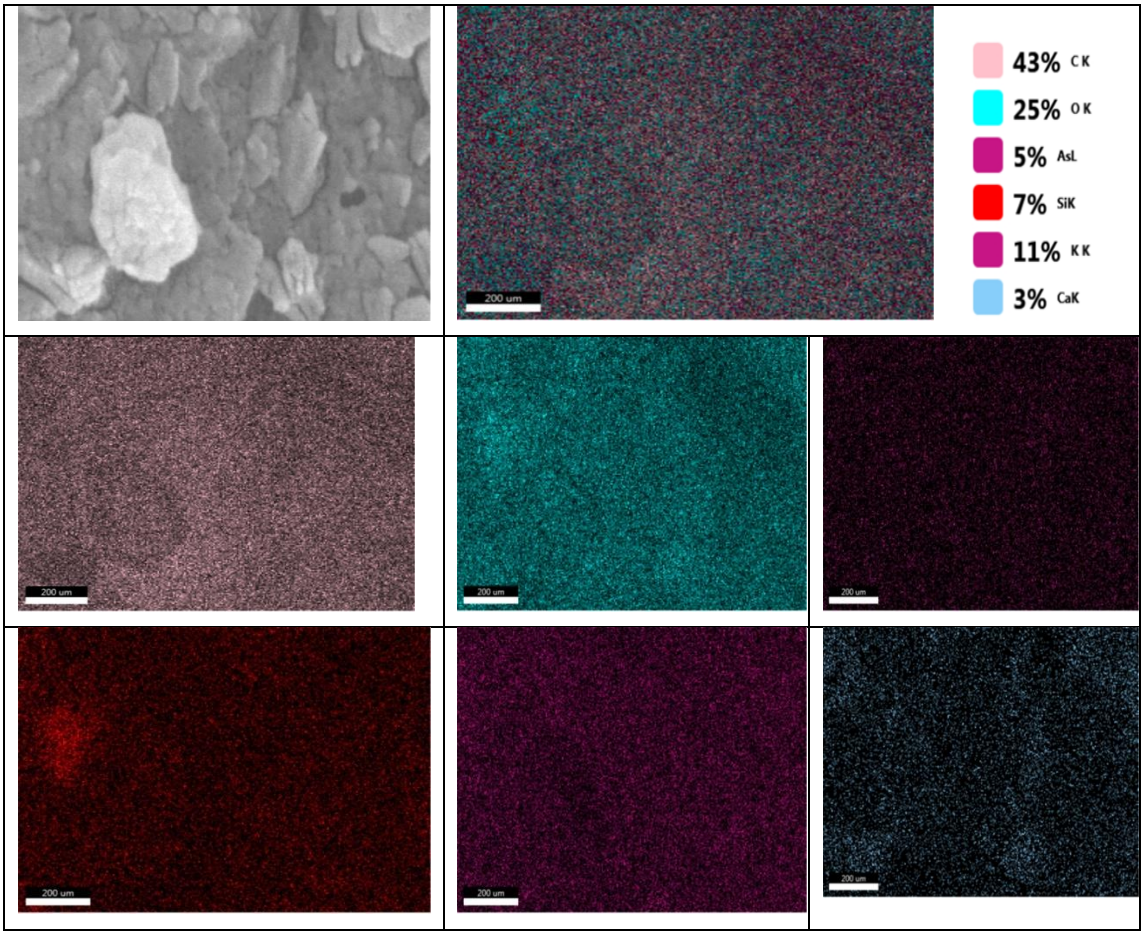


Fig (6): EDX for BPNP.

3.1.5 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was employed to conduct an analysis on the banana peels to examine the morphology of the aforementioned peels' Nanoparticles. The image depicted in Fig(7) reveals that the banana peels Nanoparticles (BPNP) is comprised of materials arranged in layers that are grouped together. Furthermore, the particles observed in the image were measured and found to possess an equivalent spherical diameter of 50.45, 47.54, and 68.93 nanometers, respectively. It is noteworthy that all the images exhibit a porous structure, rendering them highly susceptible to the adsorption of metals on their surfaces Fig (5).

3.2 Growth criteria of *Artemisia annua*plant.

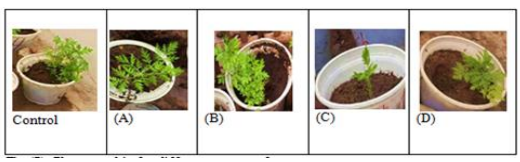


Fig (7): Photographic for different groups of treatment.

The impact of the different applications of banana Nanoparticles on *Artemisia annua* Fig (7)growth was tabulated in Table 4. Plant heights, plant fresh weight and plant dry weight as well as artemisinin as percentage of control, at level of significance $p \leq 0.01$, improved significantly all these parameters (Table 4-a) and the highest response was after 8-hours soaking in banana Nanoparticles supplied in the medium containing the used bacteria. Moreover, the impact of the different applied treatments, at level of significance $p \leq 0.05$, on all investigated growth parameter was positively affected (Table 4-b), and banana Nanoparticles in the medium containing the used bacteria recorded the best data for all parameters. In addition, the impact of the different times of the treatments was also significantly activated (Table 4-c) and the optimum application time was at 8-hours. The overall interaction between time of soaking duration and different application types of Nanoparticles was significantly corelated except the interaction impact of artemisinin parameter (Table 4-d).

Group	Type of treatment	Time of treatment (h)	Plant height (cm)	Fresh weight (g)	DRY weight (g)	Artemisinin as % of control
Control	Normal seeds	------	17 ± 0.17^{a}	14.2 ± 0.17^{a}	1.39 ± 0.017^{a}	100^{a}
A	BP NP in water	4h	$17.9\pm0.17^{a, b}$	$14.8 \pm 0.10^{b,c}$	1.45 ± 0.005^{c}	$806 \pm 5.2^{b, c}$
		8h	18.2 ± 0.34^{b}	15.1 ± 0.10^{c}	1.47 ± 0.005^{c}	$845 \pm 5.2^{b,c}$
		16 h	$17.5\pm 55.0^{a,b}$	$14.4 \pm 0.52^{a,b}$	1.42 ± 0.01^{b}	756 ± 5.2^{b}
B	BP NP in media	4h	21.5 ± 0.43^{e}	17.6 ± 0.35^{e}	1.72 ± 0.005^{f}	$1088\pm 7.0^{c, d}$
		8h	23.3 ± 0.91^{f}	19.0 ± 0.30^{f}	1.88 ± 0.01^{g}	$1177\pm 8.7^{d, e}$
		16h	19.2 ± 0.1^{d}	15.3 ± 0.47^{c}	1.55 ± 0.005^{d}	$1001\pm4.3^{b,c,d}$
C	BPNP with E in water	4h	22.9 ± 0.4^{f}	17.9 ± 0.60^{e}	1.86 ± 0.07^{g}	$1608\pm 5.1^{f, g}$
		8h	$24.2 \pm 0.20^{g, h}$	19.8 ± 0.15^{g}	1.95 ± 0.02^{h}	1725 ± 7.8^{g}
		16h	20.1 ± 0.70^{c}	16.1 ± 0.11^{d}	1.66 ± 0.005^{e}	$1381\pm 3.6^{e, f}$
D	BPNP with E in media	4h	24.6 ± 0.36^{h}	20.5 ± 0.20^{h}	2.01 ± 0.1^{i}	2817 ± 10.5^{h}
		8h	26.8 ± 0.36^{i}	22.4 ± 0.20^{i}	2.17 ± 0.02^{j}	3330 ± 10.8^{i}
		16h	23.4 ± 1.01^{g}	19.8 ± 0.26^{g}	1.92 ± 0.02^{h}	2921 ± 587.7^{h}

Table (4-a): Growth characteristics of Artemisia annua after treatment for 3 months. One way ANOVA test for growth criteria with Duncan's new multiple range tests at $p \leq 0.01$ level of significance

Parameters	Treatment	Mean ± Std. Deviation
Plant height (cm)	Control	17.00 ± 0.173^{a}
	BP NP in water	17.85 ± 0.463^{a}
	BP NP in media	21.33 ± 1.851^{b}
	BPNP with E in water	22.40 ± 1.834^{b}
	BPNP with E in media	24.94 ± 1.58^{c}
Fresh weight (g)	Control	14.20 ± 0.17321^{a}
	BP NP in water	14.76 ± 0.409^{a}
	BP NP in media	17.28 ± 1.677^{b}
	BPNP with E in water	17.83 ± 1.671^{b}
	BPNP with E in media	20.91 ± 1.177^{c}
DRY weight (g)	Control	1.39 ± 0.017^{a}
	BP NP in water	1.44 ± 0.022^{a}
	BP NP in media	1.71 ± 0.14^{b}
	BPNP with E in water	1.83 ± 0.13^{b}
	BPNP with E in media	2.03 ± 0.112^{c}
Artemisinin as % of control	Control	100^{a}
	BP NP in water	802.33 ± 38.90^{b}
	BP NP in media	1088.66 ± 76.44^{c}
	BPNP with E in water	1571.33 ± 151.55^{d}
	BPNP with E in media	3022.77 ± 376.23^{e}

Table (4-b): Growth characteristics of *Artemisia annua* after treatment for 3 months. One way ANOVA test for growth criteria among types of treatment with Duncan's new multiple range tests at $p \leq 0.05$ level of significance.

Parameters	Time of Treatment	Mean ± Std. Deviation
Plant height (cm)	0h	17.00 ± 0.17[a]
	4h	21.72 ± 2.59[b]
	8h	23.11 ± 3.28[b]
	16h	20.05 ± 2.33[b]
Fresh weight (g)	0h	14.20 ± 0.17[a]
	4h	17.62 ± 2.14 [b,c]
	8h	19.09 ± 2.74[c]
	16h	16.38 ± 2.175 [a,b]
DRY weight (g)	0h	1.39 ± 0.017[a]
	4h	1.76 ± 0.22[b]
	8h	1.86 ± 0.26[b]
	16h	1.63 ± 0.19[b]
Artemisinin as % of control	0h	100[a]
	4h	1579.75 ± 804.34[b]
	8h	1769.25 ± 996.77[b]
	16h	1514.83 ± 914.48[b]

Table (4-c): Growth characteristics of *Artemisia annua* after treatment for 3 months. One way ANOVA test for growth criteria among time of treatment with Duncan's new multiple range tests at $p \leq 0.05$ level of significance.

Parameters	Type of treatment	Time of treatment	Type of treatment * Time of treatment
Plant height (cm)	77.739**	28.136**	2.158**
Fresh weight (g)	57.307**	22.056**	1.761**
DRY weight (g)	0.547**	0.163**	0.014**
Artemisinin as % of control	8763123.8**	209704.5**	43935.9[ns]

Table (4-d): Growth characteristics of *Artemisia annua* after treatment for 3 months. Two-way ANOVA of growth criteria as dependent factors estimated for type and time of treatment. **P < 0.01; *P < 0.05; ns, non-significant

This Table presents experimental data on different groups from treatments, the timing of treatments, and various measurements related to plant growth like height and weight (dry and wet) and artemisinin content Fig (8).

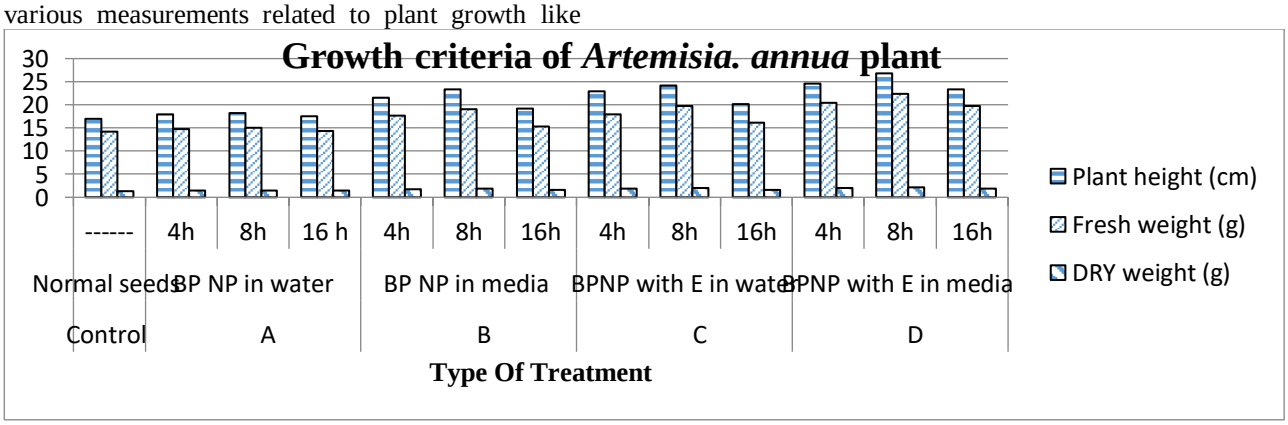


Fig (8): Growth criteria of *Artemisia annua* plant.

3.4 Gene Expression of artemisinin biosynthetic

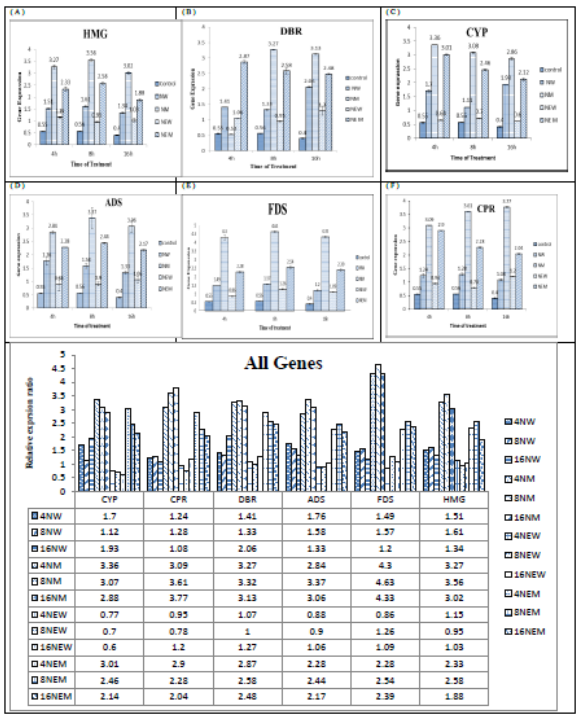
The expression of key genes in the artemisinin biosynthetic pathway is regulated by several factors, including the presence of artemisinin, the developmental stage of the plant, and the species of Artemisia.

The expression of these genes can be manipulated to increase the production of artemisinin in plants. This knowledge could be used to engineer plants to produce higher levels of artemisinin, which could help to combat the spread of malaria.

The expression of artemisinin biosynthesis genes varied depending on the species, environmental factors, and the developmental stage of the plant.

The expression of key genes affecting the content of artemisinin in five different species of Artemisia was correlated with the artemisinin content in the plants.

Expression of artemisinin biosynthetic pathway genes including HMG, DBR, CYP, ADS, FDS, and CPR by the quantitative real-time PCR technique.



	CYP	CPR	DBR	ADS	FDS	HMG
4NW	1.7	1.24	1.41	1.76	1.49	1.51
8NW	1.12	1.28	1.33	1.58	1.57	1.61
16NW	1.93	1.08	2.06	1.33	1.2	1.34
4NM	3.36	3.09	3.27	2.84	4.3	3.27
8NM	3.07	3.61	3.32	3.37	4.63	3.56
16NM	2.88	3.77	3.13	3.06	4.33	3.02
4NEW	0.77	0.95	1.07	0.88	0.86	1.15
8NEW	0.7	0.78	1	0.9	1.26	0.95
16NEW	0.6	1.2	1.27	1.06	1.09	1.03
4NEM	3.01	2.9	2.87	2.28	2.28	2.33
8NEM	2.46	2.28	2.58	2.44	2.54	2.58
16NEM	2.14	2.04	2.48	2.17	2.39	1.88

Fig (9): The expression of HMG (A), DBR(B), CYP(C), ADS(D), FDS(E), CPR(F) in quantitative real time PCR analysis at different treatment of Nano and endophyte.

3.4.1 HMG gene expression.

HMGR is one of the key regulatory enzymes involved in shunting HMG-CoA into the mevalonate arm of the isoprenoid pathway.

As shown in Fig(9A), HMG gene expression revealed the maximum level expression at media in 8h,4h,16h respectively was 6.3 ,5.9 ,7.55 fold compared to the control. about 4.6-fold when adding the bacteria to the nanoparticle mix in the media in the, but in case of culturing in water increase is not remarkable slightly increase and Nano in water 8h, 4h ,16h was 2.3 ,1.9 ,2.5 fold.

3.4.2 DBR genes expression

DBR genes expressions Fig(9B) ,revealed the maximum expression at Nano banana with endophyte in media in 8h,16 was about 5 folds, the highest increase was in the 4h (about 5.7 folds), then in Nano in media was the maximum after 8h then 16h (about 5.8-folds). The Nano water was after 16 h give the maximum expression (about 3.6 folds)

then (4h ,8h) about 2.5 folds. The only decrease in genes was in banana Nano in media at 4h after treatment.

3.4.3 CYP genes expression

As shown in Fig (9C), the CYP gene expression increased in all treatments. The maximum expression was observed at media after 4h (6-fold) and then 8h (5.6-fold) of Nano was the best than 4h Nano with endophyte in media was (5.4-fold). The increase in Nano with media is about 6 fold compared to the control and about 5.4 fold when adding the bacteria to the Nanoparticles mix in the media in the first 4 hours and nearby the same levels in the 8h was 5.6 and 16 h was 5.2 fold , also in case of culturing in water the decrease was in Nano endophyte in water not remarkable in the first 4,8,16 hour but slightly increase in case of Nano banana waste only at the first 4,8,h was around 3 folds, and in 16 h was about 4.9 folds .

3.4.4 ADS genes expression

The ADS genes expressions revealed the maximum expression at media with Nano banana wastes in 8hrs, about 7.4folds 16hrs about 6.12 folds and 4hrs about 5.68 folds.

In Nano with endophyte in media the expression in 8h,4h,16h was (4.56, 4.88 and 5.4 folds respectively). the Nano banana waste in water give expression (4h, 8h, 16h) (about 3.2, 2.8 and 2.4 folds respectively) Fig (9D).

3.4.5 FDS genes expression

FDS genes expressions revealed the maximum expression at Nano banana in media in 8h was about 9 folds and after 4h,16h was 8.6-fold, then in Nano in media was the maximum after 8h then 16h ,4h (about 4.5, 5.9 ,4.5 folds). The Nano water was after 4 h give the maximum expression (about 2.8 folds) then (4h ,16h) about 3 folds. Nano with endophyte in water increase is not remarkable Fig (9E).

3.4.6 CPR genes expression

As shown in Fig (9F), the CPR gene expression was the maximum expression observed at media in 16h,8h,4h (about 8.5, 6.5 and 5.6-folds) while Nano with Endophyte was at 4h,8h,16h the expression was (about 5.2, 4.14 and5.1 folds). In the case of culturing in water in the first 4,8, hour approximately 2.5-fold and in case of Nano with endophyte in water increase is not remarkable in the first 4 ,8 hour slightly increases but after 16 h 3-fold. Fig (9F).

In this work, the results showed that different sources of environmental factors can affect the production of artemisinin product by affecting the gene expression of specific artemisinin biosynthesis pathway genes.

4- Discussion

Plant growth and its nutritional and bioactive contents are the net result of its vital activities done under certain growth conditions. In this work, the estimated growth parameters were plant height, plant fresh weight and plant dray weight, while artemisinin was the guide of its bioactive content, and they were improved. As plants were supplied -through application of banana peels- with many necessary nutritional elements mainly nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, as well as micronutrients iron, manganese, zinc, chloride, silicon, and molybdenum (27). The beneficial impact of these elements was interpreted to the improvement occurred in their estimated scales and this impact may be due to the crucial role of these elements in photosynthesis, carbohydrate and protein biosynthesis, enzyme activity, and the biosynthesis and maintenance of cell organelles like chloroplast and membrane integrity (28). Moreover, banana peels supplied our investigated plant with some phenolic compounds (dopamine, rutin, ferulic acid, and proanthocyanidins) and some carotenoids (Alpha-carotene, beta-carotene, and lutein) (29). The enhancing effect of such phenolics and carotenoids may be ascribed to their antioxidative impact and their role in improving photosynthesis and plant anabolic activity (30 and 31).

It is worth mentioning that as these banana peels was applied in the form of Nanoparticles, so their impact was more efficient may be because of NPs not only have smaller surface area, more kinetic energy and faster absorption, but also, NNPs also shield the photosynthetic system and enhance photosynthetic efficiency, accretion of osmolytes, phenolics, antioxidant activities, hormones, and gene expression and this may be via gene expression patterns like transcriptome and proteome as well as related signaling and energy metabolism, levels of phytochelatins and metallothioneins (32).

The investigated improvement due to the application of endophytes can be discussed through many mechanisms. Endophytes take nutritive elements from the external growth medium then they delivery them to plants via symbioses causing more growth and well development of the plant, they resist oxidative stress of plant, and/or they shield plants against pathogenicity (33).

The more investigated improvement detected due to the combination between BPNPs and the nutritive elements, and between BPNPs and endophyte may be attributed to the synergism occurred between their all benefiting factors and this why the best result was obtained in the triple mixture od between BPNPs and endophyte within the nutritive medium.

The biosynthesis of Artemisinin Fig(1) has been explored for many years. However, not every detail about the regulation and biosynthesis is completely understood, but the discovery that the whole biosynthesis is in the glandular trichomes of *Artemisia annua* has facilitated in-depth regulatory studies (34).

Derived from the general terpenoid biosynthesis, two molecules of isopentenyl diphosphate (IPP) and one dimethylallyl diphosphate (DMAPP) are condensed by farnesyl diphosphate synthase (FPPS/FPS) into farnesyl diphosphate (FPP, farnesyl pyrophosphate), the C15 sesquiterpenoid precursor (35).

In the following two oxidization steps, amorpha-4,11-diene is hydroxylated into artemisinic alcohol and oxidized to artemisinic aldehyde by amorphadiene monooxygenase (CYP71AV1), a cytochrome P450 enzyme (36). Overexpression of FPS in *Artemisia annua* resulted in an increase of artemisinin production (37) which confirms the role of FPS and availability of the substrates in the regulation of artemisinin biosynthesis similar to other sesquiterpene lactones (38). FPP is converted to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) via carbocation formation and cyclization (39). The activity of CYP71AV1 has also been confirmed through a knock-out of the endogenous gene in *Artemisia annua* showing that these plants do not produce any downstream products of amorphadiene (40).

It has later been discovered that the alcohol dehydrogenaseADH1, (a dehydrogenase/reductase enzyme) is specific toward artemisinin alcohol and oxidizes this to the aldehyde. This specificity and strong expression in A. annua glandular trichomes confirm that ADH1 is responsible for oxidation of artemisinic alcohol to artemisinic aldehyde(41). Artemisinic aldehyde is further reduced to dihydroartemisinic aldehyde by artemisinic aldehyde Δ 11 (13) reductase (DBR2) and subsequently oxidized to dihydroartemisinic acid by aldehyde dehydrogenase (ALDH1), which is also expressed in the trichomes (42). Besides catalyzing the oxidation of dihydroartemisinic aldehyde to the acid, ALDH1 also catalyzes the oxidation of artemisinic aldehyde to artemisinic acid (a reaction that in yeast is catalyzed by CYP71AV1) (43).

Plants treated with MeJA (methyl jasmonate) showed only slight changes in the transcription levels of these genes compared to the control. HMGR gene expression decreased 1.5-fold at 24 h, and then increased 3-fold by the 48-h mark. It then decreased again 1.3-fold by 72 h after treatment. FDS gene expression increased 3-fold at 24 h after application, although by 48 h this dropped to an increase of only 1.5-fold over the controls. ADS transcript levels also decreased approximately 4.2-, 3.5-, and1.7- fold, respectively, 12, 24, and 72 h after MeJA application. CYP71AV1 showed a decrease at 12 h, and CPR showed a slight increase at 24 and 72 h posttreatment. DBR2 had 2 decreases, 3- and 2-fold, at 12 and 72 h, respectively, and 2 increases of 2-fold at 48 and 168 h after treatment.(44)

The transcript levels of genes in plants exposed to 2-iP (2-isopentenyladenine) were more variable. At 12 h, expression levels of HMGR,

FDS, and DBR2 increased approximately 8-, 6.5-, and 5.5-fold relative to the control, but CYP71AV1 decreased 4-fold. At 24 h, transcript levels of ADS, CYP71AV1, and DBR2 decreased 4.5-, 6-, and 7-fold, respectively, and CPR increased 2-fold. At 48 h, HMGR, ADS, and CYP71AV1 decreased 1.5-, 7.5-, and 5-fold, respectively. In contrast, gene expression levels for CPR and DBR2 increased 1.7- and 4.5-fold, respectively. At 72 h all of the studied genes increased between 2- and 17-fold. At the final measurement at 168 h, ADS and DBR2 weredownregulated 4- and 2.5-fold, respectively, and FDS was upregulated 3.5-fold.(44)

5- Conclusion

According to the present work, it can be concluded that the application of banana peels nanoparticles is good to enhance the growth of *Artemisia annua* and this application is methodically correlated with the experimental conditions. The obtained results proved that the optimum time of soaking was after 8-hours, and the optimum application was by providing these Nanoparticles within the endophytic *Kocuria artinae* bacterium grown in the bacterial medium.

Additionally, it can be concluded that the impact of this application was positive and its mode of action has multimechanisms which confirms that there are many genes acting coordinated to achieve their target.

Abbreviations

ADS (Amorpha-4,11-diene synthase). BPNP (banana peels Nanoparticles). Cpr (cytochrome P450 reductase).CYP71AV1(Cytochrome P450 monooxygenase).DBR2 (double bond reductase 2).Fds (farnesyl diphosphate synthase).Hmg (3-Hydroxy-3-Methylglutaryl-CoA Reductase).qPCR (Quantitative Real-time PCR).

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