CHITOSAN NANOPARTICLES AND ITS IMPACT ON GROWTH, YIELD, SOME BIOCHEMICAL AND MOLECULAR MARKERS IN *SILYBIUM MARIANUM*

Asmaa A. Mahdi[1*], Hamdi M. Ibrahim[1], Khaled Y. Farroh[2], Emad S. Hamed[3] and Omran Ghaly[4, 5]

[1]Biochemistry Unit, Department of Genetic Resources, Desert Research Center (DRC), Cairo, Egypt

[2]Nanotechnology and Advanced Materials Central Lab, Agricultural Research Centre, Giza, Egypt

[3]Department of Medicinal and Aromatic Plants, Desert Research Center (DRC), Cairo, Egypt.

[4]The Herbarium, Desert Research Center (DRC), Cairo, Egypt

[5]Regional Development Centers (RDC), Academy of Scientific Research and Technology (ASRT)

[*]E-mail: asmaaabdelkader@rocketmail.com

A field experimentwashelddu ringtheseasons2019/2020 and 2020/2021 in the Regional Center for Conservation and Maintenance of Endangered Plant Genetic Resources at El-Hammam city, Matrouh Governorate, Egypt. This research was conducted on wild milk thistle (*Silybum marianum* var. *albiflorum*) cultivated and subjected to two precursors (phenylalanine and p-coumaric acid at 40 mg/L) and Chitosan nanoparticles treatments (Chs NPs 50 mg/L, Chs NPs 50 mg/L conjugated with phenylalanine 40 mg/L, Chs NPs 50 mg/L conjugated with p-coumaric acid 40 mg/L, Chs NPs 200 mg/L, Chs NPs 200 mg/L conjugated with phenylalanine 40 mg/L, and Chs NPs 200 mg/L conjugated with p-coumaric 40 mg/L) compared with the control (without the previous treatments). The study aimed to determine the effect of treatments on growth, yield, some biochemical and molecular markers. The DLS, zeta potential and X-ray diffraction were assigned for several fabricated Chs NPs. The significant highest increments in dry weight/plant, seeds weight/plant and seeds yield/feddan were obtained by phenylalanine 40 mg/L followed by Chs NPs 200 mg/L. Best biochemical markers were towards the same treatment. In the molecular level, there are no noticeable polymorphic changes in the plant genomic material with polymorphism average 59.83% for all treatments. The highest increases of total silymarin content in the plant seeds were detected by Chs NPs 200 mg/L, followed by phenylalanine 40 mg/L treatments. Concerning quantity and quality estimates, application of plants with engineered Chs nanoparticles

200 mg/L, which also has the maximum antioxidant capacity and lowest toxic product malondialdehyde at the vegetative growth period to have the highest specifications and profit is recommended.

Keywords: chitosan nanoparticles, antioxidant capacity, peroxidase, DNA ISSR, seed yield, silymarin

INTRODUCTION

Cultivation of wild medicinal plants in Egypt is currently considered a strategy and action plan for biodiversity conservation for the following essential reasons. Wild medicinal plants are considered a rich source for many pharmaceuticals, so they must be propagated and cultivated. Wild medicinal plants receive increased commercial attention raising pressure on wild populations. Overharvesting and urban expansion have placed many species at risk of extinction. Overgrazing will lead to the disappearance of many plants. The climate change problem will negatively affect their distribution. Collecting wild plants that grow in different areas is difficult. Besides, the fact that wild medicinal plants have low water irrigation requirements advantages thus, it is suitable for planting in our newly reclaimed desert lands with existing limited irrigation resources (Roberson, 2008; Ministry of Environment, 2016 and Kaky and Gilbert, 2017).

Silybum marianum (L.) Gaertn. var. *albiflorum* Eig, (Family: Asteraceae), also called milk thistle, is a spiny biennial plant that grows wild in Egypt. It is also native to other Mediterranean basin countries. The basal plant leaves are alternate, large, and glabrous with spiny margins. Leaves of well-developed plants are commonly 50-60 cm long, 20-30 cm wide, and have typical white veins. The seeds are 5-8 mm long, formed at the center of a spiny white inflorescence head about 5 cm in diameter. The plant seeds' primary active ingredient is silymarin, the most frequently applied herbal remedy for liver disorders. *S. marianum* supplements are now accessible in the form of capsules, powders, and extracts. The active ingredient in this plant is silymarin. The extract made from the plant's seeds contains three isomers flavonolignans (silybin, silydianin, and silychristine). Silybin, which accounts for 50-70% of silymarin, is the component with the most biological activity. Silymarin works as an antioxidant by lowering the formation of free radicals and lipid peroxidation. Silymarin is a compound that has been used to treat alcoholic liver disease, viral hepatitis, and toxin-induced liver disorders, and it may be helpful in the treatment of hepatocellular carcinoma (Gresta et al., 2007; Karkanis et al., 2011; Keasar et al., 2016; Abenavoli and Milic, 2017 and Marmouzi et al., 2021).

Nowadays, to increase the secondary metabolites in medicinal plants by the use of biological compounds has drawn much attention. Through this, chitosan (Chs) is a naturally biopolymer derived from chitins that can be used in agriculture as a biostimulant and growth promoter. It is non-toxic,

biodegradable, and biocompatible, making it suitable for a vast array of applications. As a source of Chs, chitin is the second most abundant naturally occurring polysaccharide on the world after cellulose (Rinaudo, 2006). Chitin and cellulose are present in plant cell walls, have molecular similarities such as a linear polysaccharide chain and are neutrally charged (Kurita, 2006). Structurally, chitin is composed of repeating unit of saccharide monomer of *N*-acetylglucosamine, while cellulose consists of a linear chain of several hundred to thousands of β linked D-glucose units. After deacetylation of chitin, Chs is obtained, which is composed of a linear polymer consisting of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine linked together by glycosidic bonds. The presence of this amine group facilitates structural modifications and synthesis of functional derivatives (Shamov et al., 2002).

Biostimulants are one approach for increasing the production and quality of various crops. Chs is the second most prevalent polysaccharide after cellulose, and it is formed by partial deacetylation of chitin (poly-N-acetyl-D-glucosamine) from crustacean shells. Each year, nature recycles an estimated 10 gigatons of chitin (Ruiz-Herrera et al., 2002). Kean and Thanou (2010) define Chs as a biodegradable, renewable polysaccharide that is typically regarded biocompatible and non-toxic.

It can improve physiological responsiveness and reduce the negative effects of different stressors. Chs treatments boosts antioxidant enzymes via nitric oxide and hydrogen peroxide signaling pathways, and induces production of organic acids, sugars, amino acids, and other metabolites that are essential for osmotic adjustment, stress signaling, and energy metabolism in stress. It has also been observed to form complexes heavy metals and used as tool for phytoremediation and bioremediation of soil (Hidangmayum et al., 2019).

Chitosan nanoparticles (Chs NPs) have risen to prominence owing to their unrivaled physical and chemical properties, such as length, diameter, atomic configurations, and operation, which enable them to have tensile strength, a wide range of conductivity, elasticity, and chemical reactivity (Jackson et al., 2013). Plants treated with low concentrations of nano-chitosan can have a positive impact on plant development, yield, and water transport within the plant, with no evidence of phytotoxicity, in contrast to the negative effects that high dosage tests can produce, as they breed harmful reactionary oxygen species (Mondal et al., 2011).

Aromatic amino acids like phenylalanine (Phe) are necessary components of proteins biosynthesis in all living organisms. In plants serve as a precursor for thousands of crucial and specialized metabolites such as silymarin. Approximately 20–30% of photosynthetically fixed carbon is devoted to phenylalanine synthesis leading to lignin, the key structural component of plant cell walls (Bonawitz and Chapple, 2010). Amino acids such as Phe are required to manufacture enzymes, proteins, and other nitrogen-containing molecules that are important for plant development and

defense. Amino acids also play a role in signaling pathways by functioning as signal molecules or regulating the conjugation of amino acids and phytohormones to alter hormone levels (Tegeder and Ward, 2012). Although, the fact that Phe is an essential component of plant growth and development. Phenylalanine is a precursor of phenylpropanoids, flavonoids, anthocyanins, lignin, tannins, and salicylate. All of which are important for plant growth and reproduction and defense against abiotic and biotic stressors. Phe is vital for lignin production during the development of wood in woody plants (Pascual et al., 2016). Phenylalanine ammonia-lyase (PAL) catalyzes the conversion of Phe to trans-cinnamic acid and ammonium in the vascular cells of woody plants, which are then re-used for amino acid biosynthesis (Shi et al., 2013).

Coumarins (Cou) are a type of lactone comprising a benzene ring fused to a pyrone ring, resulting in a conjugated system with electron-rich and strong charge-transport characteristics (Murray, 1997). Biogenetically, simple Cou are generated from shikimic acid via cinnamic acid. Cou inhibited peroxidase reactivation, increased superoxide dismutase activity, lowered the activities of metabolic resumption marker enzymes, and suppressed the transcription of molecular chaperons implicated in secretory pathways (Abenavoli et al., 2006).

The utilization of NPs in plant cells can produce genetic alterations that vary by plant species, NPs type, size, and concentration. Considering very little known about the genotoxicity of Chs NPs in plants, more work should be done to investigate plant genetic responses (Yang et al., 2018). Several DNA-based techniques have been successfully used to examine the genotoxic impact of NPs on various plant species, such as Inter Simple Sequence Repeats (ISSR) and Random Amplification of Polymorphic DNA (RAPD) techniques (Plaksenkova et al., 2019 and Mahdi et al., 2020). ISSR markers are constituted of microsatellite sequences that are highly variable and widely distributed throughout the genome; through it, genomic template stability could be calculated for each treatment.

The aim of this work is to get a better understanding of the traditional legacy, pharmacological benefits especially antioxidant activity, molecular fingerprint and genomic stability of wild *S. marianum* var. *albiflorum* plants' with Chs NPs separately with two concentrations or combined with the precursors Phe or p-coumaric acid to such NPs as nano-hybrids. By increasing the secondary metabolites biosynthesis and accumulation and increasing seeds yield after application of such NPs so that, can cover the market's requirements for the active ingredient's production and consumption instead of importing it and enriching the bioactive compounds with antioxidants.

MATERIALS AND METHODS

1. Plant Material Collection and Agriculture Procedures

Aerial parts and seeds of *S. marianum* var. *albiflorum* Eig, (Family: Asteraceae) were collected from their natural habitats in wadi Om Rakham, Northwetern coast of Egypt. The collected plant specimens were identified and authenticated by Dr. Omran Ghaly, Head of Plant Taxonomy Unit, Desert Research Center. The herbariam specimen was deposited in the herbarium of Desert Research Center (CAIH) with code number CAIH-1070- R and the collected plant seeds were prepared for cultivation. The field experiment was conducted during the two successive seasons of 2019/2020 and 2020/2021 in the Regional Center for Conservation and Maintenance of Endangered Plant Genetic Resources at El Hammam city (30° 50′ N and 29° 23′ E), Matrouh Governorate, Egypt - Academy of Scientific Research and Technology (ASRT). The soil and water irrigation analyses were performed according to Durak et al. (2010) for this area is presented in table (1-3).

Sheep manure at the rate of 10 m^3/feddan was added through soil preparation. Analysis of used organic fertilizer is shown in table (4). The seeds were sown on 26th October for both seasons under a drip irrigation system. The distances between hills were 50 cm and 75 cm between rows. The plants were thinned at one per hill (11200 plants/feddan, feddan = 4200 m^2). Humic acid and a biofertilizer mixture of three bacterial strains, namely *Azotobacter chroococcum*, *Azospirillum lipoferum*, and *Bacillus megatherium*, were added as a soil drench. Irrigation scheduling was followed and good agricultural practices instructions were applied. Some photos of the grown plants are presented in fig. (1).

2. Application of Treatments

The experiment was arranged in a randomized complete block design with three replications. The trial involved nine treatments. Application of the following plant biostimulators was done on 17th January during the vegetative growth period. Spraying was carried out on the leaves of plants until the surface run-off. The investigated treatments were as follows:

- Control: without nanoparticles and precursors
- Phe: 40 mg/L (Mahdi, 2011)
- Cou: 40 mg/L
- Chs NPs: 50 mg/L
- Chs NPs: 200 mg/L (Hassanen et al. 2021).
- Chs NPs conjugated with Phe 40 mg/L (Chs NPs 50 @ Phe): 50 mg/L
- Chs NPs conjugated with Cou 40 mg/L (Chs NPs 50 @ Cou): 50 mg/L
- Chs NPs conjugated with Phe 40 mg/L (Chs NPs 200@ Phe): 200 mg/L
- Chs NPs conjugated with Cou 40 mg/L (Chs NPs 200@ Cou): 200 mg/L

Harvest took place between 28th May and 13th June, when the blossoms were dry. The flower heads were cut off from the base and placed

in paper bags in a dry place to continue the drying process. Just after drying, the seeds were separated, and any foreign materials were removed. Determination of the dry matter content was by drying at 70°C up to constancy. Manner of sampling ensured its being characteristic of an entire lot of the raw material. The subsequent measurements were reported, such as plant height (cm), dry weight/plant (g), flower heads number/plant, seeds number/flower head, 1000 seeds weight (g), seeds weight/plant (g), and seeds yield/feddan (kg).

3. Preparation of Chitosan Nanoparticles

Particulate dispersions or solid particles with a size of 1–1000 nm are referred to as nanoparticles. Chs NPs have been prepared using methods such as ionic gelation (Kataoka et al., 2000). In the method of ionic gelation, the interaction of oppositely charged macromolecules can produce Chs NPs. Because tripolyphosphate (TPP) is nontoxic, multivalent, and capable of forming gels through ionic interactions, it is frequently utilized to make Chs NPs. The charge density of TPP and Chs, which is affected by the pH of the solution, can govern the interactions.

Chs NPs were prepared by the ionic gelation method according to Calvo et al. (1997), with some modifications. The method used the electrostatic interaction between the amine groups of Chs (Sigma-Aldrich, molecular weight 50,000-190,000 Da, degree of deacetylation 75-85%) and negatively charged groups of polyanion, including sodium TPP (Sigma-Aldrich). The Chs NPs aqueous solution (0.2% w/v) was prepared by dissolving Chs NPs in the acetic acid solution (1% v/v) at room temperature. Subsequently, the sodium TPP solution (0.06% w/v) was added dropwise to the Chs NPs solution under vigorous stirring for 1 hour. The resulting Chs NPs suspension was centrifuged at 12,000 g for 30 min. The pellet was re-suspended in deionized water. The Chs NP suspension was then freeze-dried before further use.

Table (1). Mechanical analysis of the soil.

Sand (%)	Silt (%)	Clay (%)	Soil texture
91.51	2.64	5.85	Sandy

Table (2). Chemical analysis of the soil.

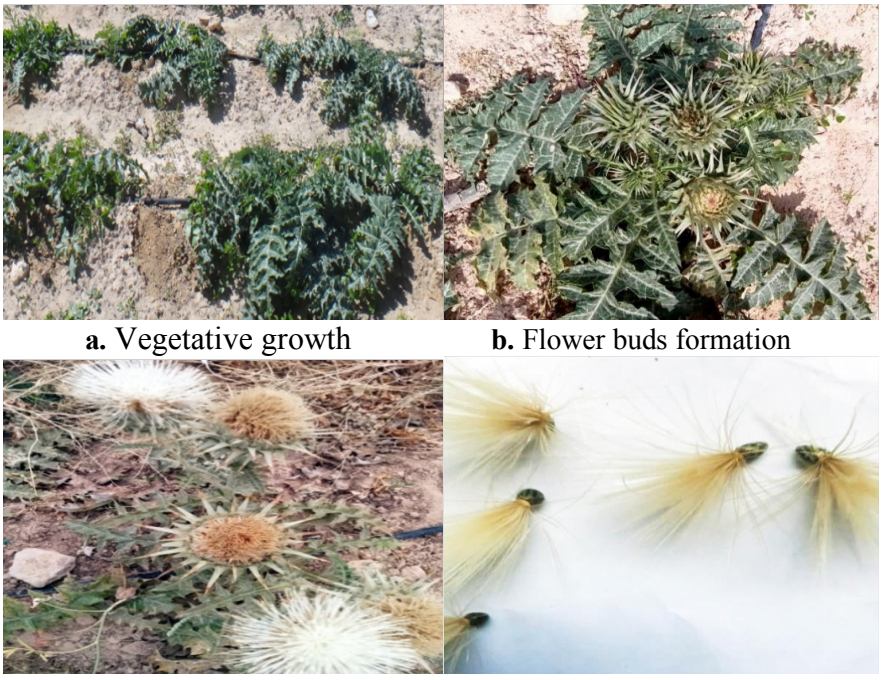
pH	E.C. (ppm)	Soluble anions (meq/l)				Soluble cations (meq/l)			
		CO_3^{--}	HCO_3^-	Cl^-	SO_4^{--}	Ca^{++}	Mg^{++}	Na^+	K^+
8.45	294.40	-	3.10	1.10	0.33	1.38	2.92	0.26	0.06

Table (3). Chemical analysis of the water irrigation.

pH	E.C. (ppm)	Soluble anions (meq/l)				Soluble cations (meq/l)			
		CO_3^{--}	HCO_3^-	Cl^-	SO_4^{--}	Ca^{++}	Mg^{++}	Na^+	K^+
7.17	409.60	-	2.75	3.07	0.53	2.53	1.89	1.86	0.14

Table (4). Chemical analysis of the sheep manure.

pH	EC (ppm)	O.M. (%)	C/N ratio (%)	N (%)	P (%)	K (%)	Fe (%)	Mn (mg/kg)	Zn (mg/kg)	Cu (mg/kg)
9.15	4902.00	44.00	18.00	1.59	0.24	1.91	0.43	105.00	26.70	12.80



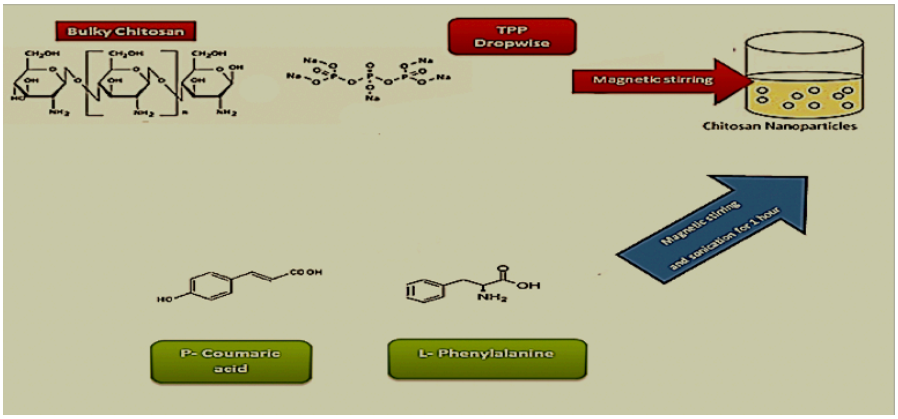
a. Vegetative growth **b.** Flower buds formation

c. Flowering **d.** Seed ripening

Fig. (1). Photos **a** to **d** show cultivated milk thistle at diverse development phases (El-Hammam farm).

4. Conjugation of P-Coumaric acid and L-Phenylalanine

Following the creation of Chs NPs, 50 mg/L and 200 mg/L concentrations were synthesized separately. Each concentration was sonicated continuously at room temperature with 40 mg/L L-Phe and 40 mg/L P-Cou until conjugation was achieved, which was confirmed by the electrostatic force between the two molecules.



5. Characterization of Chitosan Nanoparticles, Chitosan Conjugated with P-Coumaric acid and L-Phenylalanine

5.1. X-ray diffraction

A thin film of Chs NPs at a concentration of 2000 mg/L was dried and ground to fine powder for X-ray diffraction (XRD) examination, as were other nano-conjugates. XRD was used to determine the chemical structure and phase analysis of the as-prepared Chs NPs and other nano-hybrids. The accompanying XRD pattern was acquired in the scanning mode (X'pert PRO, PAN analytical, Netherlands) using a Cu K radiation tube (= 1.54 A) at 40 kV and 30 mA. The standard ICCD library loaded in the PDF4 program was used to evaluate the diffraction pattern acquired.

5.2. Size and zeta potential

The Dynamic Light Scattering (DLS) method was used to estimate the average distribution of particle size determined by the Zeta-sizer 3000HS (Malvern Instruments, ZS Nano, UK) and the zeta potential for charge and stability was also detected and could define the occurrence of the conjugation process by changing in charge of Chs NPs. The analysis was performed at a scattering angle of 90° at a temperature of 24.9°C using samples diluted to with deionized distilled water and subjected to ultrasonic to be dispersed.

6. Biochemical Markers

6.1. Lipid peroxidation content

Lipids are one of the most vulnerable cellular components to the breakdown of reactive oxygen species by peroxidation of unsaturated fatty acids in biological membranes. The lipid peroxidation test is a widely used technique for determining lipid peroxidation.

The level of lipid peroxidation in *S. marianum* fresh leaf samples was measured in terms of estimating the end product, malondialdehyde (MDA) (Heath and Packer, 1968) with some modifications. In short, was homogenized in 2.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 14,000 rpm, 4^{o}C for 15 min. Five percent

thiobarbituric acid (TBA, 2.0 mL) was mixed with 20% TCA solution and the mixture was added to 0.5 mL of the liquid supernatant of leaf sample. The mixture was heated at 95°C for 30 min in a water bath and then incubated in an ice bath for 5 min. After centrifugation, the supernatant was measured at 532 nm and 600 nm for nonspecific turbidity of each sample, and the absorbance at 532 nm was subtracted. The MDA-TBA adduct content was determined using the MDA standard curve and converted to nmol g^{-1} fresh weight.

6.2. Antioxidant capacity

The following technique was used to determine the scavenging potential of *S. marianum* fresh leaves extract against the synthetic radical DPPH (2,2-di-phenyl-1-picrylhydrazyl): 2 mL of a 0.004% solution of DPPH in absolute methanol was mixed with 0.5 mL of *S. marianum* fresh leaves methanolic extract. The reaction solution was mixed and stored in the dark for 30 min at room temperature. According to the procedure of the spectrophotometer, the absorbance of the combination was 517 nm (Oktay et al., 2003). The DPPH radical scavenging rate was calculated using the following formula:

$$RSC\ \% = (A_{blank} - A_{sample}) / (A_{blank}) \times 100$$

where RSC % = DPPH radical scavenging activity (%), Abs_{blank} is the absorbance of DPPH radical + methanol (2 mL DPPH and 0.5 mL methanol).

6.3. Peroxidase activity

About 5 mlof 0.05 M sodium phosphate buffer was used to homogenize 1.0 g of fresh leaves (pH 7.0). The homogenate was centrifuged for 10 min at 14.000 rpm at 4°C, and the supernatant was utilized to make the crude enzyme source, which was stored at −20°C until required according to Worthington Biochemical Corp. (1972). Peroxidase was measured using the O–Dianisidine procedures, a brief overview, 1.5 ml of 0.01 M phosphate buffer (pH, 6.0), 10 µl of 1% O–dianisidine in absolute methanol was added and mixed; 100 µL of enzyme was added and mixed. Then 100 µl of 0.3% H_2O_2 was added; after mixing, the increasing in absorbance was recorded kinetically at 460 nm for 3 min by a spectrophotometer as following: (change in Abs_{460}) / fresh weight / 3 min.

7. Molecular Markers

7.1. ISSR profiling for genetic variation

Six ISSR primers were used in the detection of polymorphism (Table 5). The amplification reaction was carried out in 25 µL reaction volume containing 12.5 µL master mix (sigma), 2.5 µL primer (10 pcmol), 3 µL template DNA (10 ng) and 7 µL dH_2O, according to Ibrahim et al. (2019).

Table (5). Different primers and their sequences.

Primer Name	Sequence
ISSR-1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR-2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR-3	5'-ACACACACACACACACYT-3'
ISSR-4	5'-ACACACACACACACACYG-3'
ISSR-5	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR-6	5'-CGCGATAGATAGATAGAT-3'

7.2. Thermocyling profile PCR

PCR amplification was carried out in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94oC. Each cycle consisted of a denaturation step at 94oC for 1 min, an annealing step at 45oC for 1 min, and an elongation step at 72oC for 1.5 min. The primer extension segment was extended to 7 min at 72oC in the final cycle.

7.3. Detection of the PCR products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

7.4. Data analysis

Only apparent and unambiguous bands were visually graded as present (1) or absent (0) for all samples throughout ISSR analysis and the final data sets contained polymorphic and monomorphic bands. Then, a binary statistic matrix was constructed. Dice's similarity matrix coefficients were then calculated between genotypes using the unweighted pair group method with arithmetic averages (UPGMA). Using the PAST software Version 1.91, this matrix was used to create a phylogenetic tree (dendrogram) based on the Euclidean similarity index (Hammer et al., 2001).

7.5. Genomic template stability %

The percent of genomic template stability was calculated according to the formula:

$$GTS\% = (1 - a/n) \times 100$$

Where;

a = the average number of polymorphic bands detected in each treated sample n= no. of total bands in control

8. Determination of Silymarin Compounds

Silymarin compounds of *S. marianum* seeds were determined by HPLC according to Cai et al. (2009), using the following steps:

8.1. Extraction

In a soxhlet system, silymarin components were extracted from 1.0 g of plant seeds using n-hexane for 4 hours and subsequently ethyl acetate for 8 hours. The ethyl acetate solution was evaporated on a rotary evaporator under decreased pressure at a temperature not exceeding 45°C until semi dryness. After filtration, methanolic solutions of dry film silymarin samples were utilized for HPLC analysis.

8.2. Separation

HPLC analysis was performed using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of phosphoric acid: methanol: water (0.5: 35: 65) [A] and phosphoric acid: methanol: water (0.5: 70: 30) [B] at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0-5 min (100% A); 5-30 min (30% A); 30-30.5 min (0% A); 30.5-35 min (0% A); 35-37 min (100% A). The multi-wavelength detector was monitored at 288 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 30°C. A methanolic solution of standard silymarin 1000 ug/ml was used to investigate the chromatographic behavior of the flavonolignan isomers of silymarin.

8.3. Calculation

The peak area (%) of silymarin isomers in *S. marianum* seeds samples were determined by comparing their relative retention time (min) with standard using HPLC software.

9. Statistical Analysis

The data was analyzed to a one-way ANOVA, and Duncan's multiple range test was used to detect the differences between means at the 0.05 probability level. According to Dytham (1999) the SPSS program version 16 (Richmond, USA) was used.

RESULTS AND DISCUSSION

1. Chitosan Nanoparticles Characterization

The XRD pattern of synthesized Chs NPs is represented in fig. (2a). The peaks were assigned to 56 and 54 at 2Θ 11.35 and 24.61^{o} and relative respectively, compared to the standard ICCD library installed in PDF4 program. Fig. (2c) represented the X- ray diffraction pattern of Cou with card number 00-021-1772. The coating between Chs NPs@Cou was assigned to 13, 109, 22, 70, 35, 63, 45, 321, 139, 75, 10 2Θ 6.11, 17.41, 18.35, 19.61, 20.54, 22.91, 23.97, 24.71, 26.20, 30.06 and 49.44^{o} in fig. (2e). Fig. (2d) represented Phe with card number 00-011-0827. The coating between Chs NPs@Phe was assigned to 2901, 16, 55, 22, 46, 539, 25, 631, 279, 118, 135, 115, 224, 2072, 85, 121, 58, 127, 55, 770, 63, 597, 17, 57, 10, 9, 26 at 2Θ 5.67, 6.01, 6.54,8.99, 11.30, 14.85, 16.05, 16.97, 17.80, 19.23, 19.66,

20.14,21.45, 22.70, 23.81, 24.36, 25.56, 26.24, 27.65, 28.46, 29.71, 32.97, 34.31, 36.09, 40.22, 43.07, 50.79, 58.96° as shown in fig. (2d).

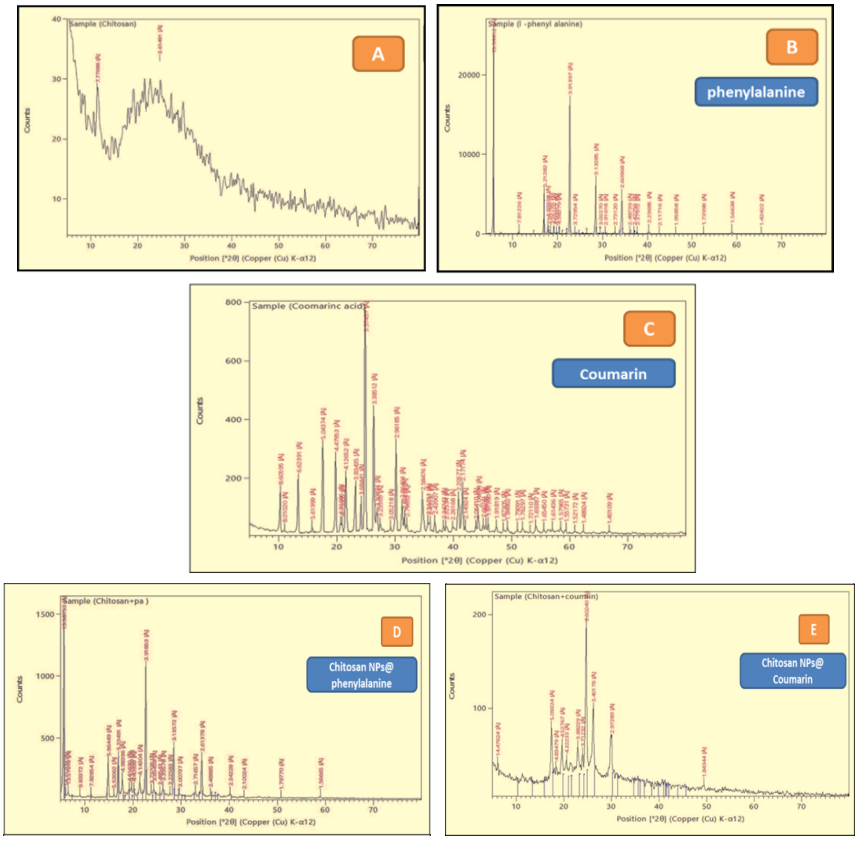


Fig. (2). X ray diffraction of several types of chitosan nanoparticles.

The size of Chs NPs was about 61 nm, the particle size distribution curve obtained from DLS measurement with good polydispersity index (PDI) of 0.632 is presented in fig. (3a and b) and the zeta potential was used to calculate hydrodynamic diameter and surface charge in the nanometric range which was +52.4 mV in fig. (3b) with good stability. Fig. (3c and d) illustrate the size and distribution of Chs NPs@Cou, which was 57.49 nm and PDI of 0.440, + 61.7 mV in fig. (3d and c, respectively) and in fig. (3e and f) illustrate the size and distribution of Chs NPs@Phe, which was approximately 43.93 nm and PDI 0.600, zeta potential about +39.8 mV in fig. (3e and f), respectively. The characterization of NPs was performed according to our previous work (Hassan et al., 2018; Mahdi et al., 2020; Farroh et al., 2020 and El-Saber et al., 2021).

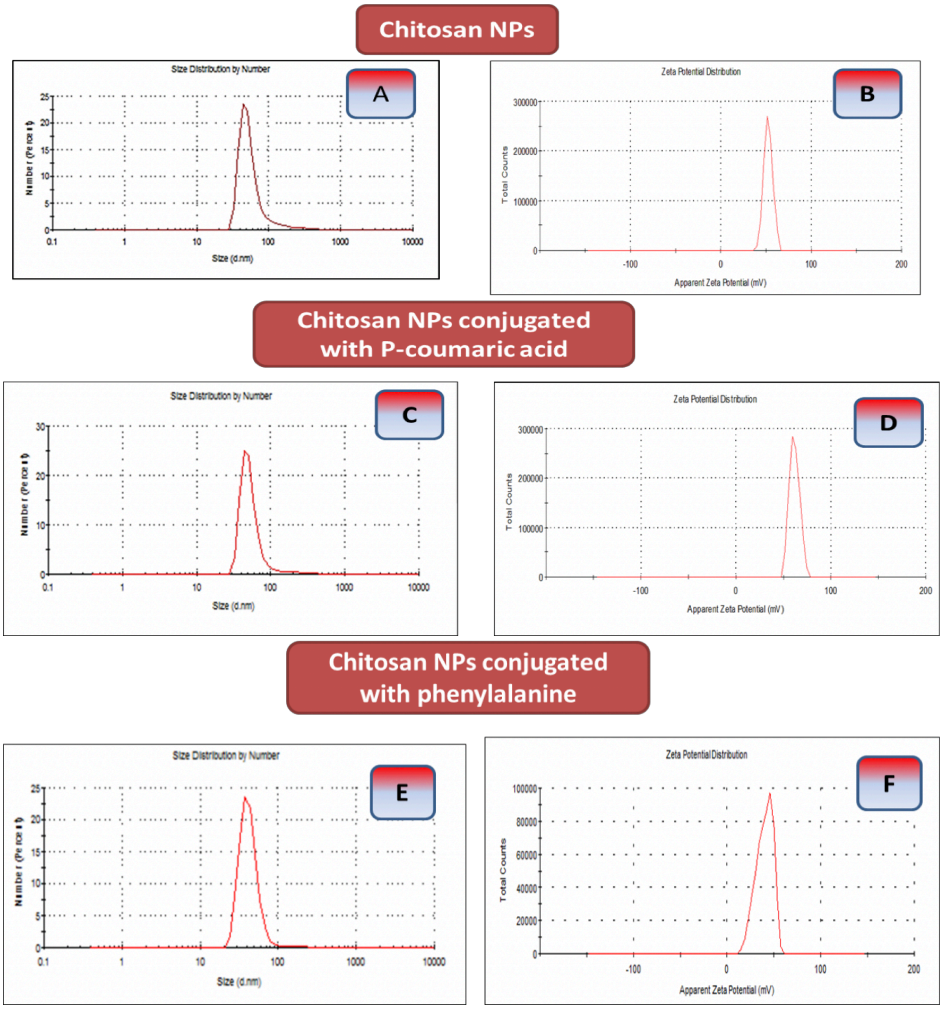


Fig. (3). Size and zeta potential of several types of chitosan nanoparticles.

2. Effect of Precursors and Chitosan NPs on Vegetative Growth and Yield Attributes

Nanotechnology, which is based on the distinctive traits of NPs, such as size, shape, and distribution, has become one of the most important study areas in current materials science. NPs are being used in a growing number of applications (Jain et al., 2009). Nanotechnology is crucial in modern agriculture for addressing global concerns such as climate change, plant disease severity, and the scarcity of critical plant nutrients (Parisi et al. 2015). Several biopolymers, including Chs NPs, have been employed in the development of innovative materials with environmentally sustainable properties and desirable functionality in recent years (Babu and Ramesh, 2017).

The effect of Chs NPs aspects on growth traits (plant height and dry weight/plant) is presented in table (6). Furthermore, data concerning the influence of treatments on yield parameters (flower heads number/plant, seeds number/flower head, 1000 seeds weight, seeds weight/plant, and seeds yield/feddan) are recorded in table (7). Generally, the data of all sprayed Chs NPs aspects surpassed the unsprayed treatment regarding vegetative growth and yield characters. The significant highest values were obtained by using the solution of Phe 40 mg/L followed by Chs NPs 200 mg/L. The variations within the treatments mentioned above were non-significant in plant height, flower heads number/plant, and seeds number/flower head. In turn, these variations were significantly related to dry weight/plant, 1000 seeds weight, seeds weight/plant, and seeds yield/feddan. The Phe 40 mg/L treatment recorded 148.00 g, 33.09 g, 42.57 g, and 476.78 kg, as well the spraying with Chs NPs 200 mg/L recorded 139.00 g, 27.46 g, 31.41g, and 351.79 kg for dry weight/plant, 1000 seeds weight, seeds weight/plant and seeds yield/feddan, respectively.

The observed growth and yield improvement of Phe on plants coincided with the literature stated by Reham et al. (2016) on Genovese basil; Aghaei et al. (2019) on hyssop; Baharlouet et al. (2019) on lemon balm; Elshorbagy et al. (2020) on lavender and Sachet et al. (2021) on dill. Also, the positive role of chitosan nanoparticles on the growth and yield of plants agrees with that of Mahdy (2019) on onion; El Shayeb et al. (2021) on coriander and Sheikhalipour et al. (2021) on bitter melon.

Table (6). Effect of treatments on plant height and dry weight/plant (mean values of the two successive seasons).

Treatments	Plant height (cm)	Dry weight/plant (g)
Control	30.33 a	41.00 a
Phe 40 mg/L	36.22 bc	148.00 b
Cou 40 mg/L	34.33 a-c	128.50 bc
Chs NPs 50 mg/L	33.00 a-c	123.50 c
Chs NPs 50 @ Phe	32.25 a-c	67.00 d
Chs NPs 50 @ Cou	31.50 a-c	92.03 e
Chs NPs 200 mg/L	35.16 c	139.00 c
Chs NPs 200 @ Phe	32.50 a-c	82.50 e
Chs NPs 200 @ Cou	32.33 a-c	46.50 a

Means with the same letter are not significantly different at 5% level of probability.

Table (7). Effect of treatments on yield attributes (mean values of the two successive seasons).

Treatments	Flower heads number/plant	Seeds number/ flower head	1000 seeds weight (g)	Seeds weight/plant (g)	Seeds yield/feddan (kg)
Control	2.67 a	240.00 a	26.75 a	17.14 a	191.97 a
Phe 40 mg/L	4.50 bc	261.67 a	33.09 b	42.57 b	476.78 b
Cou 40 mg/L	4.25 bc	206.00 bc	29.19 a	25.44 c	284.93 c
Chs NPs 50 mg/L	4.50 bc	232.00 ab	30.56 b	31.37 d	351.34 d
Chs NPs 50 @ Phe	3.00 a-c	182.00 c	20.95 c	12.39 e	138.77 e
Chs NPs 50 @ Cou	3.00 a-c	291.00 d	26.53 a	23.16 f	259.39 f
Chs NPs 200 mg/L	4.67 b	260.00 a	27.46 a	31.41 d	351.79 d
Chs NPs 200 @ Phe	3.80 a-c	182.00 b	30.44 b	22.34 f	250.21 f
Chs NPs 200 @ Cou	3.00 a-c	276.00 d	20.00 c	15.66 a	175.39 a

Means with the same letter are not significantly different at 5% level of probability.

In contrast, the Chs NPs 50 or 200 mg/L conjugated with Phe 40 mg/L or Cou 40 mg/L recorded lower values than the separated aspects as phenylalanine 40 mg/L, Chs NPs 50 or 200 mg/L, and Cou 40 mg/L. These results may be attributed to the fact that the nutrient requirements of this crop being low to moderate, since it is adapted to poor quality soils and many different growing conditions (Karkanisa et al., 2011). So, its metabolic capacity is limited even with excessive nutrients.

3. Biochemical Markers

Effect of precursors and chitosan nanoparticles on malondialdehyde content, antioxidant capacity and peroxidase activity

Lipid peroxidation product was determined for oxidative stress. The lowest content of MDA was detected in treated plants with Chs NPs 200 mg/L, which related to highest antioxidant content of carotenoids and highest antioxidant capacity of the same treatment in table (8), and this may contribute to protection for the bio-membranes and prevention of releasing to MDA toxic product. According to table (8), having antioxidant activity is an indicator of that tested plants possess certain amounts of polyphenols and flavonoids, proving them to be perfect sources of antioxidants. Highest inhibition of synthetic radical DPPH was noticed in treated plants with Chs NPs 200 mg/L. The maximum peroxidase activity was related to the treatment with Chs NPs 50 mg/L, followed by Chs NPs 50@Cou then Cou 40 mg/L and this may relate to increasing or ROS releasing and activation of peroxidase as a response to the triggered action.

To assess the physiological influence of exogenous Chs on these antioxidant enzymes, the activities of many representative antioxidant enzymes, including POD concentration, were determined in *S. marianum*. Results are compatible in some extent with our previous literature on *S. marianum* callus subjected to different aspects of Chs NPs (Hassanen et al.,

2021). In *Capsicum annuum* fruits, Chs NPs hydrogel 25 nm coated Cu induced a rise in the level of antioxidants ABTS and DPPH, total phenols and flavonoids, and a reduction in post-harvest weight loss (Pinedo-Guerrero et al., 2017). Chs NPs foliar treatments at concentrations of 10, 50, and 100 M enhanced phenolic acids, anthocyanin, and antioxidant activity in *Momordica charantia* L. (Sharifi-Rad et al., 2020).

Table (8). Effect of treatments on some biochemical markers in *S. marianum* leaves after 75 days from sowing.

Treatments	Malondialdehyde content (nmol/g fresh weight)	Antioxidant capacity %	Peroxidase Δactivity/ 3 min/ fresh weight
Control	7.96 c	65.20 bc	32.27 bc
Phe 40 mg/L	13.05 a	59.73 de	37.14 b
Cou 40 mg/L	5.59 d	62.70 cd	43.14 a
Chs NPs 50 mg/L	7.76 c	66.95 b	47.94 a
Chs NPs 50 @ Phe	12.77 a	60.00 de	12.85 f
Chs NPs 50@Co	11.28 b	61.27 de	46.99 a
Chs NPs 200 mg/L	3.34 e	74.80 a	21.40 e
Chs NPs 200@Phe	7.79 c	58.77 e	27.32 cd
Chs NPs 200 @Co	8.64 c	61.00 de	23.72 de

Means followed by the same letter in a column are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level.

4. Molecular Studies

Genomic DNA profiling by ISSR and genomic template stability

Inter simple sequence (ISSR DNA) technique has been successfully employed by many authors to confirm genetic uniformity on wheat subjected to several types of NPs and abiotic stress (Mahdi, 2017, Mahdi et al., 2020 and Fouda et al., 2021) on wheat and *Salvadora persica*. This technique was performed to evaluate the effect of different nanomaterials and substrates on the genetic material of *S. marianum* cultivated plants in comparison to control. About six primers were used for evaluating the genetic polymorphism in *S. marianum* fresh leaves as shown in table (9).

Table (9). Effect of treatments on polymorphism of *S. marianum* plant leaves with six ISSR primers.

Primer Name	bp	Monomorphic bands	Polymorphic bands			Total bands	Polymorphism (%)	Band frequency
			Non Unique	Unique	Total			
ISSR-1	810-180	3	7	0	7	10	70	0.7
ISSR-2	1200-160	7	6	0	6	13	46	0.8
ISSR-3	850-150	5	6	0	6	11	55	0.7
ISSR-4	1250-330	6	4	3	7	13	54	0.7
ISSR-5	1050-180	3	7	3	10	13	77	0.5
ISSR-6	1000-100	3	4	0	4	7	57	0.7
Total bands		27	34	6	40	67	59.83	

The six used primers were (ISSR-1, 810-180 bp), (ISSR-2, 1200-160 bp), (ISSR-3, 850-150 bp), (ISSR- 4, 1250-330 bp), (ISSR-5, 1050-180 bp) and (ISSR-6, 1000-100 bp). The appearance of unique new bands may be due to the reaction of the NPs or other treatments with the DNA strands and this may give rise to some positive effects reflected on the amazing accumulation of bioactive secondary metabolites like silymarin isomers or the increase of growth, proliferation and seed yield of the plant. According to table (9), fig. (4), the resultant amplicons were about 27 monomorphic bands, 34 polymimorphic without unique bands, 6 unique bands, 40 polymorphic with unique, with average polymorphism for all primers 59.83% scored by six used primers. The resultant unique bands were scored from primer ISSR-4 (550 bp and 880 bp in case of control and 1200 bp in case of Chs NPs200@Cou) and primer ISSR-5 (490 bp in case of Ch NPs50@Cou, 290 bp in treatment by Phe and 270 bp in case of control).

The polymorphism was changeable from primer to other as reported in table (9). The maximum polymorphism value was scored by ISSR-1 (70%), and the lowest polymorphism record was detected by ISSR-2 (46%). Data in table (10) and fig. (5) based on cluster analysis and similarity matrix showed the molecular distance among treated *S. marianum* plants. The dendogram was introduced for the plant and its applied treatments, which expressed the genetic stability of the plant as genomic template stability (GTS). The genomic template stability in graphical fig. (6) shows that all GTS% values was decreased due to all treatments but the highest GTS% was scored by Chs NPs200@Cou 95.5%, which was nearly to the control. Then followed by Phe 40 mg/L, Cou 40 mg/L, Chs NPs200@Phe and other treatments came at the next order.

In general, the size of NPs has been shown to have an inverse connection with genotoxicity, but exposure length and concentration have a direct association**.** According to Fouda et al. (2021) worked on *Salvadora persica* and like this study on *S. marianum*, the modifications in the ISSR profile showed that the NPs applied doses generated genetic variation that was

dose and NP type dependent. The emergence of new synthesized DNA bands in the ISSR profile and the absence of normal ones can be defined as a mutation, which is most likely caused by DNA damage or rearrangements caused by NP induced genetic variation. The stability of genomic templates reflects the changes in the ISSR profile. These bands are most likely created as a result of NPs ability to cause genomic variation by interfering with mitosis and modifying DNA by producing chromosomal abnormalities (Plaksenkova et al., 2019).

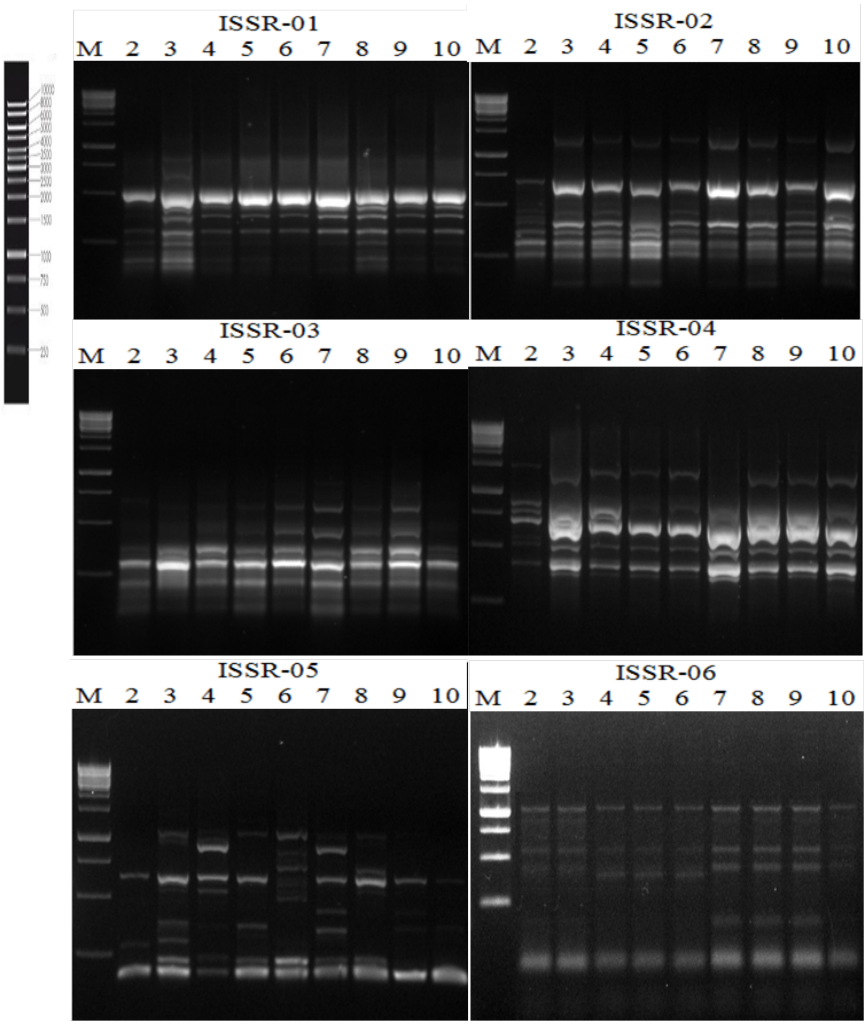


Fig. (4). Effect of precursors and chitosan nanoparticles on ISSR DNA of wild *S. marianum* where; M: marker, 2: control, 3: Phe, 4: Cou, 5: Chs NPs 50 mg/L, 6: Chs NPs50@Phe, 7: Chs NPs50@Cou, 8: Chs NPs 200 mg/L, 9: Chs NPs 200@Phe and 10: Chs NPs 200@Cou.

Table (10). Effect of treatments on cluster analysis of *S. marianum* plant leaves.

Treatments	Control	Phe	Cou	Chs NPs 50	Chs NPs 50@PA	Chs NPs 50@Cou	Chs NPs 200	Chs NPs 200@PA	Chs NPs 200@Cou
Control	100								
Phe	76	100							
Cou	75	88	100						
Chs NPs 50	73	80	85	100					
Chs NPs 50@PA	71	78	85	88	100				
Chs NPs 50@Cou	73	82	80	82	80	100			
Chs NPs 200	73	90	86	80	80	82	100		
Chs NPs 200@PA	77	86	82	80	78	92	88	100	
Chs NPs 200@Cou	75	87	82	83	78	84	82	84	100

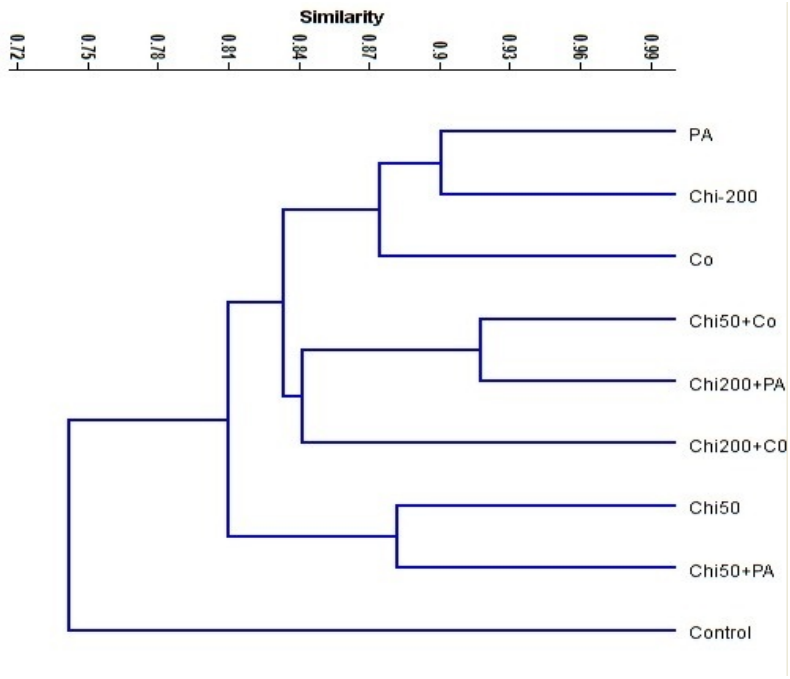


Fig. (5). Similarity matrix and cluster analysis for ISSR PCR analysis.

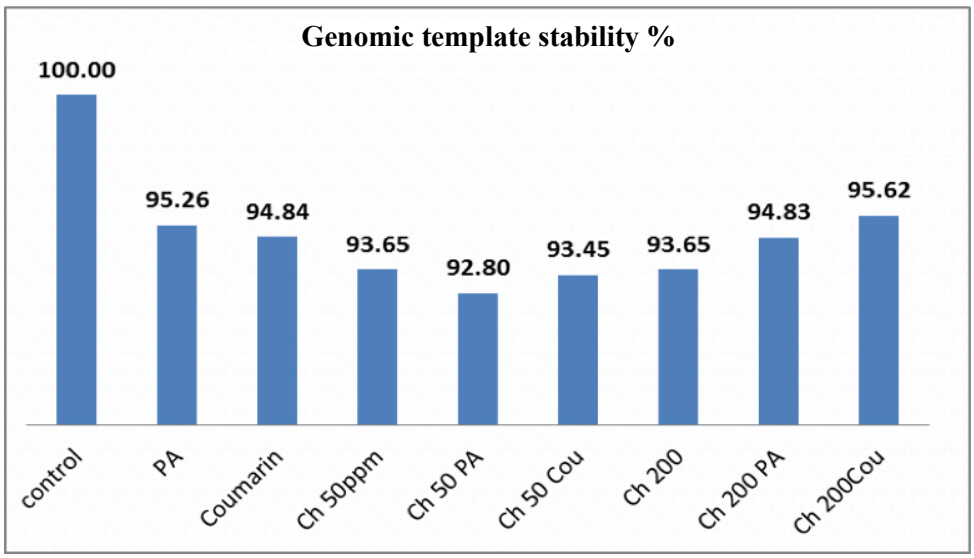


Fig. (6). Genomic template stability of all treatments.

5. Silymarin Isomers

The separated silymarin isomers by HPLC were determined as following: (taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B) with the referred retention time and almost all NPs and precursors had positive effect on their biosynthesis inside plants in table (11) and chromatograms in fig. (7) and their structure in fig. (8). The taxifolin isomer is detected only in case of Chs NPs 200@Cou and not detected in control and other treatments. A slight increase in silychristin was noticed upon the treatment with Ch NPs 50 mg/L. The conjugation of Ch NPs 50@phe gave the maximum value of silydianin. The isomer Silybin A appeared only in treatment with Ch NPs 50 mg/L, Ch NPs 200 mg/L and Ch NPs 200@Phe but not accumulated at other treatments. Silybin B, the highest accumulation was noticed in Ch NPs 200 mg/L followed by Ch NPs 200@Cou. The isosilibin A reached the highest rate in Ch NPs 200 mg/L. in case of Isosilybin B was detected only in treatment with Ch 200 mg/L and not accumulated at other treatments. The total silymarin also determined in the table and an astonishing increasing in its content by the treatment with Ch NPs 200 mg/L followed by Ch NPs 50mg/L then the amino acid precursor Phe 40 mg/L. The response of the plants is differed towards NPs. The results were compatible to Hassanen et al. (2021). Chitosan was first discovered as an elicitor in plants, activating genes involved in the biosynthesis of secondary metabolites. In vivo and in vitro, chitosan can be administered to plant aerial organs to produce the accumulation of bioactive secondary metabolites (Yin et al., 2011).

Table (11). Effect of treatments on silymarin isomers analysis of *S.marianum.*

Silymarin isomers	Retention time (min)	Treatments								
		Control	Phe 40 mg/L	Cou 40 mg/L	Chs 50 mg/L	Chs 50 @ Phe	Chs 50 @ Cou	Chs 200 mg/L	Chs 200 @ Phe	Chs 200 @ Cou
		(Peak area %)								
Taxifolin	9.50	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	0.129
Silychristin	16.12	2.194	2.352	1.722	2.530	1.705	2.092	N.D	1.662	1.623
Silydianin	18.18	0.377	0.312	0.338	0.424	0.625	0.568	N.D	0.200	0.194
Silybin A	24.69	N.D	N.D	N.D	0.031	N.D	N.D	2.621	0.055	N.D
Silybin B	25.86	0.401	0.667	0.535	0.787	0.472	0.492	45.470	0.899	1.433
Isosilybin A	28.28	0.079	0.126	0.148	0.151	0.073	0.144	0.727	0.104	0.105
Isosilybin B	28.99	N.D	N.D	N.D	N.D	N.D	N.D	2.835	N.D	N.D
Unknown 1	2.51	7.950	6.120	7.010	5.440	7.900	7.710	5.150	6.740	7.500
Unknown 2	3.45	0.000	0.000	0.000	4.630	3.700	4.440	3.680	3.570	3.750
Unknown 3	11.43	63.020	68.460	64.450	58.560	55.060	56.440	38.720	53.350	41.120
Unknown 4	14.36	0.000	0.000	0.000	4.470	3.600	3.450	0.000	4.730	2.840
Unknown 5	17.25	25.960	21.950	25.700	22.960	27.140	25.180	0.770	28.680	41.280
		Total silymarin content (mg/g)								
		1.610	3.204	2.033	4.582	2.032	1.735	52.490	1.676	1.162

- N.D (not detected) is referred to less than the detection limit of the device.

Based on the data mentioned in this research, the treatment of phenylalanine 40 mg/L yielded 476.78 kg seeds/feddan and total silymarin content of 3.204 mg/g. The treatment of chitosan nanoparticles 200 mg/L produced 351.79 kg seeds /feddan and total silymarin content of 52.490 mg/g. The silymarin content is an essential quality parameter determining the price of seeds. Since the chitosan nanoparticles 200 mg/L treatment recorded the highest total silymarin, it is recommended to follow.

CONCLUSION

1. To produce the best yield attributes (high seeds crop with richest total silymarin content and maximum antioxidant capacity) of *S. marianum* var. *albiflorum*. Spraying plants with Chs NPs at the concentration of 200 mg/L is recommended through the vegetative growth stage.
2. The present research showed the potential of nanomaterials to be used as elicitors for improving the biosynthesis and accumulation of bioactive compounds. It is necessary to use NPs strategy to enhance bioactive compounds accumulation in medicinal plants without inducting phytotoxicity especially the low polymorphism detected. Therefore, the application of nanoparticles as elicitors needs more investigation on their potential risk on humans and ecosystems and for large-scale production.

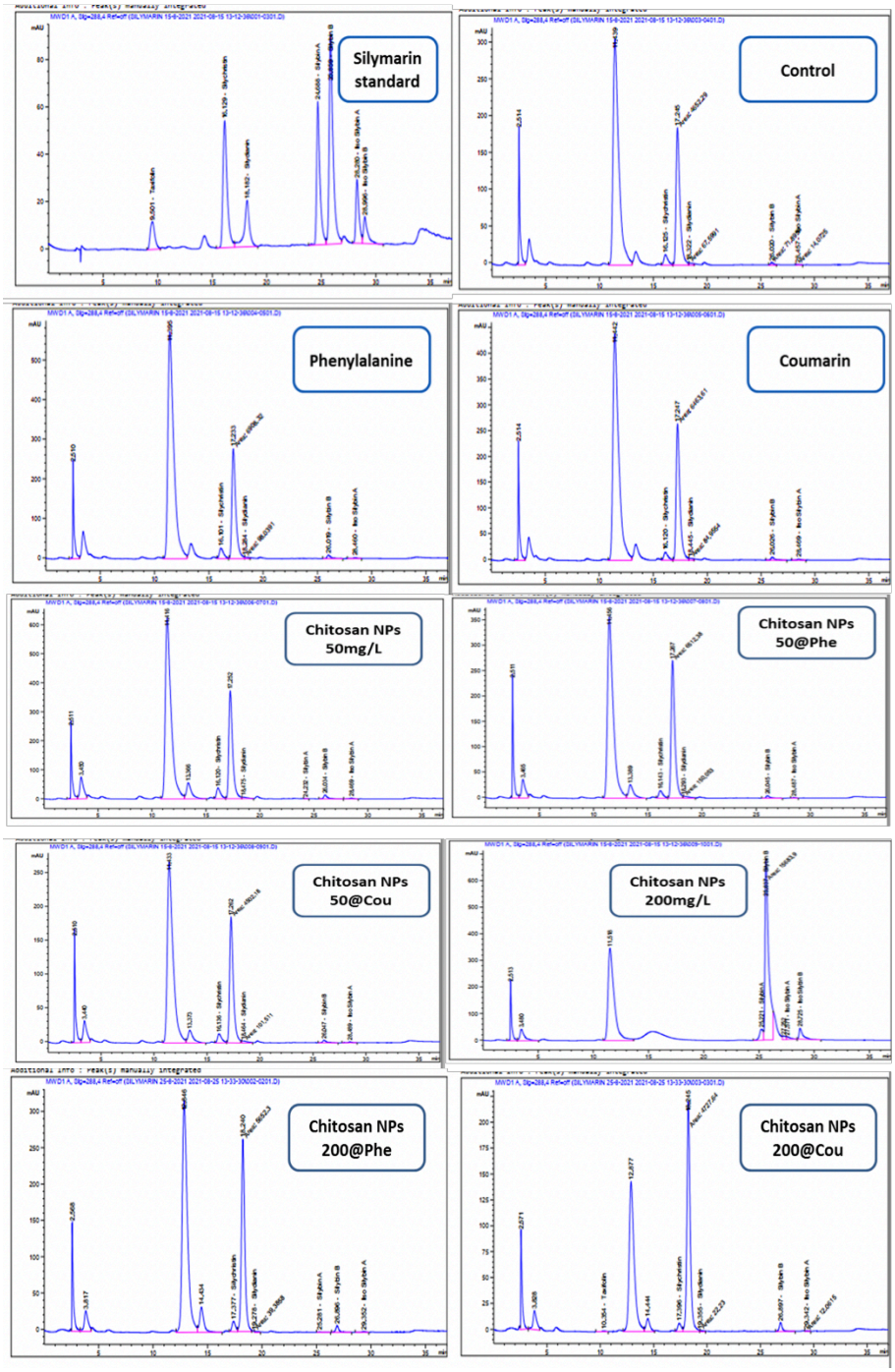


Fig. (7). Silymarin isomers structure.

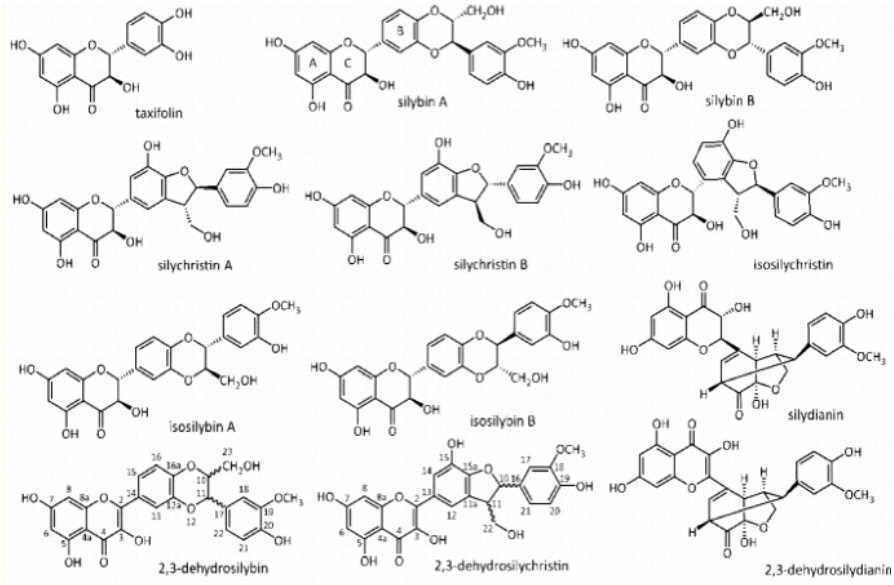


Fig. (8). Structure of silymarin isomers.

REFERENCES

Abenavoli, L. and N. Milic (2017). Silymarin for Liver Disease, Chapter 45. In: "Liver Pathophysiology" (Muriel, P. Ed.), Academic Press, p. 621-631.

Abenavoli, M.R., G. Cacco, A. Sorgonà, R. Marabottini, A.R. Paolacci, M. Ciaffi and M. Badiani (2006). The inhibitory effects of coumarin on the germination of durum wheat (*Triticum turgidum* ssp. durum, cv. Simeto) seeds. J. Chem. Ecol., 32 (2): 489-506

Aghaei, K., A.G. Pirbalouti, A. Mousavi, H.N. Badi and A. Mehnatkesh (2019). Effects of foliar spraying of L-phenylalanine and application of bio-fertilizers on growth, yield, and essential oil of hyssop [*Hyssopus offcinalis* L. subsp. *angustifolius* (Bieb.)]. Biocatal. Agric. Biotechnol., 21: 1-10.

Ministry of Environment, Arab Republic of Egypt (2016). Egyptian Biodiversity Strategy and Action Plan (2015 – 2030).

Babu, A. and R. Ramesh (2017). Multifaceted applications of chitosan in cancer drug delivery and therapy. Mar. Drugs, 15 (4): 96.

Baharlouet, M.J., A.G. Pirbalouti and F. Malekpoor (2019). Effect of different concentrations of L-phenylalanine on chemical compositions and yield of essential oil of lemon balm (*Melissa officinalis*). J. Med. Herb., 10 (4): 175-183.

Bonawitz, N.D. and C. Chapple (2010). The genetics of lignin biosynthesis: connecting genotype to phenotype. Annu. Rev. Genet., 44: 337–363.

Calvo, P., C. Remun˜A´ N-Lo´pez, J. Vila-jato and M.J. Alonsom (1997). Novel hydrophilic chitosan–polyethylene oxide nanoparticles as protein carriers. J. Appl. Pol. Sci., 63: 125–132.

Cai, X.L., D.N. Li, J.Q. Qiao, H.Z. Lian and S.Q. Wang (2009). Determination of silymarin flavonoids by hplc and lc-ms and investigation of extraction rate of silymarin in *Silybum marianum* fruits by boiling water. Asian. J. Chem., 21 (1): 63-74.

Dytham, C. (1999). In: "Choosing and Using Statistics: A Biologist's Guide". Blackwell Science Ltd., London UK., 358 p.

Durak, A., E. Buyukguner and H.M. Dogan (2010). Determination of physical and chemical properties of the soils under different land managements. Asian. J. Chem., 22 (8): 6375-6386.

El Shayeb, N.S.A., R.H.I. Hassan and M.I.A. Mohaseb (2021). Impact of nano-chitosan rate and glutamine acid concentration on growth, yield and volatile oil production of coriander plants. J. Bioagric., 1 (1): 15-24.

El-Saber, M.M., A.A. Mahdi, A.H. Hassan, K.Y. Farroh and A. Osman (2021). Effects of magnetite nanoparticles on physiological processes to alleviate salinity induced oxidative damage in wheat. J. Sci. Food. Agric., 101: 5550–5562.

Elshorbagy, A.I.M.E., A.A.A. Meawad and M.A.I. Abdelkader (2020). Effect of some amino acids concentration on growth and yield of lavender (*Lavandula officinalis*, CHAIX) plant under different NPK fertilization levels. Zagazig J. Agric. Res., 47 (4): 895-907.

Farroh, K.Y, A.A. Mahdi, A. Osman, A. Hassan and A.M. El-Saber (2020). Impact of chemically synthesized zinc oxide nanoparticles on saline stressed wheat.., 21: 117–136.

Fouda, M.S., M.H. Hendawey, Gh.A. Hegazi, H.M. Sharada, N.I. El-Arabi, M.E. Attia and E. Soliman (2021). Nanoparticles induce genetic, biochemical, and ultrastructure variations in *Salvadora persica* callus. J. Gen. Eng. Biotechnol., 19 (1): 19-27.

Gresta, F., G. Avola and P. Guarnaccia (2007). Agronomic characterization of some spontaneous genotypes of milk thistle (*Silybum marianum* L. Gaertn.) in Mediterranean environment. J. Herbs Spices Med. Plants, 12: 51–60.

Hammer, A.T., A.T.H. David and D.R. Paul (2001). PAST: Palaeontological statistics software package for education and data analysis. Palaeontologia Electronica, 4: 9.

Hassan, N.S., T.A. Salah El Din, M.H. Hendawey, A.A. Mahdi and I.H. Borai (2018). Magnetite and zinc oxide nanoparticles alleviated heat stress in wheat plants. Current Nanomaterials, 3: 32-43.

Hassanen, S.A., Gh.A. Hegazi, M.I. Diab, A.A. Mahdi, M.H. Hendawey and K.Y. Farroh (2021). Effect of coumarin and phenylalanine conjugated with chitosan nanoparticles on secondary metabolites synthesis in

callus of *Silybium marianum*. Plant Cell Biotechnol. Mol. Biol., 22 (71 & 72): 112-130.

Heath, R.L. and L. Packer (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. In. Bioch. Biophys., 125: 189-198.

Hidangmayum, A., P. Dwivedi, D. Katiyar and A. Hemantaranjan (2019). Application of chitosan on plant responses with special reference to abiotic stress. Physiol. Mol. Biol. Plants, 25 (2): 313–326.

Ibrahim, S.D., A.F. Abd El-Hakim, H.E. Ali and R.M. Abd El-Maksoud (2019). Genetic differentiation using ISSR, SCoT and DNA Barcoding for Quinoa genotypes. Arab J. Biotecnol., 22 (2): 103-118.

Jackson, P., N. Jacobsen, A. Baun, R. Birkedal, D. Kühnel, K. Jensen, U. Vogel and H. Wallin (2013). Bioaccumulation and ecotoxicity of carbon nanotubes. Chem. Central J., 7: 154-175.

Jain, D., H. Kumar-Daima, S. Kachhwaha and S.L. Kothari (2009). Synthesis of plant-mediated silver nanoparticles using papaya fruit extract and evaluation of their antimicrobial activities. Dig. J. Nanomater., 4: 557-563.

Kaky, E. and F. Gilbert (2017). Predicting the distributions of Egypt's medicinal plants and their potential shifts under future climate change. PLOS ONE, 12 (11): 1-19.

Karkanisa, A., D. Bilalisb and A. Efthimiadou (2011). Cultivation of milk thistle (*Silybum marianum* L. Gaertn.), a medicinal weed. Ind. Crops. Prod., 34 (1): 825-830.

Kataoka, K., T. Matsumoto, M.Tokohama, T. Okano and Y. Sakurai (2000). Doxorubicin-loaded poly (ethylene glycol)-poly(b-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. J. Control. Release, 64: 143-153.

Kean,T. and M. Thanou (2010). Biodegradation, biodistribution, and toxicity of chitosan. Adv. Drug. Deliv. Rev., 62: 3–11.

Keasar, T., Y. Gerchman and S. Lev-Yadun (2016). A seven-year study of flower-color polymorphism in a mediterranean annual. Basic Appl. Ecol., 17: 741–750.

Kurita, K. (2006). Chitin and chitosan: functional biopolymers from marine crustaceans. Mar. Biotechnol., 8: 203–226.

Mahdi, A.A. (2011). Biochemical changes in heat stressed wheat grown at El Wadi El Gedeed. M.Sc. Thesis, Fac. Sci., Ain Shams Univ., Egypt.

Mahdi, A.A. (2017). Biochemical role of some nanoparticles to counteract heat stress in wheat. Ph.D. Thesis, Fac. Sci., Ain Shams Univ., Egypt.

Mahdi, A.A., M.M. El-Saber, A. Osman, A.H. Hassan and K.Y. Farroh (2020). Toxicological and significant execute of biocompatible CuO nanoparticles and their influences on biochemical and molecular markers of wheat genotypes under saline conditions. IOSR. J. Biotec. Bioch., 6 (5): 58-68.

Mahdy, R.M.A. (2019). Effect of nano particles of chitosan, calcium and copper on growth, yield, quality and storability of onion (*Allium cepa* L). Ph.D. Thesis, Fac. Agric., Tanta Univ.

Marmouzi, I., A. Bouyahya, S.M. Ezzat, M. El Jemli and Kharbach (2021). The food plant *Silybum marianum* (L.) Gaertn.: phytochemistry, ethnopharmacology and clinical evidence. J Ethnopharmacol., 30 (265): 113303.

Mondal, A., R. Basu, S. Das and P. Nandy (2011): Benefcial role of carbon nanotubes on mustard plant growth: An agricultural prospect. J. Nanopar. Res., 13: 4519-4528.

Murray, R.D. (1997). Naturally Occuring Plant Coumarins. In: "Progress in the Chemistry of Organic Natural Products". Springer: NewYork, pp. 2-105.

Oktay, M., L. Gulcin and O.L. Kufrevioglu (2003). Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebeen. Wissen. Technolo., 36: 263-271.

Parisi, C., M. Vigani and E. Rodríguez-Cerezo (2015). Agricultural Nanotechnologies: What are the current possibilities? Nano Today, 10 (2): 124-127.

Pascual, M.B., J. El-Azaz, F.N. de la Torre, R.A. Cañas, C. Avila and F.M. Cánovas (2016). Biosynthesis and metabolic fate of phenylalanine in conifers. Front. Plant. Sci., 7: 1030.

Pinedo-Guerrero, Z.H., A.D. Hernández-Fuentes, H. Ortega-Ortiz, A. Benavides-Mendoza and G. Cadenas-Pliego (2017). Cu nanoparticles in hydrogels of chitosan-PVA affects the characteristics of post-harvest and bioactive compounds of jalapeño pepper. Molecules, 22: 926.

Plaksenkova, I., M. Jermaļonoka, L. Bankovska, I. Gavarāne, V. Gerbreders, E. Sledevskis, J. Sniķeris and I. Kokina (2019). Effects of Fe_3O_4 nanoparticle stress on the growth and development of rocket *Eruca sativa*. J. Nanomat., 2019: 1–10.

Reham, M.S., M.E. Khattab, S.S. Ahmed S.S. and M.A.M. Kandil (2016). Influence of foliar spray with phenylalanine and nickel on growth, yield quality and chemical composition of genoveser basil plant. Afr. J. Agri. Res., 11 (16): 1398-1410.

Rinaudo, M. (2006). Chitin and chitosan: properties and application. Prog. Polym. Sci., 31 (7): 603–632.

Roberson, E. (2008). Nature's Pharmacy, Our Treasure Chest: Why We Must Conserve Our Natural Heritage. Center for Biological Diversity. www.biologicaldiversity.org.

Ruiz-Herrera, J., J.M. Gonzalez-Prieto and R. Ruiz-Medrano (2002). Evolution and phylogenetic relationships of chitin synthases from yeasts and fungi. FEMS Yeast Res., 1: 247–256.

Sachet, T.F.K., M.H.S. Al-Mohammad and J.A. Abbass (2021). Effect of biofertilizer and spraying phenylalanine on mineral content and antioxidant compounds of dill. Indian J. Forensic. Med. Toxicol., 15 (3): 5540-5545.

Shamov, M., S.Y. Bratskaya and V. Avramenko (2002). Interaction of carboxylic acids with chitosan: effect of pK and hydrocarbon chain length. J. Colloid. Interface. Sci., 249: 316–321.

Sharifi-Rad, R., S.E. Bahabadi, A. Samzadeh-Kermani and M. Gholami (2020). The effect of non-biological elicitors on physiological and biochemical properties of medicinal plant *Momordica charantia* L. Iran. J. Sci. Technol. Trans. Sci., 44: 1315–1326.

Sheikhalipour, M., B. Esmaielpour, M. Behnamian, G. Gohari, M.T. Giglou, P. Vachova, A. Rastogi, M. Brestic and M. Skalicky (2021). Chitosan selenium nanoparticle (Cs-Se NP) foliar spray alleviates salt stress in bitter melon. Nanomaterials (Basel, Switzerland), 11 (3): 684.

Shi, R., C.M. Shuford, J.P. Wang, Y.H Sun, Z. Yang, H.C. Chen, S. Tunlaya-Anukit, Q. Li, J. Liu, D.C. Muddiman, R.R. Sederoff and V.L. Chiang (2013). Regulation of phenylalanine ammonia-lyase (PAL) gene family in wood forming tissue of *Populus trichocarpa*. Planta, 238: 487–497.

Tegeder, M. and J.M. Ward (2012). Molecular evolution of plant AAP and LHT amino acid transporters. Front. Plant. Sci., 3: 11.

Worthington Biochemical Corp. (1972). In: "Worthington Enzyme Manual". Worthington Biochemical Corp., Freehold, N.J.

Yang, A., J. Wu, C. Deng, T. Wang and P. Bian (2018). Genotoxicity of zinc oxide nanoparticles in plants demonstrated using transgenic *Arabidopsis thaliana*. Bull. Environ. Contam. Toxicol., 101: 514-520.

Yin, H., X.C. Fretté, L.P. Christensen and K. Grevsen (2011). Chitosan oligosaccharides promote the content of polyphenols in Greek oregano (*Oreganum vulgare* ssp. *hirtum*). J. Agric. Food. Chem., 60: 136-143.

جزيئات الشيتوزان النانوية وأثرها على النمو والمحصول وبعض الدلالات البيوكيميائية والجزيئية في نبات الخرشوف البري

أسماء عبد القادر مهدي[١*]، حمدي محمد إبراهيم[١]، خالد يحيى فروح[٢]، عماد صالح حامد[٣] وعمران غالي[٤،٥]

[١]وحدة الكيمياء الحيوية، قسم الأصول الوراثية، مركز بحوث الصحراء، القاهرة، مصر
[٢]المعمل المركزي للنانوتكنولوجى والمواد المتقدمة، مركز البحوث الزراعية، الجيزة، مصر
[٣]قسم النباتات الطبية والعطرية، مركز بحوث الصحراء، القاهرة، مصر
[٤]المعشبة، مركز بحوث الصحراء، القاهرة، مصر
[٥]مراكز التنمية الإقليمية، أكاديمية البحث العلمي والتكنولوجيا، مصر

أجريت تجربة حقلية خلال موسمي ٢٠١٩ /٢٠٢٠ و٢٠٢٠/٢٠٢١ في المركز الإقليمي لحفظ وصيانة الموارد الوراثية النباتية المهددة بالانقراض في مدينة الحمام، محافظة مطروح، مصر. تم إجراء هذا البحث على نبات الخرشوف البري المزروع ومعاملته بالآتي: فينيل ألانين وحمض الكيومارين عند ٤٠ ملجم/لتر ومعاملات الشيتوزان النانوية المختلفة مقارنة مع مجموعة الكونترول. تهدف هذه الدراسة إلى تحديد تأثير المعاملات على النمو والمحصول وبعض الدلالات البيوكيميائية والجزيئية المختلفة. تم تقدير DLS وZeta وحيود الأشعة السينية للعديد من مركبات الشيتوزان النانوية. أظهرت النتائج أن أعلى الزيادات المعنوية في الوزن الجاف/نبات ووزن البذور/نبات ومحصول البذور/فدان تم الحصول عليها بإستخدام الفينيل ألانين ٤٠ ملجم/لتر يليها الشيتوزان ٢٠٠ ملجم/لتر. كانت أفضل الدلالات البيوكيميائية في نفس المعاملة. وعلى المستوى الجزيئي، لا توجد تغيرات ملحوظة في المادة الوراثية وكانت بمعدل ٨٣.٥٩٪ لجميع المعاملات. تم الكشف عن أعلى زيادة في محتوى السيليمارين الكلي في بذور النبات بواسطة الشيتوزان النانوي ٢٠٠ ملجم /لتر، تليها معاملة الفينيل ألانين ٤٠ ملجم /لتر . وفقًا لقياسات الكم والجودة، توصي الدراسة بتطبيق معاملة الشيتوزان النانوي ٢٠٠ ملجم /لتر في المرحلة الخضرية للنباتات و لها أيضًا أعلى معدلات مضادات الأكسدة والأقل سمية للحصول على أعلى المواصفات والربح.