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Silver nanoparticles and *Chlorella* treatments induced glucosinolates and kaempferol key biosynthetic genes in *Eruca sativa*

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Abstract

Background: Microalgae and nanoparticles are currently considered promising tools for numerous agricultural and biotechnological applications. The green microalga *Chlorella* sp. MF1 and its biosynthesized silver nanoparticles (AgNPs) were used in this study as biofortification agents to enhance glucosinolate and kaempferol levels in *Eruca sativa*. UV–visible spectroscopy, XRD, FTIR and TEM were comprehensively used for characterizing *Chlorella*-based AgNPs.

Results: The biosynthesized AgNPs were found to be spherical in shape, with size ranging from 1.45 to 5.08 nm. According to FTIR measurements, silver ions were reduced to AgNPs by functional groups such as amide, hydroxyl and carboxylate. Different experimental treatments were conducted, including either soaking seeds of *E. sativa* or foliar spray with various concentrations of *Chlorella* suspension (1, 2, 3 and 4 g L^{-1}) and AgNPs (5, 10, 20 and 40 mg L^{-1}). Expression levels of five key genes in the biosynthetic pathway of glucosinolates (*MAM1*, *SUR1*, *MYB34* and *MYB51*) and kaempferol (*CHS*) were assessed using qRT-PCR. The results indicated an upregulation in the gene expression levels in all treatments compared to control, recording the highest level at 40 mg L^{-1} AgNPs and 4 g L^{-1} *Chlorella* suspension. In addition, high glucosinolates and kaempferol content was detected in plants whose leaves were sprayed with AgNPs and *Chlorella* suspension (40 mg L^{-1} and 4 g L^{-1}) based on HPLC analysis. Sequence analysis of amplified *CHS* fragments from *E. sativa* plants treated with AgNPs (40 mg L^{-1}) showed high sequence similarity to *A. thaliana CHS* gene. However, there were several *CHS* regions with sequence polymorphism (SNPs and Indels) in foliar sprayed plants.

Conclusions: Results of this study evidenced that the application of AgNPs and *Chlorella* suspension increased glucosinolates and kaempferol content in *E. sativa* through upregulation of key genes in their biosynthetic pathway.

Keywords: AgNPs, Marine microalga, *Eruca sativa*, Glucosinolates, Kaempferol, *Chalcone synthase*, Real-time PCR

1 Background

The interest in the bioavailability and biological effects of secondary metabolites in plants has been increased in the past two decades [78, 102]. These are identified by their direct free radical-scavenging and antioxidant activities. They can also induce expression of various genes encoding metabolic enzymes, which are thought to decrease the risk of various diseases and disorders [7, 57, 66].

Eruca sativa Mill. (arugula or rocket) is an edible annual plant from the Brassicaceae family. Because of its fresh peppery taste, it is utilized as a leaf vegetable. It is native to the Mediterranean area, but it is found all over the world [32]. Rocket leaves are a source of various bioactive compounds such as carotenoids, fibers, flavonoids, glucosinolates, polyphenols and vitamin C [25, 97]. Glucosinolates are nitrogen- and sulfur-containing plant

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secondary metabolites present in plants of the order Brassicales [40]. Glucosinolates and numerous decomposition products possess several biological activities [55]. They are frequently studied as part of the plant defense system against insects, herbivores, and microbial pathogens [64]. However, glucosinolates have recently attracted great research attention because of their potential health benefits in degenerative diseases such as cancer, cardiovascular, and neurodegenerative diseases [11, 83].

Enhanced glucosinolates accumulation in plants has been related to high expression levels of genes encoding the glucosinolate biosynthesis enzymes or transcription factors associated with the regulation of these genes, such as *methylthioalkylmalate synthase 1* (*MAM1),* myeloblastosis transcription factor *34* (*MYB34),* myeloblastosis transcription factor *51* (*MYB51) and SUPER-ROOT 1* (*SUR1)* [51, 92]. *MAM1* and *SUR1* are aliphatic glucosinolate biosynthetic genes, whereas *MYB34* and *MYB51* regulate genes encoding enzymes in the indolic glucosinolate biosynthetic pathway in *E. sativa* [49]. Glucosinolates biosynthesis occurs in three successive steps: amino acid elongation for aliphatic GSLs, core structure formation, and side chain modification. In the first step, methionine is de-aminated to a 2-oxo-acid, which in turn is condensed with acetyl-CoA. A subsequent decarboxylation step leads to the net gain of one methyl group, and this elongation cycle can be repeated two to six times [23]. These reactions are catalyzed by enzymes belonging to the methylthioalkylmalate synthases (MAMs) family [36]. In the second step, the resulting chain elongated products are oxidized to the corresponding aldoximes by cytochrome P450 enzymes [72]. A C-S lyase (SUR1) catalyzes the cleavage of the S-alkylthiohydroximates and the formation of thiohydroximates [71]. Then, thiohydroximates are glucosylated for the formation of desulfoglucosinolates. The core structure formation is terminated by the sulfation reactions of desulfo-GSLs [80]. The final step of GSL biosynthesis includes the side chain modification reactions, which are responsible for the differences in biological activity of GSL by-products found in plants [52]. Transcriptional regulation of GSL biosynthetic genes is reportedly under the control of MYB-family transcription factors [96].

On the other hand, kaempferol is a dominant flavonoid found generally in vegetables and fruits. *E. sativa* has been reported as one of the major sources of kaempferol [70, 87]. Dietary kaempferol has attracted extensive attention because of the beneficial effects on human health [13, 15, 16, 35, 42]. The first enzyme in flavonoid biosynthesis, chalcone synthase, is tightly regulated in response to a variety of environmental and developmental stimuli [22, 98, 104].

Plants exposed to different metals have been found to stimulate essential enzymes in the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL) and chalcone synthase [94]. Recently, total phenolic compounds: tannins and flavonoids, in roots of red radish were affected by foliar application of ZnO and FeO nanoparticles [65]. Besides their role in improving the effectiveness and bioavailability of new fertilizers, nanofertilizers are used to reduce the amount of these materials lost to the surrounding environment [90]. The most interesting metal nanoparticles are silver nanoparticles (AgNPs) due to their strong biological activity [38, 60]. They affect plants at many different levels [69, 106]. Their positive effects include stimulation of germination [79], growth invigoration [43], and increment of biomass accumulation [37]. In wheat plants, the application of AgNPs enhanced the agro-morphological criteria including photosynthesis and yield [86].

Microalgae are ubiquitous photosynthesizing microscopic phototrophs: autotrophs or heterotrophs that grow in a wide range of aquatic habitats [14]. They provide organic matter for plant growth [82] and improve soil structure and porosity through polysaccharides and mucilage secretions [47]. Also, microalgae have been reported to associate with higher plants in a symbiotic relationship [34]. Moreover, they were used as a biofertilizer for a variety of crops, tomato [77], cucumber, eggplant and lettuce [29], spinach [28], wheat [88, 93] and maize [26]. Furthermore, using microalgae in crops has been shown to increase biomass, fruit quality and vegetable yield. *Chlorella vulgaris* as a biofertilizer has been found to be cost-effective in ameliorating soil nutrients for increasing the productivity of *Hibiscus esculentus* [3].

Currently, both microalgae and nanoparticles have been reported as promising tools for many biotechnological applications via enhancing production of diverse phytochemicals, especially medicinal natural products [43, 50, 68]. The main goal of this study is to investigate the impact of AgNPs and *Chlorella* sp. MF1 suspension on expression of five key genes in the biosynthetic pathway of two highly important groups of secondary metabolites (glucosinolates and kaempferol) in *E. sativa* plants as well as to assess their effect on glucosinolates and kaempferol content. In addition, amplification and sequencing of *chalcone synthase* (*CHS*) PCR fragments were conducted to investigate the potential sequence variation in *CHS* PCR products of *E. sativa* plants treated with high level of AgNPs compared with control.

2 Methods

2.1 Cultivation of *Chlorella*

The marine microalga *Chlorella* sp. MF1 was procured from the Taxonomy and Biodiversity Lab of Aquatic

Biota, National Institute of Oceanography and Fisheries, Egypt. It was cultivated in BG11 broth medium [89]. Before autoclaving, the pH of the medium was adjusted to 7.5. Then, microalga was incubated for 15 days at room temperature with light/dark condition of 16:8 h and gently regular agitation in sterile 1000-mL Erlenmeyer flasks containing 500 mL medium and 10% inoculum. *Chlorella* biomass was collected by centrifugation at 4500 rpm for 10 min at the end of the logarithmic phase, washed twice with distilled water and dried in an oven at 80 °C for 16 h.

2.2 Biosynthesis and characterization of AgNPs

Algal extracts were prepared by powdering the dried algal biomass with a pestle and mortar, then suspending 10 mg in 100 mL of double distilled water and filtering with Whatman No.1 filter paper. Finally, the filtrate was utilized for biosynthesis of AgNPs by mixing of 10 mL $AgNO_3$ solution (10 mM) and 100 mL of algal extract, the reduction reaction of silver nitrate was started. Then, the reaction mixture was heated at 60 °C for 1 h. Nanoparticles formation was observed using color alteration. The produced AgNPs were centrifuged at 15,000 rpm for 20 min at 4 °C, and the pellet was washed several times with dist. H_2O and finally with 90% ethanol to get pure AgNPs powder. The UV–Vis Spectrophotometer (Lambda 25, PerkinElmer, UK) was used to characterize AgNPs. The wavelength ranged between 300 and 800 nm. FTIR Spectroscope (Bruker Optics, GmBH, Germany) was used to measure the FTIR spectra of AgNPs using KBr pellet. In addition, the morphology of AgNPs was examined using a TEM (Jeol, Japan), and the XRD pattern was evaluated using a Diffractometer (Xpert-Pro, England) at room temperature using 30 mA and 40 kV. The peaks were recorded at 2θ range of 20°–80°.

2.3 Plant treatments

Seeds of *E. sativa* Mill. (Sementi Da Orto Prato Flore Andria, Milan, Italy) were surface sterilized with 10% commercial bleach [5% (w/v) sodium hypochlorite] for 10 min and rinsed three times with distilled water before sowing. Three principal treatments were carried out as follows: Controls: 20 seeds were sown in a plastic pot, filled with 2 kg of sieved air-dried clay and sand (2:1) soil and left to grow in the greenhouse of the Botany and Microbiology Department, Faculty of Science, Tanta University, Egypt. Soaking treatments: 200 seeds were soaked for 4 h in 10 mL of 5, 10, 20, and 40 mg L^{-1} AgNPs or 1, 2, 3 and 4 g L^{-1} *Chlorella* suspension and then, seeds of each treatment were sown in 3 pots as described above. Foliar treatments: 200 seeds were sown in pots as described in controls. After 14 days, leaves were sprayed twice a week with 5, 10, 20, and 40 mg L^{-1} AgNPs or 1, 2, 3, and 4 g L^{-1} *Chlorella* suspension. After 35 days, leaves were gathered and frozen in liquid nitrogen, then stored at − 80 °C for further analysis. For both treatments and controls, three replicates were used.

2.4 Measurement of plant Ag content

The mixed acid digestion method was used for determination of Ag content in *E. sativa*-treated plants (sprayed or soaked) according to Allen et al. [5]. An amount (0.1 g) of plant dry powder was mixed in a 150-mL digestion flask with 69% Nitric acid and 30% H_2O_2 (5:2 v/v). The mixture was heated gently until the disappearance of nitric acid fumes, and the whole mixture turned into a pale-yellow clear solution. Silver was evaluated by using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), PerkinElmer/Optima 7000 apparatus at the Central Lab of Tanta University.

2.5 Gene expression analysis

Quantitative real-time PCR (qRT-PCR) was used to assess the mRNA transcript levels of the five studied genes. To extract total RNA, 50–100 mg of leaves were homogenized in liquid nitrogen and then extracted using the TRIzol™ Reagent (Invitrogen, Germany) according to the manufacturer's instructions. A DNase I treatment was performed to eliminate DNA contamination. Then, 1 µg of total RNA was utilized as a template for cDNA synthesis using the QuantiTects Reverse Transcription Kit (Qiagen, USA). cDNA aliquots were used as a template for qRT-PCR analysis using gene-specific primers and β-actin as a house-keeping gene (Table 1). qRT-PCR was conducted to assess gene expression using Rotor-Gene Q (Qiagen, USA). cDNA amplicons were amplified with Maxima SYBR Green/Fluorescein qPCR Master Mix according to the manufacturer's protocol. The following procedure for reactions was applied: 1 cycle of 50 °C 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 60 s. Relative gene expression was estimated as fold change using $2^{-\Delta\Delta ct}$. The house-keeping gene was used for normalization and determination of the relative amount of gene expression [63].

2.6 HPLC analysis of glucosinolates and kaempferol

2.6.1 Glucosinolates extraction and quantification

For glucosinolates extraction, leaf samples were ground in liquid nitrogen and pulverized into a fine powder. Isolation of glucosinolates was conducted according to Kliebenstein et al. [52]. Twenty milligrams of powdered leaf material were used for glucosinolates extraction. The sephadex/sulfatase protocol was followed with slight changes. To extract glucosinolates, the following mix was used: 400 µL of methanol, 10 µL of 0.3 M lead acetate, 120 µL of water and 12 µL of glucotropaeolin as internal

Table 1 Sequences and names of forward and reverse primers of five key genes related to glucosinolates and kaempferol synthetic pathway as well as primers used for *Eruca sativa CHS* PCR amplification and sequencing

Gene name	Primer sequence (5′–3′)	References
MAM1 (*E. sativa*)	MAM1-F: 5′-ACTCCACCGCAGAAGATTG-3′ MAM1-R: 5′-CCTCATCCACCTCGTTTCC-3	[49]
SUR1 (*E. sativa*)	SUR1F: 5′-CATTGTTGTGATTAATCCCAAC-3′ SUR1R: 5′-TCCGAATATAGTCTGATCATAA-3	[49]
MYB34 (*E. sativa*)	MYB34-F: 5′-CTACATGGTGAAGGTGGATGG-3′ MYB34-R: 5′-ATCGGGTCTTAGATAGTTAGCC-3	[49]
MYB51 (*E. sativa*)	MYB51-F: 5′-CCTTCACACCAACCTCATCAG-3′ MYB51-R: 5′-AACACAAGACTCTCCGAACATC-3	[49]
CHS (*Arabidopsis thaliana*)	CHS-F: 5′-AAGCTCTCACTCTCCGGT-3′	
	CHS-R: 5′- TCGTGTGAGTCCCTTGCT-3′	[61] (*used for qRT-PCR analysis*)
β-actin	Actin-F: 5′-CGCCGCTTAACCCTAAGGCTAACAG-3′ Actin-R: 5′-TTCTCTTTAATGTCACGGACGATTT-3′	[9]
CHS (*Arabidopsis thaliana*)	CHS-F1: 5′-ACTTCCGCATCACCAACAGT-3′ CHS-R1: 5′-AGAGGGACCACGGAAGGTAA-3′ CHS-F2:5′-TTACCTTCCGTGGTCCCTCT-3′ CHS-R2: 5′-TGTTCTTGGAGATGAGGCCG-3′	This study (*used for PCR amplification and sequencing*)

MAM1: *Methylthioalkylmalate synthases 1*, *SUR1*: *SUPERROOT 1*, *MYB34*: *Myeloblastosis transcription factor 34. MYB51*: *Myeloblastosis transcription factor 51*, *CHS*: *Chalcone synthase*, *β-actin*: Reference gene

standard were added. 10 µL of water and 10 µL of sulfatase (Sigma-Aldrich, St. Louis) solution were added to each column to desulfate glucosinolates and then incubated overnight at room temperature. Desulfoglucosinolates were eluted with 100 µL of 60% (v/v) methanol and twice with 100 µL of distilled water. This experiment was carried out five times for each sample. Then, the extracts were utilized for the separation and identification of the glucosinolates.

For quantification of glucosinolates, 20 µL of the glucosinolates extract were used. The chromatographic analyses were conducted using a high-performance liquid chromatography (HPLC; Waters, Milford, MA) equipped with a 2695 Waters Separations Module and a 996 Waters photodiode array detector. The HPLC column was a C18 Waters Symmetry Shield RP18 (5 µm particle size, 150 × 4.8 mm i.d.) and protected with a C18 guard column (Waters). The oven temperature was pre-heated to 30 °C. The glucosinolates were identified at 229 nm and separated by using the following program with a flow rate of 0.8 mL min^{-1}: three min. at 100% H_2O, 23 min. gradient from 0 to 25% (v/v) acetonitrile, 1 min. at 25% (v/v) acetonitrile, 9 min. gradient from 25 to 0% (v/v) acetonitrile and a final 4 min. at 100% H_2O.

2.6.2 Kaempferol extraction and quantification

Frozen ground samples (2.0 g) were mixed with 80 mL of methanol/water (62.5:27.5, v/v) in a 500-mL flask with a round bottom and spiked with an internal standard of kaempferol (Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H1-benzopyran-4-one, 95%, Sigma-Aldrich St. Louis, MO, USA) to a final concentration of 0.005 mg mL^{-1} and then refluxed for 1.5 h at 100 °C [53]. After cooling, a 2.0 mL aliquot was centrifuged and a sub-aliquot of 200 mL of hydrolysate was mixed with 200 mL of methanol. After that, the mixture was sonicated for 10 min. before being filtered through 0.45 mm Millex-FH (PTFE) SLFH 013 NL (Millipore, Bedford, MA, USA) filters and quantified as described in the case of glucosinolates.

Glucosinolate and kaempferol contents are given as µg per gram dry weight (DW) leaf powder calculated from HPLC peak areas using response factor computed for standard at 229 nm [52].

2.7 *Chalcone synthase* (*CHS*) amplification and sequencing

In order to evaluate the influence of seed soaking and foliar spraying of 40 mg L^{-1} AgNPs on amplified *CHS* PCR products of *E. sativa*, PCR was used to amplify the *CHS* gene sequence using gene-specific primers (Table 1). The *A. thaliana CHS* gene sequence (GenBank Accession No.: AY612787.1) was used to design these two primer sets. The primer set (CHSF1R1 and CHSF2R2) was designed to amplify 488 and 252 bp, respectively. First, total genomic DNA was isolated from AgNPs-treated and control *E. sativa* plants using Thermo Scientific GeneJET plant genomic DNA purification mini Kit (K0792/Thermo Fisher, USA) following the manufacturer's instructions.

DreamTaq PCR Master Mix (2×) (K1071/Thermo Fisher USA) was used for PCR amplification. The thermal cycler conditions were as follows: denaturation at 94 °C for 30 s followed by annealing at 55 °C for 30 s. and extension at 72 °C for 30 s. There was an initial delay of 15 min at 95 °C at the beginning of the first cycle and a 10 min delay at 72 °C at the end of the last cycle as a post extension step. PCR products were resolved on 1.5% agarose using TBE as running buffer and at 80 V for 100 min. The expected PCR bands were extracted from agarose gel and purified with E.Z.N.A.® Gel Extraction Kit (V-spin) (Omega Bio-TEK, USA) Microspin filters and quantified spectrophotometrically. The sequencing of the purified PCR bands was conducted using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp, Korea).

2.8 *Chalcone synthase* (*CHS*) sequence analysis

The sequences of all PCR products were compared against sequences in the NCBI database using BLASTn software. Sequence alignment of *E. sativa* and sequences showing high similarity was conducted with MEGAX (version10.1.8) software. The phylogenetic tree was obtained automatically by applying neighbor-joining algorithms to the matrix of pairwise distances estimated using the Jukes–Cantor model. The phylogenetic tree was generated automatically using a matrix of pairwise distances by applying neighbor-joining algorithms. 1000 was used as the bootstrap value.

2.9 Statistical analysis

All results were presented as the mean of 3 replicates ± standard error (SE). Differences among treatments for all measured variables were tested by one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test when significant differences were found ($p < 0.05$). To test whether an equal effect of AgNPs and *Chlorella* sp. suspension on glucosinolates and kaempferol content was observed in a test combination, Chi-square test was performed. JMP statistical analysis software ver. 13.2.0 (Statistical Discovery From SAS©, USA) was used.

3 Results

3.1 Characterization of biosynthesized AgNPs

3.1.1 Ultraviolet–visible spectrophotometer analysis

One of the most frequently used approaches for structural characterization of nanoparticles is UV–visible spectroscopy. The absorption spectrum of biosynthesized AgNPs revealed that AgNPs have a strongly symmetric single band absorption with a peak limit at 449 nm signifying Ag nanoparticles (Fig. 1a).

3.1.2 Transmission electron microscopy (TEM)

To complement the above optical and spectroscopic characterization and assign size and morphology of the produced silver nanoparticles, TEM was carried out. The TEM images showed that the particles exhibit small size disparity. The spherical shape of AgNPs was observed and their size ranged from 1.45 to 5.08 nm with a mean of 3.19 ± 0.14 (Fig. 1b).

3.1.3 Fourier-transform infrared (FTIR) spectroscopy

FTIR spectroscopy was employed to identify the functional groups responsible for the biosynthesis of

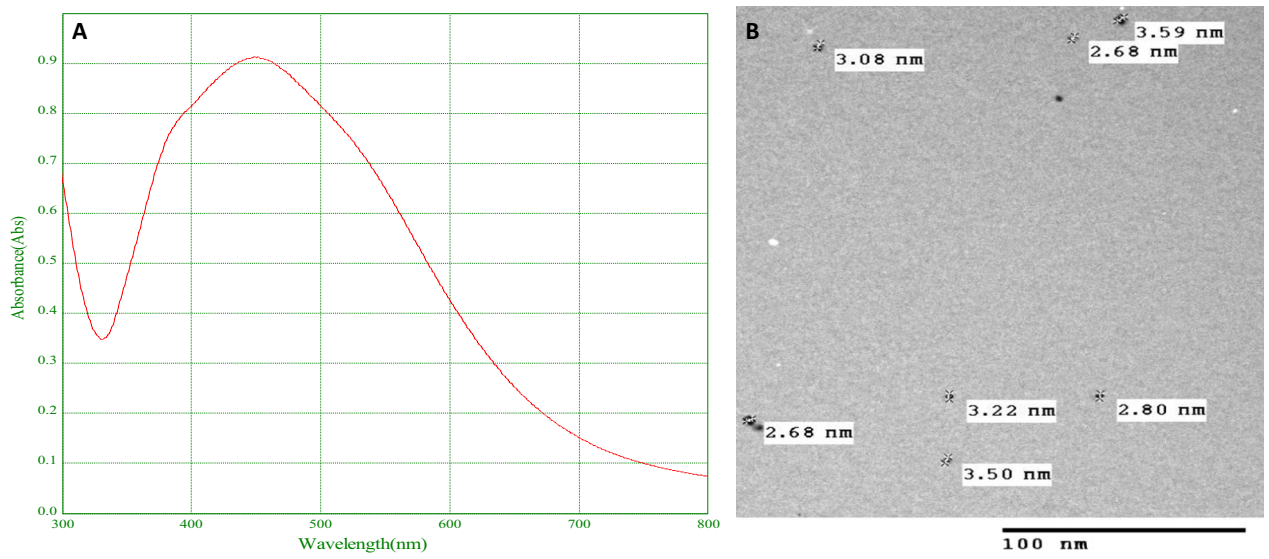

Fig. 1 UV–visible spectrum (**a**) and TEM analysis of biologically synthesized silver nanoparticles. (**b**) *TEM* transmission electron microscopy

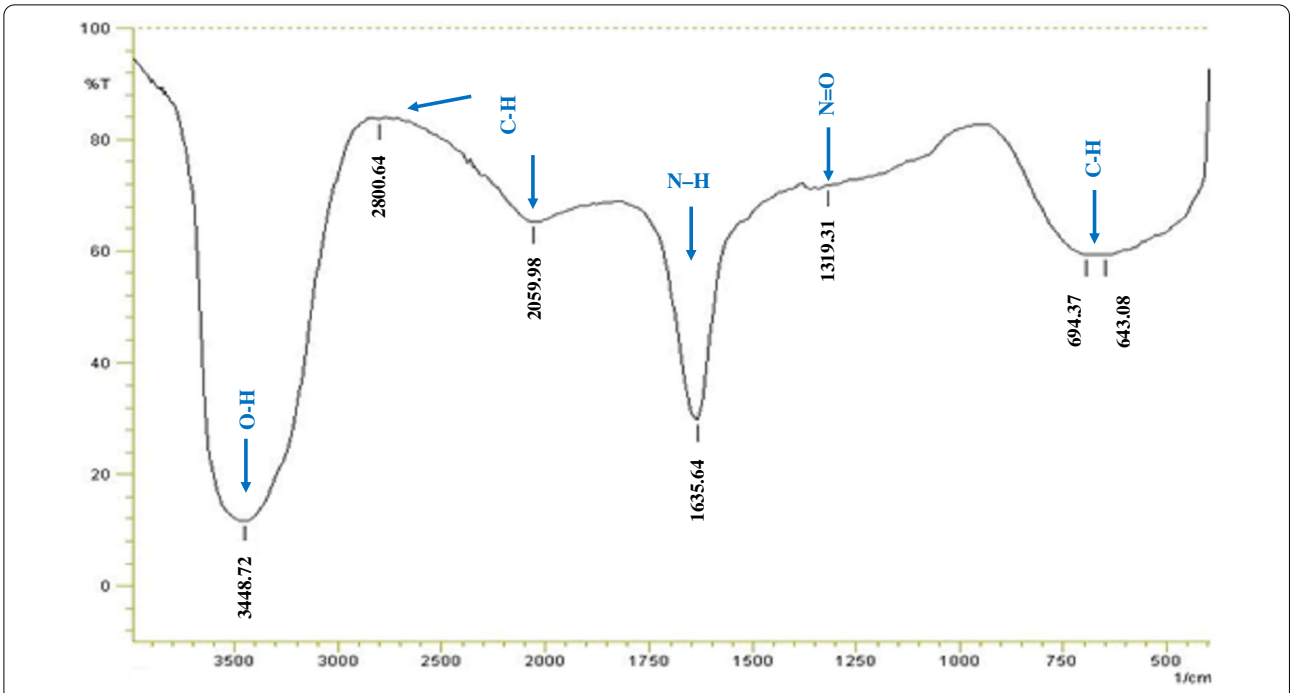

Fig. 2 FTIR spectrum of biologically synthesized silver nanoparticles (AgNPs) using *Chlorella* sp.

silver nanoparticles. The FTIR spectra of AgNPs are illustrated in Fig. 2. FTIR spectra showed a shift in the following peaks: 3488.72 (due to O–H stretching of phenol or carboxylic acids), 2059.8–2800.64 (due to C–H stretching of alkane), 1635.64 (due to N–H stretching mode in amide group which is commonly found in the protein), 1319.31 (due to N=O symmetry stretching typical of the nitro compounds) and 694.37–648.08 (due to C–H stretching of alkane).

3.2 Plant silver content

The accumulation of silver (Ag) in *E. sativa*-treated plants was measured using an ICP-OES apparatus. As shown in Table 2, no Ag (0.0 µg g^{-1}) was detected in control plants. On the contrary, Ag content increased gradually with increasing AgNPs level in *E. sativa* plants (spraying or soaking). This means that there is a positive relationship between the dose (spraying or soaking) of AgNPs used and Ag content in *E. sativa*-treated plants. Also, results indicate that spraying of *E. sativa* plants with AgNPs significantly increased Ag content, especially in plants sprayed with the high (40 mg L^{-1}) AgNPs.

3.3 Gene expression analysis

In this study, the expression levels of four key genes in the biosynthesis pathway of glucosinolates in *E. sativa* (*methylthioalkylmalate synthase 1 (MAM1), myeloblastosis transcription factor 34 (MYB34), myeloblastosis transcription factor 51 (MYB51*), *and SUPERROOT 1 (SUR1)*) were determined in response to treatment with AgNPs and *Chlorella* sp. suspension. In addition, the expression level of *chalcone synthase* (*CHS*) that is involved in biosynthesis of kaempferol was examined. The results revealed that the key genes (*CHS, MAM1, MYB34, MYB51 and SUR1*) responded differently to various treatments of AgNPs and *Chlorella* sp. suspension and their expression levels were significantly different from control plants, exhibiting the highest expression level of

Table 2 Means of silver content (µg g^{-1} DW), measured in *Eruca sativa* plants treated with different levels of silver nanoparticles (AgNPs), either spraying or soaking

Ag content (µg g^{-1})	Control	Sp. Nano-5	Sp. Nano-10	Sp. Nano-20	Sp. Nano-40	So. Nano-5	So. Nano-10	So. Nano-20	So. Nano-40
Mean ± SE	0.00 ± 0.0	0.79^{abc} ± 0.24	0.88^{abc} ± 0.18	0.92^{ab} ± 0.24	1.22^{a} ± 0.14	0.35^{c} ± 0.08	0.38^{bc} ± 0.20	0.53^{bc} ± 0.22	0.59^{bc} ± 23

Different letters indicate a statistically significant difference at $p < 0.05$

Sp. Nano- and So. Nano-: spraying or soaking with AgNPs (5, 10, 20, 40 mg L^{-1})

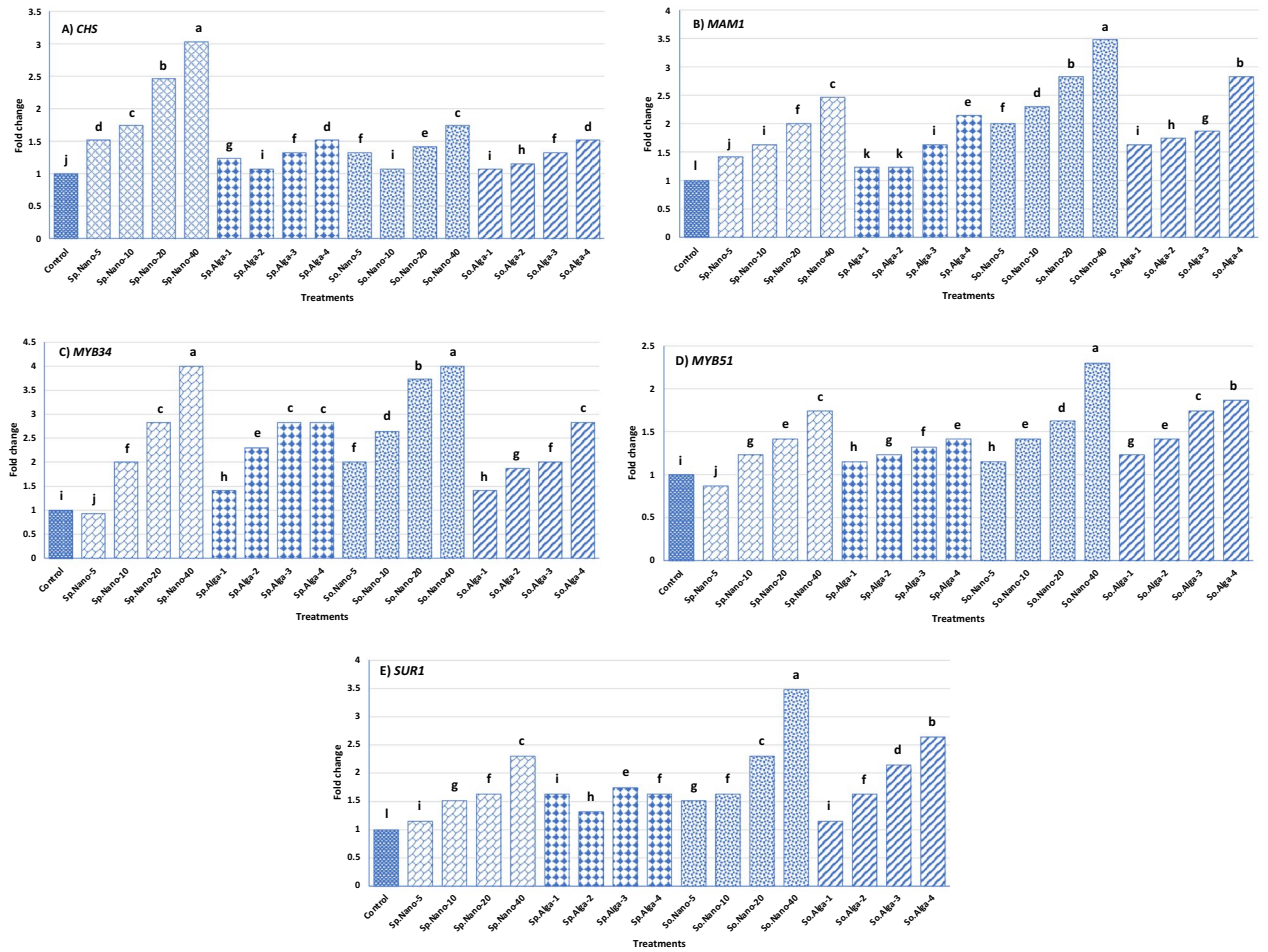

Fig. 3 Genes expression analysis of five glucosinolates and kaempferol-related genes in *Eruca sativa* plants treated either soaking or spraying with different concentrations of AgNPs and *Chlorella* sp. suspension. Different letters indicate statistically significant difference at $P < 0.05$. **a** *Chalcone synthase CHS*, **b** *methylthioalkylmalate synthases 1 (MAM1)*, **c** *myeloblastosis transcription factor 34* (*MYB34*), **d** *myeloblastosis transcription factor 51* (*MYB51*), E: *SUPERROOT 1* (*SUR1*). Sp. Nano- and So. Nano-: spraying or soaking with AgNPs (5, 10, 20, 40 mg L^{-1}), Sp. Alga- and So. Alga-: spraying or soaking with *Chlorella* sp. suspension (1, 2, 3, 4 g L^{-1})

these genes at 40 mg L^{-1} AgNPs and 4 g L^{-1} *Chlorella* sp. suspension (seed soaking and foliar spray) (Fig. 3).

The higher upregulation of *MAM1, MYB34, MYB51* and *SUR1* genes was observed in plants whose seeds were soaked with 40 mg L^{-1} AgNPs than with 4 g L^{-1} *Chlorella* sp. suspension. The highest fold change of these genes was found in plants whose seeds were soaked with 40 mg L^{-1} AgNPs was 2.48-, three-, 1.9- and 2.48-fold, respectively (Fig. 3b–d). Similarly, the highest fold change was observed in the case of plants whose leaves were sprayed with 40 mg L^{-1} AgNPs was 1.46-, three-, 0.74- and 1.29-fold, respectively.

On the other hand, the highest fold change (2.03-fold) of the *CHS* gene was observed in plants whose leaves were sprayed with 40 mg L^{-1} AgNPs (Fig. 3a). However, the highest *CHS* expression was found in plants whose seeds were soaked with 4 g L^{-1} *Chlorella* sp. suspension (Fig. 3a).

3.4 HPLC analysis of glucosinolates and kaempferol

Data in Table 3 and Fig. 4 showed that treating *E. sativa* plants with AgNPs and *Chlorella* sp. suspension (soaking and spraying) led to alteration of glucosinolates and kaempferol content compared with the control plants. The highest glucosinolates and kaempferol contents were detected in plants sprayed with AgNPs (40 mg L^{-1}) and with *Chlorella* sp. suspension (4 g L^{-1}), 81.92 µg g^{-1} DW and 80.70 µg g^{-1} DW, respectively (Table 3). Also, foliar spray with *Chlorella* sp. suspension (4 g L^{-1}), seeds soaking with AgNPs (40 mg L^{-1}) and seeds soaking with *Chlorella* sp. suspension (4 g L^{-1}) exhibited high glucosinolates and kaempferol

Table 3 Retention time, absorbance and content (μg g^{-1} DW) of glucosinolates and kaempferol in *Eruca sativa* plants treated with AgNPs (40 mg L^{-1}) and *Chlorella* sp. suspension (4 g L^{-1}) either spraying or soaking

	Control	Foliar spray (AgNPs)	Seed soaking (AgNPs)	Foliar spray (*Chlorella* sp. suspension)	Seed soaking (*Chlorella* sp. suspension)
		40 mg L^{-1}	40 mg L^{-1}	4 g L^{-1}	4 g L^{-1}
Glucosinolates					
Retention time (min)	13.2	13.3	13.32	13.11	10.41
Absorbance (229 nm)	23	391	194	230	66
Content (μg g^{-1} DW)	6.42	81.92	28.91	36.14	4.81
Kaempferol					
Retention time (min)	7.7	6.7	7.2	7.81	7.33
Absorbance (229 nm)	195	593	311	565	198
Content (μg g^{-1} DW)	33.11	42.42	57.94	80.70	30.17
Chi-square test					
χ^2	90.49				
p value	< 0.0001				

DW dry weight

contents compared with the control plants (Fig. 4). Chi-square test revealed that glucosinolates and kaempferol content depends on the type of treatment (Table 3). In other words, there is a significant variation in the concentration of glucosinolates and kaempferol among different treatments (p value < 0.0001).

3.5 Sequencing of *Eruca sativa CHS* gene fragments

In order to evaluate the effect of AgNPs on DNA of *E. sativa CHS*, specific primers were designed and used to amplify *E. sativa CHS* DNA fragments. *E. sativa*-specific *CHS* gene bands were eluted from agarose gel and were sequenced. The length of amplified DNA fragment from untreated *E. sativa CHS* (control) was 674 bp, while the length of *CHS* gene amplicon from AgNPs (40 mg L^{-1})-soaked plants was 677 bp. Finally, the length of the *CHS* gene fragment in the case of spraying AgNPs (40 mg L^{-1}) was 673 bp. The sequences of all 6 PCR amplicons were compared against sequences in the NCBI database using BLASTn software.

The results of NCBI BLASTn showed that all *CHS* sequences were strongly conserved and shared high sequence identity (> 99%) with *A. thaliana CHS* gene sequences. Also, NCBI BLAST showed that the two amplified sequences were highly similar to *A. thaliana chalcone synthase* (*CHS*) gene, partial cds (Accession no.: AY612787) with 99.77% sequence identity and 98% query coverage. The *CHS* sequences of untreated (control) and treated *E. sativa* (soaking AgNPs and spraying AgNPs treatments at 40 mg L^{-1}) were aligned with *CHS* sequences of *A. thaliana* and other *Arabidopsis* species using MEGAX (version 10.1.8) software. The multiple sequence alignment (MSA) analysis indicated that there are several single nucleotide polymorphisms (SNPs) and Indel (insertion and deletion) variations mainly in the *CHS* fragment sequence of *E. sativa* plants sprayed with AgNPs (40 mg L^{-1}) compared with control (untreated *E. sativa*), AgNPs (40 mg L^{-1})-soaked *E. sativa* plants and *Arabidopsis CHS* gene sequences.

The phylogenetic analyses revealed that the *CHS* fragment sequences of untreated *E. sativa* (control) were closely related to the *A. thaliana CHS* gene (AY612787.1), while the *CHS* fragment sequence of *E. sativa* plant sprayed with AgNPs (40 mg L^{-1}) and the *CHS* fragment sequence of *E. sativa* plant soaked with AgNPs (40 mg L^{-1}) were grouped together into a separate major cluster (Fig. 5).

4 Discussion

Glucosinolates (GSLs) and kaempferol are two highly important groups of secondary metabolites, which are found at high levels in *Brassica* species [6]. In this study, application of abiotic and biotic elicitors (AgNPs and *Chlorella* sp. suspension) was found to increase glucosinolates and kaempferol in *E. sativa* plants sprayed with 40 mg L^{-1} AgNPs and 4 g L^{-1} *Chlorella* sp. suspension. Such elicitors have modified plant primary and secondary metabolites, as reported by Marslin et al. [67], Chanda et al. [14] and Anjum et al. [6]. Nanoelicitation was found to be a powerful technique for increasing secondary metabolite synthesis, which can boost metabolism and induce physiological responses in plants [76].

In the present study, biosynthesis of silver nanoparticles (AgNPs) using suspension of the marine chlorophyte

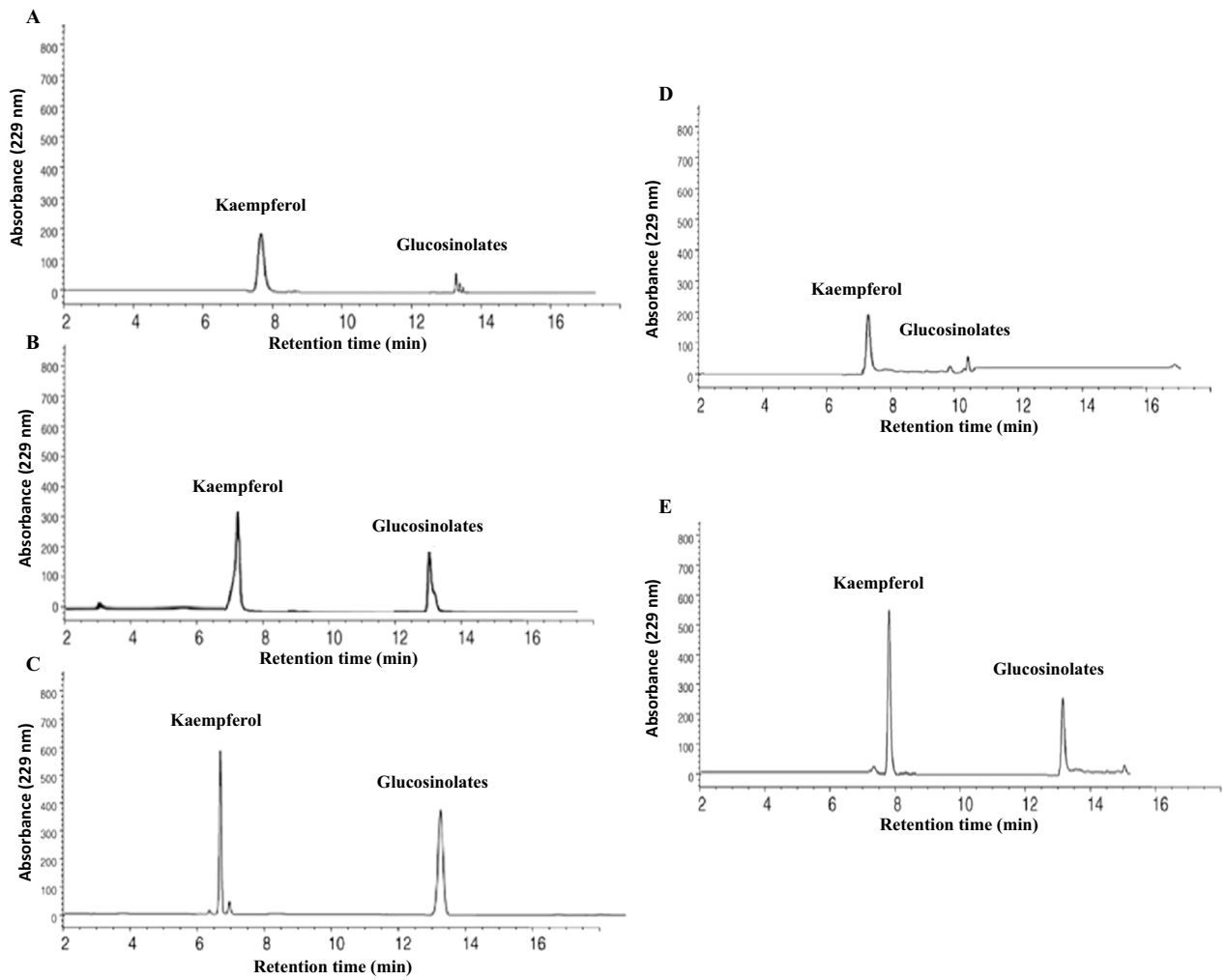

Fig. 4 HPLC chromatograms of glucosinolates and kaempferol showing their contents in *Eruca sativa* **a** control plants, **b** AgNPs (40 mg L^{-1}) seeds-soaked plants, **c** AgNPs (40 mg L^{-1}) leaves-sprayed plants, **d** *Chlorella* sp. suspension (4 g L^{-1}) seeds-soaked plants, **e** *Chlorella* sp. suspension (4 g L^{-1}) leaves-sprayed plants

Chlorella sp. MF1 was performed. For characterization and confirmation of biosynthesized AgNPs, UV–Vis spectroscopy, TEM and FTIR analyses were utilized. UV–Vis spectroscopy is regularly used to investigate AgNPs surface plasmon resonance (SPR). In this study, the UV–Vis spectrum revealed a single strong and large SPR peak at 449 nm (Fig. 1), indicating that AgNPs were synthesized successfully. Similarly, various studies have identified a surface plasmon resonance peak in the range of 410–450 nm as an indicator of AgNPs biosynthesis [21, 100, 105]. Also, TEM images of AgNPs biosynthesized in the present study indicated that AgNPs are spherical and have a diameter that ranged between 1.45 and 5.08 nm (Fig. 1). According to Pirtarighat et al. [81], spherical nanoparticles have only a single SPR band, and the number of peaks increases as the diversity of particle shapes increases. However, various forms of biologically synthesized nanoparticles, including rectangular, cubic, and spherical, have been observed in a study by Soleimani and Habibi-Pirkoohi [95].

In addition, FTIR analysis was used in the present study to classify potential biomolecules present in the algal extract, which are involved in AgNPs biosynthesis, stabilization and capping, according to Moldovan et al. [73]. The results showed the existence of various functional groups of phytochemicals such as amides, phenols and carboxylic groups in algal water extract, which were involved in the reduction of silver ions to AgNPs as well as their stabilization (Fig. 2). The presence of amides indicated that AgNPs were capped and

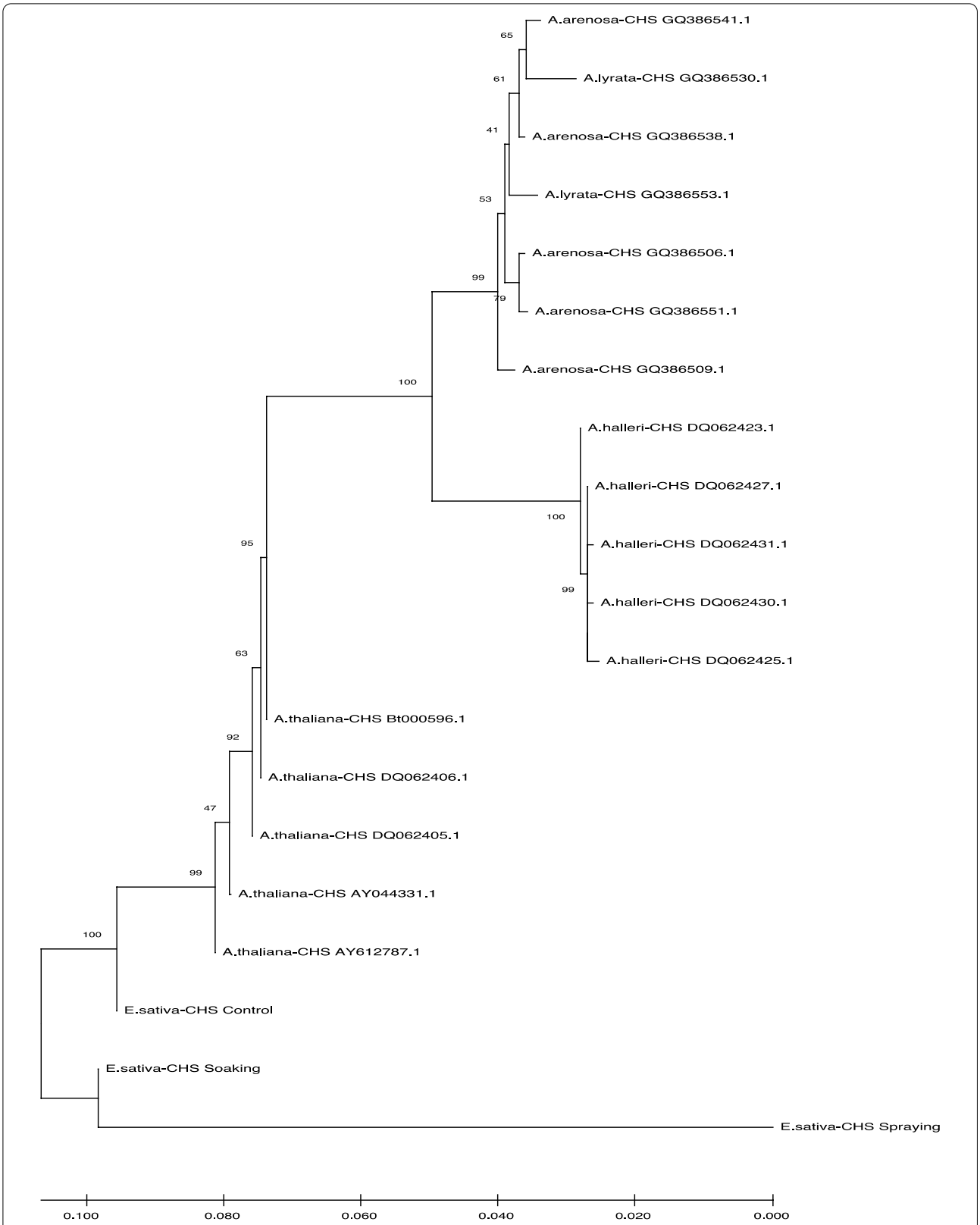

Fig. 5 Maximum likelihood phylogenetic tree of *CHS* fragment sequences of untreated, AgNPs (40 mg L^{-1}) soaked, AgNPs (40 mg L^{-1}) sprayed *Eruca sativa* plants and *CHS* gene sequences of four different *Arabidopsis* species

protected by the proteins present in the algal extract. This result is in accordance with Rajeshkumar et al. [84] and Abdul Razack et al. [1], who suggested that proteins are the major constituents in the algal extract involved in reducing Ag ions into nanoparticles, capping and stabilizing AgNPs by electrostatic forces. Moreover, negatively charged groups present in the algal extract, such as carboxylate (COO^-) and hydroxyl groups (OH^-), have a high tendency to bind Ag ions, contributing to both reduction (AgNPs biosynthesis), and stabilization of Ag ions [4, 48].

In this study, HPLC results exhibited higher content of glucosinolates and kaempferol in plants foliar-sprayed with AgNPs (40 mg L^{-1}) and *Chlorella* sp (4 g L^{-1}) than with the seed soaking method (Table 3 and Fig. 4). Similarly, the foliar application of AgNPs (20 and 40 ppm) increased total phenolics and carotenoids in oakleaf lettuce (*Lactuca sativa* L. var. *foliosa* Bremer) seedlings [45], while ZnO and FeO green synthesized nanoparticles increased total tannins and flavonoids in roots of red radish [65]. Moreover, foliar application of TiO_2 nanoparticles has significantly elevated carotenoids and phenolic compounds in the maize crop [74]. Furthermore, CuONPs-treated seedlings of *Brassica rapa* ssp. rapa also had substantially higher levels of glucosinolates and phenolic compounds than control seedlings [18].

Also, in this study, the expression levels of four key genes in the glucosinolate biosynthetic pathway (*MAM1, MYB34, MYB51* and *SUR1*) increased significantly in response to treatment with AgNPs or *Chlorella* sp. suspension. The highest expression level of these genes was observed when seeds were soaked with 40 mg L^{-1} AgNPs or 4 g L^{-1} *Chlorella* sp. suspension. In addition, the fold change was higher in the case of AgNPs treatment, 2.48-, three-, 1.9- and 2.48-fold, respectively (Fig. 3b–e). It has been reported that high expression levels of genes encoding glucosinolate biosynthesis pathway enzymes or transcription factors involved in their regulation, such as MAM1, MYB34, MYB51 and SUR1, have been linked to glucosinolate accumulation in plants [51, 92], which is similar to the finding of this study. In addition, *MAM1* and *SUR1* are aliphatic glucosinolate biosynthetic genes, whereas *MYB34* and *MYB51* regulate genes encoding enzymes in the indolic glucosinolate biosynthetic pathway in *E. sativa* [49]. As described above, there was an upregulation of the four key genes in the glucosinolate biosynthetic pathway in this study, which is in agreement with the finding of Thiruvengadam et al. [99], who detected that expression of aliphatic GSLs (*BrMYB28* and *BrMYB29*) and indolic GSLs (*BrMYB34* and *BrMYB51*) as well as aliphatic GSL biosynthetic genes (*St5C* and *SUR1*) transcripts was upregulated in plants treated with high concentrations (5 and 10 mg L^{-1}) of AgNPs.

Similarly, the expression of aliphatic GSLs (*BrMYB28* and *BrMYB29*) and indolic GSLs (*BrMYB34* and *BrMYB122*) transcripts of Chinese cabbage was upregulated in CuONPs-elicited hairy roots [17]. Moreover, higher concentrations of CuONPs (250 and 500 mg L^{-1}) increased the expression of aliphatic GSL (BrMYB28 and BrMYB29) and indolic GSL (BrMYB34 and BrMYB51) transcripts in *Brassica rapa* ssp. rapa seedlings [18]. Recently, Ramezani et al. [85] discovered that AgNPs function as elicitors and strongly increase the transcription of key genes (UGT85C2 (UDP-glycosyltransferases), KAH (Kaurenoic acid-13 hydroxylase), UGT74G1 and UGT76G1) of *Stevia rebaudiana* B. responsible for secondary metabolites involved in the pathway of glycosides biosynthesis. Similar to the finding of this study, microalgal extracts from *Chlorella vulgaris* and *Scenedesmus quadricauda* had bio-stimulant effects on the expression of sugar beet genes involved in various biological pathways and processes, such as primary and secondary metabolites [10]. Recently, Farid et al. [30] found that crude polysaccharides from *Chlorella sorokiniana* had an important stimulatory effect on *PAL* gene function, suggesting an upregulation of main genes in the phenolics pathway.

In contrast to the results of glucosinolate genes, plants treated with 40 mg L^{-1} AgNPs (foliar spray) as 4 g L^{-1} algal suspension (foliar spray) did not induce a high expression of *CHS*, 2.03- and 0.74-fold, respectively (Fig. 3a). It is well known that *CHS* is the first enzyme in the biosynthetic pathway of all flavonoids [98, 104]. As mentioned above, the results of this study indicated that *CHS* gene expression levels were upregulated in most treatments. It has been shown that *CHS* expression causes accumulation of flavonoids such as the natural flavonol kaempferol [12], which is in accordance with the finding of this study. Similarly, AgNPs upregulated the key genes of the flavonoid and anthocyanin biosynthetic pathway in *A. thaliana* as reported by Garcia-Sanchez et al. [31]. Moreover, the total phenolic and flavonoid contents, as well as the expression levels of their genes (*PAL, CHI* and *FLS*), were significantly high in CuONPs-elicited hairy roots [17].

The precise mechanism by which nanoparticles (NPs) regulate plant secondary metabolism is not yet fully understood. The upregulation of mitogen-activated protein kinase (MAPK) cascades, reactive oxygen species (ROS) and cytoplasmic Ca^{+2} efflux was thought to be increased as the plant's early responses to NPs treatment [46, 54]. In general, MAPK phosphorylation and activation of downstream transcription factors generally lead to the transcriptional reprogramming of secondary metabolism in plants [103].

In order to investigate the uptake and accumulation of AgNPs in *E. sativa* plants (soaked or sprayed with different AgNP levels), Ag content was measured in their leaves. The results showed a positive relationship between the dose of AgNPs and Ag content. However, the highest Ag content was 1.22 μg g^{-1} in plants sprayed with 40 mg L^{-1} (Table 2). This result suggests that AgNPs uptake in the case of foliar application through stomatal pores is higher than that penetrated into endosperm and embryonic tissues through the seed coat. Similar findings have been observed by Larue et al. [59] in *Lactuca sativa*, Hong et al. [41] in *Cucumis sativus*, Cocozza et al. [19] in tree plants and Noori et al. [75] in tomato. This finding explains the higher glucosinolates content in plants sprayed with AgNPs compared to soaked plants (Table 3 and Fig. 4) due to higher Ag bioaccumulation, which is similar to the finding of Li et al. [62].

It was found that treatment of *E. sativa* plants with AgNPs showed no phytotoxicity, where *E. sativa* plants sprayed with high levels of AgNPs (40 or 20 mg L^{-1}) exhibited the highest significant increase in both fresh weight and dry weight, indicating that the application of silver nanoparticles enhanced plant vegetative growth [91]. Regarding the potential risk of toxicity to humans who will feed on plants treated with nanoparticles, results in Table 2 revealed that the highest Ag content was 1.22 μg g^{-1} in plants sprayed with 40 mg L^{-1}, which is a very small amount compared with the maximum daily intake of silver, which should be less than 350 μg according to the US Environmental Protection Agency (EPA) reference dose [27]. Also, the European Food Safety Authority (EFSA) has considered a total life-time oral intake of 10 g Ag (0.39 mg/day/person) as the human "no observed adverse effect level" [2].

To detect if AgNPs induced sequence variation in *CHS* sequences of AgNPs-treated plants (sprayed or soaked) compared to control and *CHS* sequence of *A. thaliana*, multiple sequence alignment (MSA) analysis was performed. MSA results reflected high sequence similarity between *CHS A. thaliana* and *CHS E. sativa* sequences of control and treated plants (seed soaking and foliar spray treatments). Besides, MSA analysis showed several single nucleotide polymorphisms (SNPs) and Indel (insertion and deletion) variations in the *E. sativa CHS* fragment sequence of plants that were foliar sprayed with AgNPs (40 mg L^{-1}) compared to control plants (untreated *E. sativa*). Treatments with NPs have affected DNA integrity, proteins and DNA expression in plants [8, 58, 101], which is in accordance with the findings of this study. Cu and CdNPs acted as conventional mutagens and affected chromosomal attributes [56]. They induced several types of aberrations in *Lathyrus sativus* L. [33]. Treatment with silver and gold NPs resulted in some specific chromosomal abnormalities in *Allium cepa* roots [24].

It has been shown that chemical interaction of NPs with DNA during interphase can influence DNA replication and transcription of DNA [20]. In addition, silver ions have been discovered to bind to DNA in a localized binding mode, which means that the metal ions are in direct contact with the DNA structure, causing a significant shift in DNA conformation [44]. CeO_2NP treatment of soybean (*Glycine max* L.) resulted in the appearance of new bands in a random amplified polymorphic DNA pattern indicating NP-induced mutations [39]. The phylogenetic tree showed that the sequence of *CHS* of *E. sativa* control was closely related to the *A. thaliana CHS* gene, while the sequence of *CHS* fragment of *E. sativa* plants sprayed with AgNPs (40 mg L^{-1}) was clustered into a separate major cluster (Fig. 5) indicating the variation that was induced by high AgNPs level in the *CHS* sequence of the treated plants.

5 Conclusions

The biosynthesized silver nanoparticles (AgNPs) were spherical and had a slight size disparity. Glucosinolates and kaempferol key genes responded differently when plants were treated with AgNPs and *Chlorella* sp. suspension. However, upregulation of studied genes was observed and the highest expression level of these genes as well as glucosinolates and kaempferol content was at 40 mg L^{-1} AgNPs and 4 g L^{-1} *Chlorella* suspension (seed soaking or foliar spray). *E. sativa* plants sprayed with AgNPs (40 mg L^{-1}) showed several single nucleotide polymorphisms (SNPs) and Indel (insertion and deletion) variations in the *CHS* fragment sequence. Therefore, more attention should be paid when using AgNPs.

Abbreviations

AgNPs: Silver nanoparticles; CdNPs: Cadmium nanoparticles; CeO_2NPs: Cerium dioxide nanoparticles; *CHS*: *Chalcone synthase*; CuONPs: Copper oxide nanoparticles; FTIR: Fourier-transform infrared; GSLs: Glucosinolates; HPLC: High-performance liquid chromatography; Indels: Insertions and deletions; *MAM1*: *Methylthioalkylmalate synthase 1*; MAPK: Mitogen-activated protein kinase; MSA: Multiple sequence alignment; *MYB34*: *Myeloblastosis transcription factor 34*; *MYB51*: *Myeloblastosis transcription factor 51*; NPs: Nanoparticles; *PAL*: *Phenylalanine ammonia-lyase*; qRT-PCR: Quantitative real-time PCR; *RAS*: *Rosmarinic acid synthase*; ROS: Reactive oxygen species; SNPs: Single nucleotide polymorphisms; *SUR1*: *SUPERROOT 1*; TEM: Transmission electron microscopy; UV–Vis: Ultraviolet–visible.

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Authors' contributions

RG, AE and AH conceived the research idea and designed the experiments. DE performed the experiments, DE and RG analyzed the data. RG, DE and HSA

wrote and revised the manuscript. All authors have read and approved the final manuscript.

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