International Journal of Advanced Biological and Biomedical Research
Available online at http:www.ijabbr.com
Volume 9, Issue 2 (2021) pp. 190-203 **DOI**: 10.22034/ijabbr.2021.523524.1343

Original Article

The effects of chitosan nanoparticles on genes expression of artemisinin synthase in suspension culture of *Artemisia annua* L: A comparative study

Bita Ghassemi[1], Fatemeh Dehghan Nayeri[1]*, Ramin Hosseini[1]

[1] Department of Agricultural Biotechnology, Faculty of Agricultural and Natural Sciences, Imam Khomeini International University (IKIU), Qazvin, Iran

*Corresponding Author E-mail: nayeri@eng.ikiu.ac.ir

Received: 21 January 2021, Revised: 25 February 2021, Accepted: 05 April 2021

ABSTRACT

Background: Despite significant efforts, the artemisinin-based drugs are still very expensive due to the limited production of this metabolite within wild *Artemisia spp*. plants. Therefore, the current work set out to evaluate the effect of chitosan nanoparticles, as a novel elicitor to characterize the expression of genes functioning in artemisinin synthesis pathway using a comparative experimental investigation.
Methods: The suspension cultures of *A. anuua* were exposed to 5, 10, 15 mg/L of chitosan nanoparticles (during 8, 24, 48 and 72 h upon treatment). The expression of *DBR$_2$*, *SQS*, *CYP*, *ADS*, *CPR* and *ALDH* genes were quantified by qRT-PCR technique.
Results: Chitosan nanoparticles were effective in inducing artemisinin production at 15 mg/L after 8 h, and 5 and 10 mg/L after 72 h of elicitation, in which all the *ADS*, *CYP*, *CPR, DBR$_2$* and *ALDH* genes were upregulated except *SQS*.
Conclusion: The treatment of 5 mg/L after 72 h, when cells entered the stationary and then death phases, is recommended because it seems chitosan nanoparticles require more time to up-regulate the *ADS, CYP* and *ALDH* genes and thereby probably enhance the artemisinin content. The results suggest that chitosan nanoparticles can be used as a novel effective elicitor for artemisinin production.
Keywords: Artemisinin, chitosan, gene expression, nano elicitor.

1. Introduction

Today, medicinal plants have a pivotal role in the cornerstone of human disease prevention [1-3]. The growing body of evidence suggests that medicinal plants contain various chemical metabolites that exhibit potential health benefits in a dose-dependent manner [4-6]. *Artemisia annua* L., as a medical plant, has drawn researchers' attention because of its beneficial effects for clinical purposes. It has a specific metabolite called artemisinin, a sesquiterpene lactone with an unusual endoperoxide structure [7].

The accumulated body of evidence speculates that this metabolite displays different functionalities from antibacterial to anticancer activities [8]. Various genes are involved in the biosynthesis of artemisinin. The most important ones are *DBR$_2$*, *SQS*, *CYP*, *ADS*, *CPR* and *ALDH* genes and studies suggested that the overexpression of these genes could increase the total level of artemisinin within *A. annua* L. Each

gene is responsible for production of a specific intermediate that later will be converted into another biochemical metabolite to produce artemisinin constituents [9].

Biochemical investigations have unraveled that naturally occurring artemisinin has been produced in lower amounts among those plants that have a route for biosynthesis of this secondary metabolite; therefore, many efforts have been conducted to increase the overall production of artemisinin either through genetic engineering and plant tissue culture or inducing its biosynthesis routes by chemical stimulants [10, 11]. Still, the applied method to elevate the total production of artemisinin could not produce large-scale quantity of this product, thus further investigations should be performed to boost the production of artemisinin within target plants.

Studies have shown that the application of nanoparticles to induce the production of valuable secondary metabolites is a trustworthy strategy to obtain bulk production of these metabolites. Using coupled policies such as co-utilization of plant cell culture in combination with nanoparticles can outstandingly increase the level of secondary metabolites production within medicinal plants [12].

A variety of nanoparticles have been used as complementary elicitors within plant tissue culture medium by which researchers could change the way of gene expression and metabolic profile among target plants. According to the review of the literature, using metal nanoparticles such as AgNPs and plant tissue culture could increase the level of artemisinin production [12, 13]. Combinatory treatments such as the use of nanoparticles and biochemical agents like methyl jasmonate have also showed effective functionality when they are used under plant cell cultures to increase artemisinin production [14].

The application of chitosan nanoparticles to improve metabolic profile of plants has been investigated and the results have suggested that these particles could improve the secretion or biosynthesis of valuable metabolites. Chitosan is a natural sugar and it is highly bioactive, exhibiting a variety of biological functionalities from antibacterial properties to use as cell-compatible drug carrier [15, 16]. In the case of artemisinin, there is no report on the effects of chitosan particles to increase the level of the above-mentioned metabolite; therefore, using chitosan nanoparticles within plant tissue culture medium might induce the production of artemisinin. Considering what stated above, this study addressed the effect of chitosan nanoparticles on artemisinin biosynthetic pathway genes to find its functionality for elevation of artemisinin production.

2. Material and methods

2.1. Plant material

The seeds of *Artemisia annua* were obtained from the Forest, Rangeland and Watershed Organizations of Iran. Seeds were surface sterilized with 96% ethanol for 30 seconds and 0.1% $HgCl_2$ for 5 min. Then, they were rinsed 3 times by autoclaved distilled water. Sterilized seeds were cultured in the basal MS medium [17]. Calli were developed from chopped leaves of aseptically germinated seedlings and maintained on the MS medium supplemented with 0.5 mg/L NAA, 0.5 mg/L BAP and 30 g/L sucrose. All experiments were conducted with 3 replicates. Plates were kept in a growth chamber with a 16/8 h light/dark photoperiod and the temperature was set at 25 ± 2 °C. The explants were sub-cultured every 3 weeks.

2.2. Establishment of cell suspension culture and treatments with nano elicitor

The white, fresh, and friable calli obtained from the MS medium containing 30 g/L sucrose, 0.5 mg/L NAA, 0.5 mg/L BAP and 8 g/L agarose, were used (2 g of FW) for establishing suspension culture. The 500 ml Erlenmeyer flasks containing 100 mL of the liquid MS medium, containing either 0.5 mg/L NAA + 0.5 mg/L BAP or 0.1 mg/L NAA + 0.1 mg/L Kin or 0.5 mg/L NAA+0.05 mg/L Kin with 30 g/L sucrose or glucose placed on a rotary shaker at 25±2 °C and a 16/8 h light/dark photoperiod (Sahand Azar Co, Iran) with 120 rpm. They were sub-cultured every three weeks. Growth was measured during a month in order to select the best medium. The nano chitosan solution at 5, 10 and 15 mg/L concentrations were added to the 14-day-old cell suspensions cultured in 100 ml Erlenmeyer flasks containing 20 ml of the liquid MS medium and 5 ml of fresh cell suspension. Nano elicitor solution was ultra-sonicated for 15 min and pH was adjusted to 5.8. Samples were taken after 8, 24, 48 and 72 h, then filtered and kept at -80 °C. Control samples were 14-day-old suspension cells without any treatment. All samples were in three replicates [18]. Chitosan nanoparticles colloidal solution was purchased from NanoZino Company (Iran). Its purity was 99.9%.

2.3. RNA extraction and cDNA synthesis

The cell samples treated by elicitors were harvested at 8, 24, 48 and 72 h after chitosan nanoparticles treatments, immediately frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted by using Trizol (Sigma, Germany) according to the manufacturer's recommendations. RNA samples (2 µg) were treated with *DNase* I (Fermentas, Germany) to eliminate DNA impurities. The concentration and purity of RNA were monitored by measuring the ratio of A_{260}/A_{280} and A_{260}/A_{230} (Thermo nanodrop 1000, USA). The quality of RNA was assessed by separation on 1.2% formaldehyde agarose gels. cDNA was prepared with M-MuLV reverse transcriptase (Vivantis, Malaysia) using 1 µg DNase-treated total RNA as the template in a 20 µL reaction volume with oligo (dT) primer (Vivantis, Malaysia). Twenty-fold dilution of cDNA samples were used as templates for the quantitative real-time PCR [19].

2.4. Primer design and quantitative real-time PCR

In order to examine the expression of six genes involved in artemisinin biosynthesis (*DBR_2*, *SQS*, *CYP*, *ADS*, *CPR* and *ALDH*), seven primer pairs, including one pair for the house keeping gene (*ACT*) designed by Primer 3 online software (www.embnet.sk/cgi-bin/primer3) and synthesized as listed in Table 1. In this study, the utilized primer pairs for *Artemisia* genes were designed based upon the available gene sequences in the NCBI database. For each primer set, the complete coding sequence (CDS) of target genes was selected as input for the primer design procedure. Using the primer-Blast tool in NCBI, the similarity of primers/target sequences was checked and sent for further experimental synthesis. The specificity of primers was checked by standard PCR and electrophoresis on a 0.8% agarose gel. Quantitative real-time PCR reactions were performed using SYBR Green on an iQ5 System (BioRad, USA). The SYBR Green PCR Master Mix (SYBR Biopars, GUASNR, Iran) was used with a final concentration of 1 × SYBR Green PCR Master Mix, in a total volume of 20 µL, containing 1 µL of 10 pico mole of each primer and 12.5 ng of cDNA. Quantitative real-time PCR cycles were set as follows:

94 °C for 30 s, 94 °C for 10 s, 60 °C for 10 s, 72 °C for 20 s (40 cycles). The temperature increased from 55 °C to 94 °C with 0.5 °C/s ramping. Gene expression was calculated by Pfaffl formula [20]. The relative expression was normalized against *Actin* and calculated using the untreated samples as a calibrator. Each sample was evaluated in three technical and biological replications. The ratio between the target and housekeeping genes was analyzed by REST software [21]. Melting curve was used to check primer specification.

Table 1. Nucleotide seq uence of primers used in quantitative real-time PCR

Gene name	Primer Name	Primer sequence F/R [5'-3']	Primer Tm [°C]	% GC	Amplicon length [bp]
SQS	AF302464	F- TTTGAAAGCAGTATTGAAACAC R- CAGACAGCATCACGAAGC	51.3 52.8	31.8 55.6	192
DBR2	EU704257	F- CATCAACAAGCAAGCCCATTTC R- GCGATAGTCTTCAACCACCTC	56.5 55.7	45.5 52.4	125
CYP	DQ315671.1	F- TTGGAGCTGGGAGAAGGATG R- CGACGTGCATTCGTGACATA	62.61 61.71	55 50	273
ADS	AF138959	F- GTCGAATGGGCTGTCTCTGC R-CCATCAATAACGGCCTTGGA	63.28 63	60 50	256
CPR	EF197890	F- TTCTTCGGATGCAGGAATCG R- GCTCCGCCTTTGAGGAGTCTA	63.48 63.13	50 57.1	292
ALDH	FJ809789	F- GGTGGTAAGCCATTTGGGAAG R- CATCCCGGTCGAGTGCTAAA	62.78 63.37	52.3 55	280
ACT	U36376	F- AGTGCTCCTGGTTAGTTGTC R- CTTGTTGCCTCGTAATCTTCG	54.1 54.7	50 47.6	166

3. Results

3.1. Cell suspension culture

The *A. annua* seeds were germinated after 2 weeks. The chopped fresh leaves of two-month-old *in vitro* grown plants were used for callus induction. Callus induction initiated after seven days. The white friable calli was obtained from the treatment containing 0.5 mg/L NAA, 0.5 mg/L BAP and 30 g/L sucrose were used (2 g FW) to establish the suspension culture. Medium containing 30 g/L glucose induced brown compact calli with a slow growth rate (Fig. 1). Among different treatments analyzed for obtaining suspension culture, cells had the fast growth in the medium containing 0.5 mg/L NAA and 0.5 mg/L BAP with glucose (Figure 2). The medium containing sucrose also induced suitable suspension culture. However, the medium containing glucose (0.5 mg/L NAA, 0.5 mg/L BAP) was selected to proceed the elicitation tests. According to the cell growth curve (Figure 3) cells were in the exponential phase during 13th and 15th day and the maximum biomass obtained was 23.5 mg/L. Therefore, the 14th day was chosen for treating the cells with the elicitor when the cell number and viability were in their maximum levels.

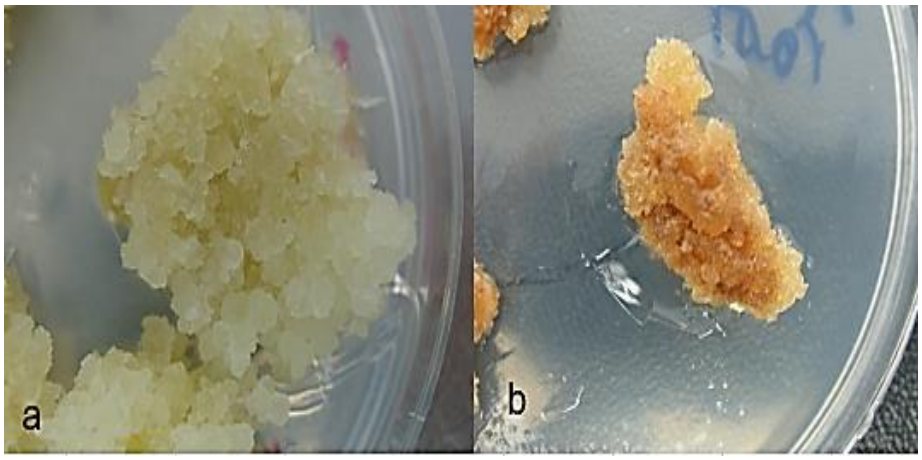


Figure 1. (a) White and friable calli obtained from 0.5 mg/L NAA+ 0.5 mg/l BAP with sucrose (b) brown calli with low growth rate obtained from 0.5 mg/L NAA+ 0.5 mg/L BAP with glucose

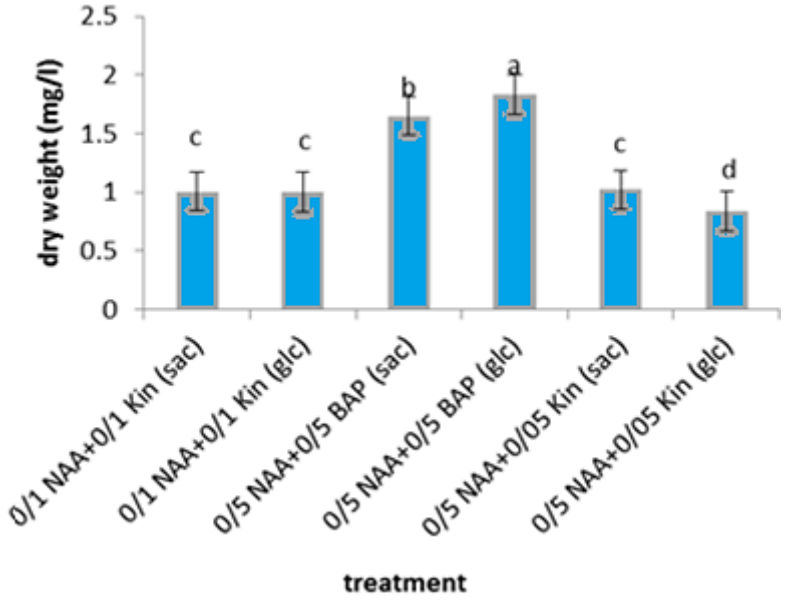


Figure 2. Different combinations of plant growth regulators and carbon resource to obtain the optimum treatment for suspension culture of *A. annua*.

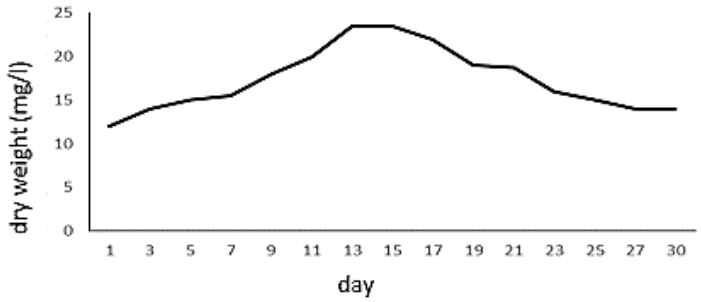


Figure 3. Time course of cell growth of suspension cultures of *A. annu* sub-cultured in the MS medium containing 0.5 mg/L NAA+ 05 mg/L BAP and glucose during 30 days of growth. The values are the means of three independent experiments ±SD

3.2. Expression of artemisinin biosynthetic genes

Expression of artemisinin biosynthetic pathway genes including *ADS*, *ALDH*, *DBR*$_2$, *CYP*, *CPR* and *SQS* was measured in 24 treatments and the control sample in three technical and biological was replicated by the quantitative real-time PCR technique.

3.2.1. *CYP* and *CPR* genes expression

The only decrease in *CYP* gene was in 10 mg/L of nano chitosan at 24 h after treatment, and the maximum expression was observed at 5 mg/L in 72 h. No decrease was observed in *CPR* gene expression at 72 h after nano chitosan treatment. The *CPR* gene expression increased in all treatments except in 5 mg/L at 24 h, 10 mg/L at 24 h and 5 and 10 mg/L at 48 h after treatment. We can postulate that 5, 10 and 15 mg/L of chitosan nanoparticles at 8 and 10 mg/L after 72 h can increase the artemisinin production because *DBR*$_2$ and *ALDH* were upregulated as well, and *SQS* was down-regulated (Figure 4 and 5). The maximum increase of *CPR* gene expression was observed using 5 mg/L, 8 h after treatment.

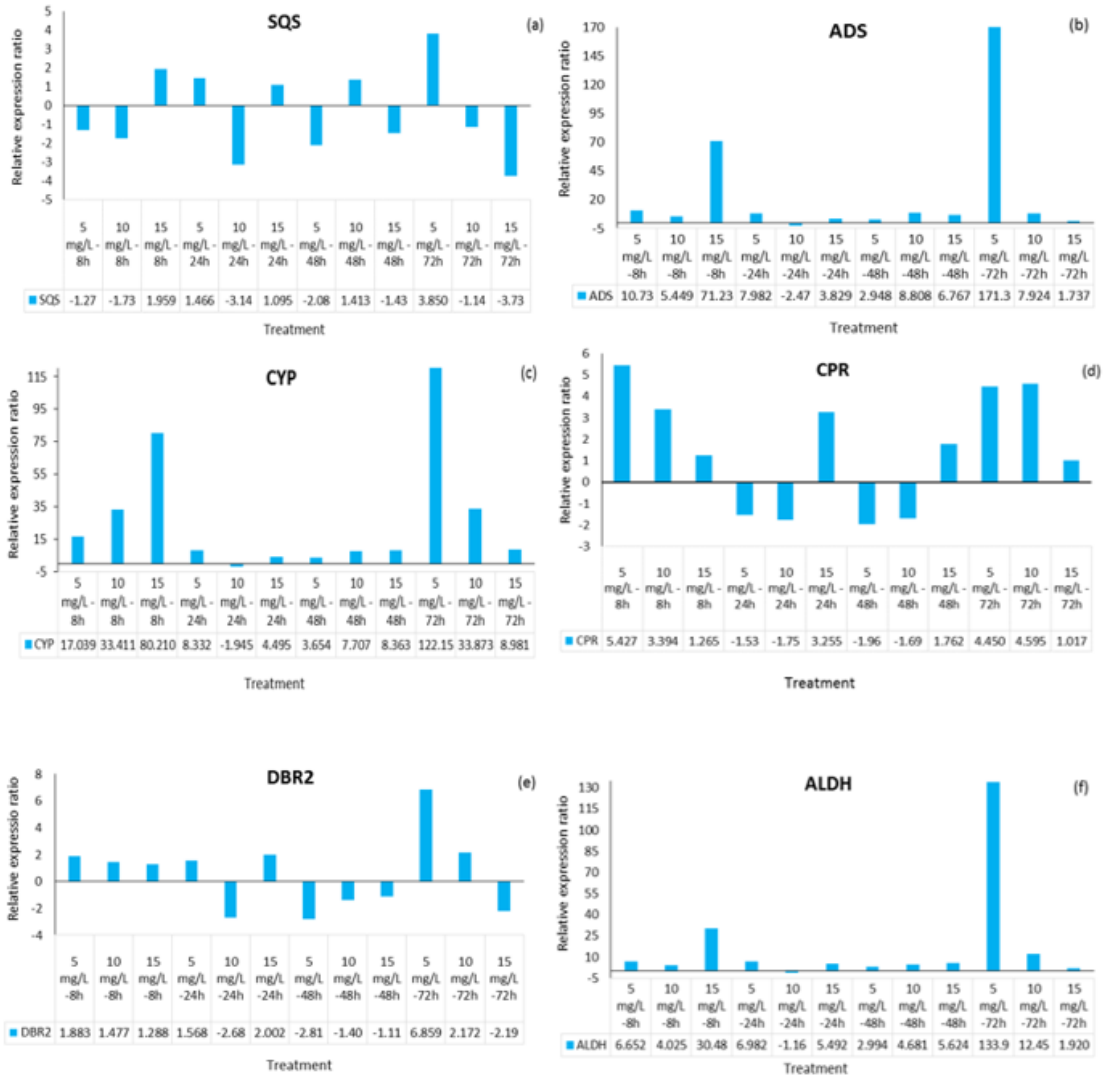
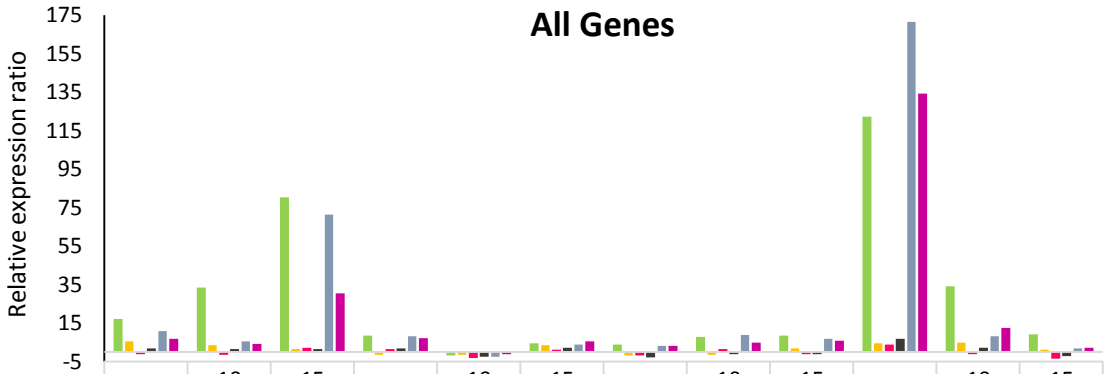


Figure 4. The expression of *SQS* (a), *ADS* (b), *CYP* (c), *CPR* (d), *DBR*$_2$ (e) and *ALDH* (f) in quantitative real-time PCR analysis at different treatments of nano chitosan.



	5 mg/L -8h	10 mg/L - 8h	15 mg/L - 8h	5 mg/L -24h	10 mg/L - 24h	15 mg/L - 24h	5 mg/L -48h	10 mg/L - 48h	15 mg/L - 48h	5 mg/L -72h	10 mg/L - 72h	15 mg/L - 72h
CYP	17.039	33.411	80.210	8.332	-1.945	4.495	3.654	7.707	8.363	122.15	33.873	8.981
CPR	5.427	3.394	1.265	-1.537	-1.759	3.255	-1.961	-1.693	1.762	4.450	4.595	1.017
SQS	-1.270	-1.735	1.959	1.466	-3.145	1.095	-2.086	1.413	-1.431	3.850	-1.145	-3.737
DBR2	1.883	1.477	1.288	1.568	-2.681	2.002	-2.811	-1.406	-1.112	6.859	2.172	-2.190
ADS	10.731	5.449	71.234	7.982	-2.472	3.829	2.948	8.808	6.767	171.32	7.924	1.737
ALDH	6.652	4.025	30.489	6.982	-1.169	5.492	2.994	4.681	5.624	133.99	12.459	1.920

Nano chitosan treatment

Figure 5. The expression of all genes involved in artemisinin production pathway in different nano chitosan treatments

3.2.2. *ADS* and *SQS* genes expression

ADS gene was upregulated in all chitosan nanoparticles treatments except at 10 mg/L after 24 h, and the maximum expression was obtained using 5 mg/L at 72 h and then with 15 mg/L at 8 h. In different treatments of chitosan nanoparticles, *SQS* gene was significantly upregulated only using 15 mg/L after 8 h and 5 mg/L after 24 or 72 h (Fig. 4a). It means that chitosan nanoparticles in low concentrations (5 and 10 mg/L), when cells do not enter the growth phase, could enhance the artemisinin content because *SQS*, as an inhibitor of Artemisinin production, was dramatically down-regulated (Fig. 3 and 4a).

3.2.3. *DBR*$_2$ and *ALDH* genes expression

The maximum up-regulation of *DBR*$_2$ was observed in 5 mg/L of nano chitosan at 72 h after treatment, and declined after 48 h. The expression of *ALDH* remarkably increased in 5 mg/L at 72 h after treatment. Except this treatment, *ALDH* gene could slightly be up regulated by various nano chitosan treatments (Fig. 4f). The significant effect of chitosan nanoparticles on stimulating *ALDH* expression was observed in the high concentration (15 mg/L) at early stage (8 hours) and also in the low concentration (5 mg/L) at the late stage of cell viability (72 h after the treatment) (Fig. 4f).

4. Discussion

Although the white and well growing suspension cells were obtained from the medium containing sucrose, a proper treatment containing glucose was opt for the further elicitation test because, according to the Ali et al.'s (2017) report, *A. anuua* cells grown in the medium containing glucose produced more artemisinin compared with the cells grown in the medium containing fructose, and fructose was recognized as the determining factor to inhibit artemisinin production due to the presence of sucrose [22]. Moreover, Wang and Weathers (2007) also

reported that artemisinin production was two folds higher when glucose was added to the medium rather than sucrose [23]. Therefore, the medium fortified with 0.5 mg/L NAA, 0.5 mg/L BAP and glucose was the best option to proceed for the elicitation of artemisinin production.

The only decrease in *CYP* gene was in 10 mg/L of nano chitosan at 24 h after treatment while no decrease was observed in *CPR* gene expression at 72 h after nano chitosan treatment. It was predictable because *CPR* and *CYP* participate in different parts of artemisinin biosynthetic pathway. Cytochrome P450 monooxygenase (*CYP*) is a multifunctional sesquiterpene oxidase with a key role in the biosynthesis of artemisinin. In three steps, it converts amorpha-4,11-diene to artemisinic acid via artemisinic alcohol and artemisinic aldehyde middle metabolites [24]. Cytochrome P450 reductase (*CPR*) was identified as a redox partner of *CYP71AV1* that helps *CYP71AV1* to catalyze the conversion of amorpha-4,11- diene to more oxygenated products in vivo [25]. The *CPR* gene expression increased in all treatments except in 5 mg/L at 24 h, 10 mg/L at 24 h and 5 and 10 mg/L at 48 h after treatment.

Previous studies demonstrated that co-overexpressing genes *CYP71AV1* and *CPR* could increase artemisinin content in *A. annua* [26], accordingly, the results here suggested that 5, 10 and 15 mg/L of chitosan nanoparticles at 8 and 10 mg/L after 72 h could increase the artemisinin production because DBR_2 and *ALDH* were upregulated as well, and *SQS* was down-regulated (Fig. 4 and 5). The maximum increase of *CPR* gene expression was observed using 5 mg/L, 8 h after treatment. It has been reported that expression of *CPR* was also promoted slightly by chitosan elicitation in the leaf of *A. annua* [27]. *ADS* gene was not upregulated only after 24 h (10 mg/L), and the maximum expression was detected at 72 h (5 mg/L) and 8 h (15 mg/L), respectively. Lei et al. (2011) reported that using chitosan the expression of *ADS* as the first committed gene of artemisinin biosynthesis was transiently and significantly induced at 2 h and returned to its initial level at 8 h in the leaf of *A. annua* plant [28].

Our results showed that the ADS gene was dramatically up-regulated at higher concentrations of nano chitosan (15 mg/L) when cells had not yet entered the stationary phase (8 h after treatment). On the other hand, ADS also upregulated at the low concentration of chitosan nanoparticles (5 mg/L) when cells had entered the death phase (72 h after treatment) (Fig. 3 and 4b). Therefore, it can be inferred that ADS was mostly affected by chitosan nanoparticles instantly after the chitosan treatment or when cells entered the death phase (72 hours).

Squalene synthase (SQS) is an enzyme which catalyzes the condensation of two FDP molecules to form squalene, as a main precursor for sterol synthesis. SQS is the key enzyme catalyzing the first step of the sterol biosynthetic pathway, which is in competition with artemisinin biosynthetic pathway [29]. We found that *SQS* was significantly upregulated only using 15 mg/L (after 8 h) and 5 mg/L (after 24 and 72 h) (Fig. 4a). It means that chitosan nanoparticles in low concentrations (5 and 10 mg/L) can be used as an inhibitor of *SQS* before cells enter the growth phase, thereby it could enhance the artemisinin content (Fig. 3 and 4a). The inhibition of sesquiterpene synthesis resulted in enhanced sterol synthesis.

In *A. annua*, when *SQS* was inhibited with miconazole, artemisinin yield increased [30]. Moreover, studies using either an antisense or interference strategy in *A. annua* [31], or promoter

replacement in Saccharomyces cerevisiae [32], showed that when *SQS* was inhibited, both artemisinin content and transcripts of genes involved in the artemisinin biosynthetic pathway increased. Salehi et al. (2018) further showed that down-regulation of *SQS* resulted in a preferential up-regulation of *ADS* instead of other competing sesquiterpene cyclase [33]. However, the quantitative real-time PCR analysis of artemisinin biosynthetic pathway genes could not support this idea in all treatments of chitosan nanoparticles (Fig. 4a, b and 5), suggesting that chitosan nanoparticles might behave differently from other non-nano elicitors because of their molecular structure.

The maximum up-regulation of *DBR2* was observed in 5 mg/L of nano chitosan at 72 h. Lei et al. (2011) reported that *DBR2* expression began to increase at 4 h and declined by 24 h during the foliar application of chitosan [28]. Here, DBR2 declined after 48 h of treatments with chitosan nanoparticles. Although the expression of *ALDH* gene increased by all nano chitosan treatments (except 10 mg/L at 72 h), these increases were not significant (Fig. 4f). The results showed that chitosan nanoparticles did not significantly influence the expression of *ALDH* at mRNA level. The expression of *ALDH* remarkably increased in 5 mg/L at 72 h after treatment. At this treatment, the *ADS* and *CYP* were also highly up-regulated, but *DBR2* gene expression did not increase with the same amount (Figure 5).

The significant effect of chitosan nanoparticles on stimulating *ALDH* expression was observed in the high concentration (15 mg/L) at early stage (8 hours) and in the low concentration (5 mg/L) at the late stage of cell viability (72 h after the treatment) (Fig. 4f). It seems that chitosan nanoparticles could be potentially used as a suitable elicitor for enhancing artemisinin production at 5 and 10 mg/L after 8h and 10 mg/L after 72 h of treatments with chitosan nanoparticles because important genes involving in artemisinin production such as *ADS*, *CYP*, *CPR* and *ALDH and DBR2,* were highly upregulated and *SQS*, as the competitor of *ADS* gene and artemisinin production, was down-regulated (Fig. 5).

In this research, the results showed that different concentrations of chitosan nanoparticles had a low impact on *ADS*, *CYP, CPR, ALDH* and *DBR2* when cells were in the exponential growth phase (24 and 48 hours after treatment) (Fig. 3 and 5). According to the cell growth curve (Figure 3), cells entered the death phase at 17th day. In such a condition, the pivotal genes were up-regulated when a low concentration of chitosan nanoparticles was utilized (5 mg/L after 72 h), so it can be seen that 5 mg/L of chitosan nanoparticles could be effective on artemisinin enhancement when cells entered the stationary and then death phases. Additionally, 15 mg/L chitosan nanoparticles had no significant elevated effect on gene expression except for *CYP* gene when cells entered the death phase (at 72 hours). The *CYP* gene is involved in three reactions in artemisinin biosynthetic pathway [34]; therefore, its higher expression might increase the level of artemisinin production *in vitro*. Amorpha-4,11- diene is gradually oxidized to artemisinic alcohol, artemisinic aldehyde, and artemisinic acid through cytochrome P450 enzyme *CYP71AV1* (*CYP*) [25]. Our results displayed that the exposure of prepared medium to chitosan nanoparticles could enhance the expression of this gene as its functional role highlighted in the literature to trigger the production of artemisinin.

In this respect, Yin *et al*. (2012) studied the effect of chitosan oligosaccharide and salicylic acid on the leaf of *A. annua* by monitoring the genes involved in artemisinin biosynthetic

pathway. They reported that the expression of *TTG1*, as a transcription factor in this signaling pathway, was upregulated by chitosan treatment revealing the possibility of a positive effect of chitosan on glandular trichome formation. In addition, their outcomes also suggested that chitosan significantly promoted the expression of some artemisinin synthesis related genes (*ADS*, *CPR*), 24 and 48 h after chitosan application through which slightly induced the higher artemisinin concentration in leaves [35]. They suggested that elicitors, however, were not a critical method for enhancing artemisinin or these compounds might need extra time (more than 48 h) for affecting artemisinin content [35].

Lei *et al.* (2011) did not investigate the effect of chitosan application on the gene expression after 48 h while herein, we showed the effect of chitosan nanoparticles up to 72 h and differential gene expression was observed for studied genes. The results unraveled that the highest up-regulation of important gene including *ADS*, *CYP* and *ALDH* were at 72 h after treatment (at 5 and 10 mg/L) (Fig. 5). Therefore, it can be concluded that chitosan nanoparticles need more time to promote artemisinin content [28]. Putalun et al. (2007) reported that artemisinin production in hairy roots increased 6-fold to 1.84 mg/mg dry wt over 6 days by adding 150 mg/L chitosan [36]. In Jiao et al.'s (2018) study after chitosan treatment , the total flavonoids were increased 7.08-fold *Isatis tinctoria* hairy root culture [37]. It was demonstrated that the increase in secondary metabolite production after chitosan treatments was related to significant changes in the expression of the PAL enzymes pathway [38]. Lei et al. (2011) showed the leaf artemisinin content was enhanced by treating the leaf of *A. annua* with 100 mg/L chitosan. According to their results, artemisinic acid and artemisinin were also increased 72 % and 53%, respectively. In addition, their results demonstrated that H_2O_2 and O^{-}_2 levels were 1.4 and 3-fold higher than those of the control group; therefore, they might lead to convert the dihydroartemisinic acid to artemisinin. Studies also reported that there was no negative effect on plant growth after the application of chitosan treatment [28].

Interestingly, in a study by Putalun *et al.* (2007) on hairy root cultures of *A. annua*, artemisinin production was promoted 6-fold higher (1.8 µg/mg DW) compared with the control group by application of 150 mg/L chitosan (polymer of COS) [36]. In contrast, Baldi et al. (2008) reported that chitosan had no effect on artemisinin accumulation in *A. annua* suspension cultures [39]. Positive effects of chitosan treatment on artemisinin production and even other metabolites have been reported in previous studies, but there are no reports on the chitosan nanoparticles effect as the elicitor. The effect of other nanoparticles has been approved, and their positive effect as elicitors such as AgNPs, CuSO4 and cobalt nanoparticles were reported [18, 40].

By and large, medicinal plants are available pools of natural products with beneficial health benefits and finding a way to enhance the production of these metabolites can improve the knowledge of researchers to find the most important secondary metabolites for human disease prevention [41]. Over the past decades, many chronic diseases have affected world nations, caused millions of deaths yearly and modern medicine could not find an ultimate medicine for controlling of these diseases [42]. Therefore, production of beneficial secondary metabolites using the application of biochemical agents, nanoparticles and natural stimulants could decrease the cost of drug design for developing effective drugs against human diseases.

Since a holistic view on the interaction of nanoparticles and plant metabolic pathways, soil rhizobia and human body has not been reported [43, 44], further studies on artemisinin production using natural and environmentally friendly nanoparticles can pave the way for further research to entirely unravel the effectiveness of these compounds for enhancing plant metabolites production.

5. Concluding remarks

In conclusion, our results showed that different concentration of chitosan nanoparticles could trigger the production of artemisinin metabolite. In a time-dependent manner, these particles affected the gene expression profile of target explants, leading to an elevated production of artemisinin. Therefore, these outcomes suggested that using natural nanoparticles is an effective strategy to increase the expression of pivotal genes that are involved in the production of artemisinin metabolite. Further studies should be conducted on these particles to unravel their beneficial effects for large-scale production of artemisinin for medicinal and industrial applications.

Acknowledgment

This work was done in Agricultural Biotechnology Department of Imam Khomeini International University. The authors appreciate all staffs on their good collaborations.

Abbreviations:

ADS: Amorpha-4,11-diene synthase
AgNPs: Silver nanoparticles
ALDH: Aldehyde dehydrogenase
BAP: 6-Benzylaminopurine, benzyl adenine
COS: Chitosan oligosaccharide
CPR: Cytochrome P450 reductase
CYP: Cytochrome P450 monooxygenase
DBR2: Artemisinic aldehyde delta-11(13) reductase
DW: Dry weight
KIN: Kinetin
NAA: 1-Naphthaleneacetic acid
NPs: Nanoparticles
MS: Murashige and Skoog
PAL: Phenylalanine ammonia lyase
qRT-PCR: Quantitative real-time PCR
SQS: Squalene synthase
TTG1: Transparent testa glabra 1
WT: Weight

Conflict of interest statement

The authors declare no competing interests.

Consent for publications

The authors have read and approved the submitted manuscript.

Availability of data and material

The authors declare that all the data is embedded in the manuscript.

Authors' contributions

All authors contributed in conceptualization, methodology, software and writing.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Funding information

This study was supported by International University of Imam Khomeini.

References

1. Rasouli H, Hosseini-Ghazvini S M-B, Adibi H, Khodarahmi R. (2017). Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic

compounds: a virtual screening perspective for the treatment of obesity and diabetes. *Food & Function*, 8(5): 1942-1954. https://doi.org/10.1039/C7FO00220C

2. Rasouli H, Hosseini-Ghazvini S M-B, Khodarahmi R. (2019). Therapeutic potentials of the most studied flavonoids: highlighting antibacterial and antidiabetic functionalities Studies in natural products chemistry (Vol. 60, pp. 85-122): *Elsevier*.
3. Keikhaie K R, Fazeli-Nasab B, Jahantigh H R, Hassanshahian M. (2018). Antibacterial Activity of Ethyl Acetate and Methanol Extracts of Securigera securidaca, Withania sominefra, Rosmarinus officinalis and Aloe vera Plants against Important Human Pathogens. *Journal of Medical Bacteriology*, 7(1-2): 13-21.
4. Rasouli H, Hosseini Ghazvini S M B, Yarani R, Altıntaş A, Jooneghani S G N, Ramalho T C. (2020). Deciphering inhibitory activity of flavonoids against tau protein kinases: a coupled molecular docking and quantum chemical study. *Journal of Biomolecular Structure and Dynamics*: 1-14.
5. Rasouli H, Parvaneh S, Mahnam A, Rastegari-Pouyani M, Hoseinkhani Z, Mansouri K. (2017). Anti-angiogenic potential of trypsin inhibitor purified from Cucumis melo seeds: Homology modeling and molecular docking perspective. *International journal of biological macromolecules*, 96: 118-128.
6. Sobhanizade A, Solouki M, Fazeli-Nasab B. (2017). Optimization of callus induction and effects of biological and non-biological elicitors on content of phenol/flavonoid compounds in *Nigella sativa* under in-vitro conditions. *Cell and Tissue Journal*, 8(2): 165-184.
7. Judd R, Bagley M C, Li M, Zhu Y, Lei C, Yuzuak S, Ekelöf M, Pu G, Zhao X, Muddiman D C. (2019). Artemisinin biosynthesis in non-glandular trichome cells of Artemisia annua. *Molecular plant*, 12(5): 704-714.
8. Sebola T E, Uche-Okereafor N C, Mekuto L, Makatini M M, Green E, Mavumengwana V. (2020). Antibacterial and Anticancer Activity and Untargeted Secondary Metabolite Profiling of Crude Bacterial Endophyte Extracts from Crinum macowanii Baker Leaves. *International Journal of Microbiology*, 2020.
9. Shi P, Fu X, Liu M, Shen Q, Jiang W, Li L, Sun X, Tang K. (2017). Promotion of artemisinin content in Artemisia annua by overexpression of multiple artemisinin biosynthetic pathway genes. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 129(2): 251-259.
10. Wang J, Li J-l, Li J, Li J-x, Liu S-j, Huang L-q, Gao W-y. (2017). Production of active compounds in medicinal plants: from plant tissue culture to biosynthesis. *Chinese Herbal Medicines*, 9(2): 115-125.
11. Nielsen E, Temporiti M E E, Cella R. (2019). Improvement of phytochemical production by plant cells and organ culture and by genetic engineering. *Plant cell reports*, 38(10): 1199-1215.
12. Mahendran D, Geetha N, Venkatachalam P. (2019). Role of Silver Nitrate and Silver Nanoparticles on Tissue Culture Medium and Enhanced the Plant Growth and Development In vitro Plant Breeding towards Novel Agronomic Traits (pp. 59-74): *Springer*.
13. Chung I-M, Rajakumar G, Thiruvengadam M. (2018). Effect of silver nanoparticles on phenolic compounds production and biological activities in hairy root cultures of Cucumis anguria. *Acta Biologica Hungarica*, 69(1): 97-109.
14. Alam P, Albalawi T H. (2020). In vitro alteration of artemisinin biosynthesis in Artemisia annua L during treatment with methyl jasmonate. *Tropical Journal of Pharmaceutical Research*, 19(1): 33-37.
15. Zarayneh S, Sepahi A A, Jonoobi M, Rasouli H. (2018). Comparative antibacterial effects of cellulose nanofiber, chitosan nanofiber, chitosan/cellulose combination and chitosan alone against bacterial contamination of Iranian banknotes. *International Journal of Biological Macromolecules*, 118: 1045-1054.
16. Babaei Z, Solouki M, Fazeli-Nasab B. (2018). Investigating The Effect of Biological and non-Biological Elicitor on Expression of Hyp-1 Gene in Hypericum perforatum. *Modern Genetics*, 13(4): 543-549. Article Code: MGJ-17-B-00487

17. Murashige T, Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*, 15(3): 473-497.
18. Ghasemi B, Hosseini R, Dehghan Nayeri F. (2015). Effects of cobalt nanoparticles on artemisinin production and gene expression in Artemisia annua. *Turkish Journal of Botany*, 39(5): 769-777.
19. Ghassemi B, Hosseini R, Dehghan nayeri F. (2015). The Effect of Nano Cobalt and Nano Chitosan on Artemisinin production and expression of SQS and DBR2 genes in Artemisia annua. [Research]. *Genetic Engineering and Biosafety Journal*, 4(1): 25-39.
20. Pfaffl M W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, 29(9): e45-e45.
21. Mallona I, Díez-Villanueva A, Martín B, Peinado M A. (2017). Chainy: an universal tool for standardized relative quantification in real-time PCR. *Bioinformatics*, 33(9): 1411-1413.
22. Ali M, Abbasi B H, Ahmad N, Khan H, Ali G S. (2017). Strategies to enhance biologically active-secondary metabolites in cell cultures of Artemisia–current trends. *Critical reviews in biotechnology*, 37(7): 833-851.
23. Wang Y, Weathers P. (2007). Sugars proportionately affect artemisinin production. *Plant Cell Rep*, 26(7): 1073-1081.
24. Shen Q, Zhang L, Liao Z, Wang S, Yan T, Shi P, Liu M, Fu X, Pan Q, Wang Y. (2018). The genome of Artemisia annua provides insight into the evolution of Asteraceae family and artemisinin biosynthesis. *Molecular plant*, 11(6): 776-788.
25. Firsov A, Mitiouchkina T, Shaloiko L, Pushin A, Vainstein A, Dolgov S. (2020). Agrobacterium-Mediated Transformation of Chrysanthemum with Artemisinin Biosynthesis Pathway Genes. *Plants*, 9(4): 537.
26. Tang K, Shen Q, Yan T, Fu X. (2014). Transgenic approach to increase artemisinin content in Artemisia annua L. *Plant Cell Rep*, 33(4): 605-615.
27. Saifi M, Khan S, Kiran U, Fatima S, Abdin M Z. (2020). Transgenic technology to improve therapeutic efficacy of medicinal plants. *Transgenic Technology Based Value Addition in Plant Biotechnology*: 207.
28. Lei C, Ma D, Pu G, Qiu X, Du Z, Wang H, Li G, Ye H, Liu B. (2011). Foliar application of chitosan activates artemisinin biosynthesis in Artemisia annua L. *Ind Crops Prod*, 33(1): 176-182.
29. Ma Y N, Xu D B, Yan X, Wu Z K, Kayani S I, Shen Q, Fu X Q, Xie L H, Hao X L, Hassani D. (2021). JA and ABA activated AaGSW1-AaTCP15/AaORA transcriptional cascade promotes artemisinin biosynthesis in Artemisia annua. *Plant Biotechnology Journal*.
30. Perassolo M, Cardillo A B, Busto V D, Giulietti A M, Talou J R. (2018). Biosynthesis of sesquiterpene lactones in plants and metabolic engineering for their biotechnological production Sesquiterpene Lactones (pp. 47-91): *Springer*.
31. Xie L, Yan T, Li L, Chen M, Ma Y, Hao X, Fu X, Shen Q, Huang Y, Qin W. (2020). The WRKY transcription factor AaGSW2 promotes glandular trichome initiation in Artemisia annua. *Journal of Experimental Botany*.
32. Du Y, Yang B, Yi Z, Hu L, Li M. (2020). Engineering Saccharomyces cerevisiae coculture platform for the production of flavonoids. *Journal of agricultural and food chemistry*, 68(7): 2146-2154.
33. Salehi M, Karimzadeh G, Naghavi M R, Badi H N, Monfared S R. (2018). Expression of key genes affecting artemisinin content in five Artemisia species. *Scientific reports*, 8(1): 1-11.
34. Ma D, Li G, Zhu Y, Xie D-Y. (2017). Overexpression and suppression of Artemisia annua 4-hydroxy-3-methylbut-2-enyl diphosphate reductase 1 gene (AaHDR1) differentially regulate artemisinin and terpenoid biosynthesis. *Front Plant Sci*, 8: 77.
35. Yin H, Kjaer A, Fretté X C, Du Y, Christensen L P, Jensen M, Grevsen K. (2012). Chitosan oligosaccharide and salicylic acid up-regulate gene expression differently in relation to the biosynthesis of artemisinin in Artemisia annua L. *Process Biochem*, 47(11): 1559-1562.

36. Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y. (2007). Improvement of artemisinin production by chitosan in hairy root cultures of Artemisia annua L. *Biotechnology letters*, 29(7): 1143-1146.
37. Jiao J, Gai Q-Y, Wang X, Qin Q-P, Wang Z-Y, Liu J, Fu Y-J. (2018). Chitosan elicitation of Isatis tinctoria L. hairy root cultures for enhancing flavonoid productivity and gene expression and related antioxidant activity. *Industrial crops and products*, 124: 28-35.
38. Ahmed K B M, Khan M M A, Siddiqui H, Jahan A. (2020). Chitosan and its oligosaccharides, a promising option for sustainable crop production-a review. *Carbohydrate polymers*, 227: 115331.
39. Baldi A, Dixit V. (2008). Yield enhancement strategies for artemisinin production by suspension cultures of Artemisia annua. *Bioresour Technol*, 99(11): 4609-4614.
40. Darki B S, Shabani L, Pourvaez R, Mostafa S. (2019). Effects of CuSO4 and AgNO3 on artemisinin and phenolic compound in shoot cultures of Artemisia annua L. *Journal of Plant Process and Function*, 8(31): 2.
41. Rasouli H, Norooznezhad A H, Rashidi T, Hoseinkhani Z, Mahnam A, Tarlan M, Moasefi N, Mostafaei A, Mansouri K. (2018). Comparative in vitro/theoretical studies on the anti-angiogenic activity of date pollen hydro-alcoholic extract: Highlighting the important roles of its hot polyphenols. *BioImpacts: Bi*, 8(4): 281.
42. Jahani M, Azadbakht M, Rasouli H, Yarani R, Rezazadeh D, Salari N, Mansouri K. (2019). L-arginine/5-fluorouracil combination treatment approaches cells selectively: rescuing endothelial cells while killing MDA-MB-468 breast cancer cells. *Food and Chemical Toxicology*, 123: 399-411.
43. Rasouli H, Popović-Djordjević J, Sayyed R, Zarayneh S, Jafari M, Fazeli-Nasab B. (2020). Nanoparticles: a new threat to crop plants and soil Rhizobia? Sustainable Agriculture Reviews 41 (pp. 201-214): *Springer*.
44. Rasouli H. (2019). Devil's hand conceals behind the obscure side of AgNPs: A letter to the editor. *International Journal of Biological Macromolecules*, 125: 510-513.

How to cite this article: Bita Ghassemi, Fatemeh Dehghan Nayeri, Ramin Hosseini. The effects of chitosan nanoparticles on genes expression of artemisinin synthase in suspension culture of *Artemisia annua* L: A comparative study. *International Journal of Advanced Biological and Biomedical Research*, 2021, 9(2), 190-203. Link: http://www.ijabbr.com/article_243302.html