

## The effect of titanium dioxide nanoparticles on the relative expression of catalase, P450, SOD, diTDS and WRKY genes of *Vitex agnus-castus* L.

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### Abstract

Each environmental factor is able to change the way genes are expressed. Application of nanoparticles also affects the expression of different genes in plants. The aim of this study was to investigate the effect of three different concentration of titanium dioxide nanoparticles, TiO<sub>2</sub> (zero, 200 and 800 micrograms per milliliter) on the relative expression of catalase, P450, SOD, diTDS and WRKY genes in *Vitex* plant leaf tissue using qRT-PCR. Plant cultivation was carried out in 2018 in the greenhouse of Islamic Azad University of Mashhad. The experiment was arranged as completely random design with 5 replications. XRD measurements showed that applied TiO<sub>2</sub> nanoparticles were in the form of anatase. Statistical analysis of gene expression in treated leaves of *Vitex* plant with TiO<sub>2</sub> nanoparticles showed that this nanoparticle significantly affected the expression of catalase, P450, SOD, diTDS and WRKY genes. A concentration of 800 micrograms per milliliter of TiO<sub>2</sub> nanoparticle increased the expression of catalase, P450, SOD and WRKY genes and decreased the expression of diTDS gene. In contrast, concentrations of 200 micrograms per milliliter only increased the expression of catalase and WRKY genes. The expression of the diTDS gene under treatments of 200 and 800 micrograms per liter of TiO<sub>2</sub>, compared with control, decreased by 2.1 and 0.46, respectively. Overall, the nanoparticle was able to influence the expression of genes in the biosynthetic pathway of terpenoids, as well as the plant's antioxidant enzymes, depending on the concentration of nanoparticles.

**Keywords:** gene expression; quantitative RT-PCR; *Vitex* plant; TiO<sub>2</sub> nanoparticles

### Introduction

Research on nanoparticles is increasing due to distinct physical and chemical properties such as particle morphology, larger surface area, pore size, high reactivity, and physical and chemical properties related to particle size (Siddiqui *et al.*, 2015). It has been reported that most nanoparticles show toxic effects even at very

Received: 20 Feb 2021. Received in revised form: 02 Jun 2021. Accepted: 19 Jul 2021. Published online: 30 Nov 2021.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

low concentrations and affect their morphophysiological, biochemical and molecular properties. In recent years, application of TiO<sub>2</sub> nanoparticles due to biological properties has been highly considered by plant physiologists (Qi *et al.*, 2013). One of the applications of nanoparticles is to use them as elicitor. Elicitors are biotic or abiotic compounds, which induces biosynthesis and accumulation of secondary metabolites by inducing defensive responses (Zhao *et al.*, 2005; Namdeo, 2007; Shakya *et al.*, 2019). TiO<sub>2</sub> nanoparticles through enhancing the nitrate reductase and glutamate dehydrogenase functioning affect the nitrogen metabolism and thus improve the crop growth and development. Due to their small size, they can also easily penetrate into cells induce protein production and improve the gene expressions (Yang *et al.*, 2008).

Various plants species that have been used in traditional medicine for thousands of years are rich source of biologically active compounds (Wubshet *et al.*, 2016). *Vitex* is the largest genus in the Lamiaceae family, containing 250 species worldwide (Rani and Sharma, 2013). Species of this genus are shrubby. One of the most important species used in medicine is the *Vitex* plant or chaste tree with the scientific name (*Vitex agnus-castus* L.), which grows in the Mediterranean region of southern Europe and Central Asia as volunteer on the banks of rivers and along waterways. (Rani and Sharma, 2013). The *Vitex* plant essential oils mainly contain sabinene, pinene, and sesquiterpene and various compounds including alkaloids as vicine, isoflavones derivatives (mostly casticin) and flavonoids like penduletin and chrysophanol. The essential oils of this plant contain cineol and pinene (Chen *et al.*, 2011a). The fruit of this plant is used to treat menstrual problems in women, including premenstrual syndrome, imbalance of estrogen and progesterone levels, jaundice, menopausal complications, hyperprolactinemia (Carmichael, 2008; Dugoua *et al.*, 2008). In traditional Iranian medicine, its leaves and fruits are used to increase milk production (Azadbakht *et al.*, 2005). According to the studies, the main medicinal properties of this plant are due to the presence of diterpenoids and their effect on dopamine receptors in the brain (Jarry *et al.*, 2006; Brattström and Max Zeller, 2014). Numerous types of labdane-related diterpenoids have been extracted from the vitex plant, and a recent study indicated of 114 unique structures covering a wide range of groups, including labdanes, abietanes, and clerodanes (Yao *et al.*, 2016). Most extracted diterpenoids from vitex plant are combined with the hydroxyl group on carbon 9 (C-9) carbon 13 (C-13) (Hoberg *et al.*, 1999; Ono *et al.*, 2008, 2009, 2011). Biosynthesis of terpenes in angiosperm plants is performed by terpene synthases (TPSs) enzymes of the geranylgeranyl diphosphate (GGPP) (Peters, 2010; Chen *et al.*, 2011a). These enzymes, which are found in all plants, separate the phosphate group and form the skeletons of monoterpenes, sesquiterpene, and diterpenes (Chen *et al.*, 2011b). Two types of diTPS enzymes class I and II have been identified in the biosynthesis pathway of the *Vitex* plant diterpenes. In the first stage, class II diTPS produces bicyclic intermediates from GGPP and by de-phosphorylation them by class I diTPS, produces two or three labdane-related bicyclic diterpenes. The oxidation steps followed by cytochrome P450 enzymes (CYP) in the biosynthesis pathway result in highly functional diterpenes of *Vitex* plant (Ignea *et al.*, 2016; Scheler *et al.*, 2016; Pateraki *et al.*, 2017). Over the past 20 years, WRKY transcription factor (TF) family genes have been well known for their role in regulating the tolerance of biotic and abiotic stresses (Schluttenhofer and Yuan, 2015). Studies confirm that transcription factors such as WRKYs may play a role in regulating the biosynthetic pathways of terpenoids in plants (Xu *et al.*, 2004; Cheng *et al.*, 2007; Suttipanta *et al.*, 2011). Catalase (CAT) and superoxide dismutase (SOD) enzymes are important antioxidants that play a role in the response of plants to abiotic stresses, and TiO<sub>2</sub> nanoparticles enhance their activity (Mohammadi *et al.*, 2014).

The *Vitex* plant has important medicinal properties for the pharmaceutical industry, however there are not many studies on the expression of the genes of this plant for these properties. Nanoparticles appear to be able to affect the expression of some of the genes involved in the biosynthesis of secondary metabolites and antioxidants. Therefore, the aim of this study was to investigate the relative expression of five important genes in the *Vitex* plant under treatment with TiO<sub>2</sub> nanoparticles by performing Real-Time PCR reaction.

## Materials and Methods

### *Plants and TiO<sub>2</sub> nanoparticles*

Vitex plant seeds were obtained from Dashtiar Company of Isfahan in 2018. Cultivation in greenhouse conditions was done as a completely randomized design with 5 replications. Each repetition consisted of 5 pots and each pot contained 10 seeds of a *Vitex* plant that was planted at a depth of 1 cm above the soil surface. The pots were placed on the platform in front of the sunlight and were irrigated on a daily basis. Greenhouse temperatures ranged from 21 to 28 °C, with light conditions 16 hours a day, 8 hours as night, and relative humidity of 75%.

The concentrations of TiO<sub>2</sub> nanoparticles used in this experiment included of 200 and 800 micrograms per milliliter and distilled water as control treatments (chosen concentration was due to the finding that the 200 and 800 micrograms per milliliter of TiO<sub>2</sub> nanoparticles showed the highest and lowest positive effects on germination factors and the seedling growth of the *Vitex* plant, respectively (Farahi *et al.*, 2019)) TiO<sub>2</sub> nanoparticles were produced by Notirino Company in Tehran. In order to prepare a homogeneous solution of TiO<sub>2</sub> nanoparticles, further to the regular shaker, the solution was placed in an ultrasonic cleaner bath for 30 minutes. The nanoparticle solution was sprayed as a leaf spray by piset on 30th day of experiments on plants, and control plants were sprayed with distilled water. The duration of the experiment was 65 days.

### *Investigation of nanoparticle properties*

The nanoparticle characteristics including particle motion, zeta potential and TiO<sub>2</sub> nanoparticle size were analyzed by Cordouan-Vasco Particle Size Analyzer with Nano Q software in the central laboratory of Ferdowsi University of Mashhad. To perform the experiment, the nanoparticle, which was dissolved in a suitable solvent (ionized water at pH of 6), was first placed in an ultrasonic device. The ultrasonic device was used to create uniform dispersion for a maximum of 20 minutes at ambient temperature. After the nanoparticles were dispersed into the solvent, the sample was transferred to the Zetasizer device without interruption. The Zetasizer device first determines the particle size by measuring the Brownian motion of the particles in a sample by Dynamic Light Scattering (DLS) and then by interpreting the size using proven theories. The crystalline properties of TiO<sub>2</sub> were also investigated by X-ray diffraction (XRD).

### *Investigating gene expression*

Design of primers: To investigate the expression of genes studied in response to nanoparticles, primers were designed for CAT, P450, SOD, diTPS and WRKY genes and Alpha-tubulin gene, and generated by cell probe gene research company (Table 1) The primers were designed in Geneious IR9 software based on primer3 and accompanied by Oligoanalyzer software.

Extraction of RNA, quantitative production of cDNA and PCR: Sampling of plants was performed 24 hours after application of the nanoparticles. Total cell RNA extraction was performed by Total RNA Extraction Kit according to the instructions of Pars Tous Manufacturing Company. Nano drop was used to determine the concentration and ensure the desired quality of the extracted RNA. The concentration of extracted RNAs after reading the treatment in the samples was read at a wavelength of 260 nm. The concentration of each sample was calculated and for the qualitative and quantitative control of the total extracted RNA, the electrophoresis method was used on agarose gel (1.2%) In order to eliminate possible contamination with genomic DNA in RNA samples extracted from DNase I treatment was used according to the proposed method of Fermantaz Company and the tubes were stored at -80 °C for long-term storage.

To make cDNA, according to the results of the nano-drop, one microgram of RNA was taken from each RNA sample and cDNA was used for all samples using Easy™ cDNA Synthesis Kit (New) according to the manufacturer's instructions (Pars Tous, Iran) In order to confirm the production of cDNA, the method of replication of homogenous gene (Actin1) on cDNA (after diluting and increasing the concentration of cDNA made to 200 ng / μl) was used by PCR and its electrophoresis on 1% agarose gel.

Real-time PCR was used to evaluate gene expression. This method is one of the most accurate methods for examining gene expression in which the changes in the number is not important, but the increase or decrease of gene expression is important. These changes will be compared with an internal control that is the standard or reference gene and normalized CT value will be matched It against an untreated sample (Pfaffl, 2001). For this purpose, the PCR response conditions for the housekeeping gene (Actin1) (by changing the annealing temperature, the time of each cycle, as well as the number of reaction cycles) were optimized. The PCR reaction was then performed for other genes under the specified conditions. For all samples, each PCR-RT reaction was performed in four repetitions (two biological repetitions and two technical repetitions). Finally, the results were analyzed using 2-DDCT comparison method (Livak and Schmittgen, 2001). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) and mean comparison by Duncan's test using SPSS software version 16.

**Table 1.** Primer sequences used in polymerase chain reaction in RT-PCR reaction

Gene name	Sequence primer 5' → 3'	Annealing temperature (°C)	Replicated fragment length (bp)
diTPS	F: ATGATACAGTAAATGAGTTCGCAGA	60	130
	R: TCGTCGGTCCATGAATCCAA	60	
ViAgP450	F: AAGGCCAAGATTTCGTGCTT	60	140
	R: TGTCACGCCTGGTTCAAATT	60	
WRKY	F: TGGGTGGTTGTGGTTGCC	60	176
	R: CGGATGGATGCCAATGGCG	60	
ViAgSOD	F: CCACTGGGGGAAGCATCACAG	60	153
	R: GCCTGAGCTGCATTGTTGAA	59	
ViAgCat	F: TGTGC(CT)GATTCCTTCGAGC	58.6	149
	R: AAATCAAAGTTTCCCTCTCTGGTAT	58	
Alpha-tubulin	F: GCTAACAACTTTGCCCGTGGAC	61	166
	R: CCAGCAGAAGAGATCCAAGACC	59.5	

## Results and Discussion

### *Characteristics of titanium dioxide nanoparticles*

X-ray diffraction is a method of studying the crystal structure of materials. X-rays used for diffraction usually have a wavelength of about 0.5 to 2.5 angstroms. In order to perform this experiment, electromagnetic radiation with a wavelength between 0.01 and 10 nanometers was used. This wavelength is shorter than the ultraviolet wavelength and longer than the gamma ray. The crystalline properties of TiO<sub>2</sub> nanoparticles were investigated by X-ray diffraction (XRD) and the result is presented in (Figure 1). XRD measurements showed that TiO<sub>2</sub> nanoparticles were used in the anatase form (Figure 1). The size of TiO<sub>2</sub> nanoparticles was measured by a nano-particle size device and is presented in scattered intensity (Figure 2 a), scattered volume (Figure 2 b) and scattered number (Figure 2 c), average. Nanoparticles diameter 98.87: (D mean), particle hydrodynamic diameter: 197.01 (Zaverage), particle scattering index: 0.2050 (PDI) (Figure 3 a) Particle motion and (Figure 3 b) show the potential of Zeta TiO<sub>2</sub> nanoparticles measured by the Zetasizer device (Figure 3).

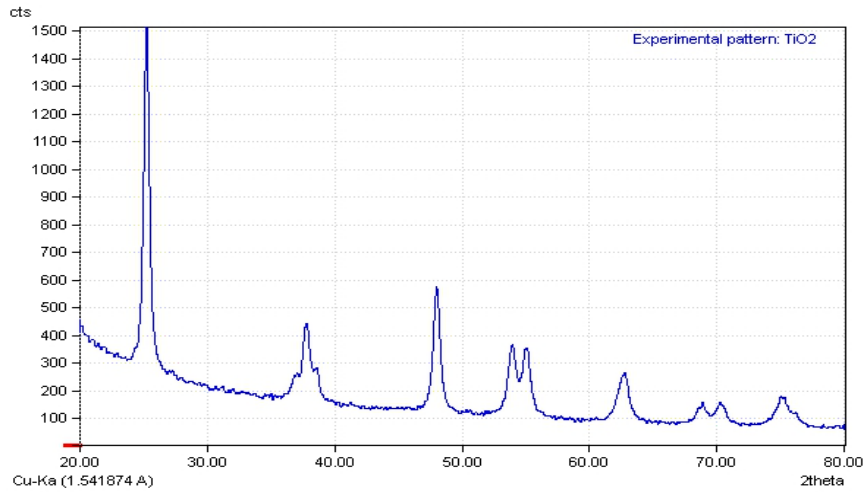


Figure 1. XRD pattern of TiO<sub>2</sub> nanoparticles

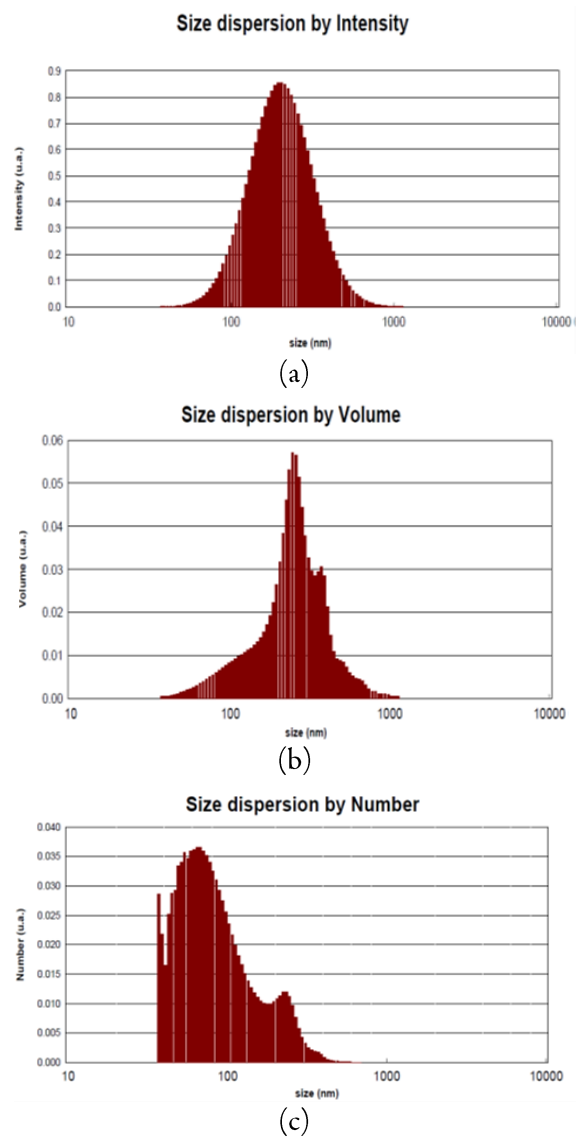
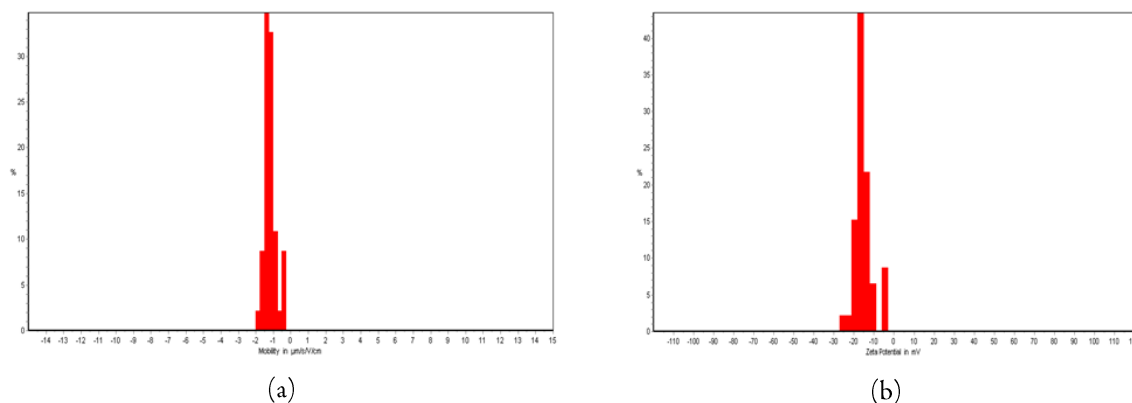


Figure 2. Size of titanium dioxide nanoparticles measured by nanoparticle sizer device with: a) scattered intensity, b) scattered volume and c) scattered number



**Figure 3.** a) Particle motion and b) Zeta potential of titanium dioxide nanoparticles by Zeta Sizer device

#### *Relative expression of genes*

Statistical analysis of gene expression in treated *Vitex* leaves with TiO<sub>2</sub> nanoparticles showed that this nanoparticle significantly affected the expression of Catalase, P450, SOD, diTPS and WRKY genes. In other words, treatment with TiO<sub>2</sub> nanoparticles significantly altered the accumulation of transcripts of the studied genes. The greatest change in gene expression due to the use of TiO<sub>2</sub> nanoparticles compared to the control treatment was observed in WRKY gene and concentration of 800 µg/ml nanoparticle (5.69 times) and the lowest change was in diTPS gene expression and in concentration of 800 µg/ml nanoparticle (0.46 times) (Figure 4) According to the results, the expression of WRKY and CAT genes with increasing use of TiO<sub>2</sub> nanoparticles had an increasing trend. However, different responses were found for genes in different concentrations of nanoparticles (Figure 4) Previous studies have shown the inducive or inhibitory effect of TiO<sub>2</sub> nanoparticles on the physiological and biochemical responses of plants depending on the species, cultivar, texture and concentration of nanoparticles (Nair *et al.*, 2010; Kurepa *et al.*, 2010) The results of this study also showed that the use of nanoparticles on *Vitex* medicinal plant may participate in re-programming the genome. The presence of nanoparticles in the plant can act as a stressor and increase the activity of the plant's defense system at different concentrations of nanoparticles, thereby stimulating the expression of certain genes and the production of secondary plant metabolites.

The relative expression of the diTPS gene decreased with the use of TiO<sub>2</sub> nanoparticles. The expression of this gene under treatments of 200 and 800 µg/ml TiO<sub>2</sub> decreased compared with control treatment, by 2.1 and 0.46 respectively (Figure 4 a) According to studies on the Verbenaceae family species, there are different classes of diTPS that play a role in the biosynthesis of the plant's highly valuable diterpenes (Zhou and Pichersky, 2020) Given the importance of the expression of the diTPS gene in the biosynthesis pathway of phytonutrient diterpenes, it is likely that the production of these compounds will decrease with the use of TiO<sub>2</sub> and reduced expression of the diTPS gene, although some researchers believe this is not always the case and some enzymes and final products are not close. This is because there are time and space gaps between genes and the final product. Even TPS genes, which account for 60 to 80 percent of similar protein and nucleotide specifications, produce different product ranges (He *et al.*, 2018) Following the application of nanoparticle, rapid defense responses occur in plant cells, such as increased ionic currents across the plasma membrane, production of reactive oxygen species, activation of defense-related genes, structural changes in the cell wall, and synthesis of phytoalexins. Decreased diTPS gene expression may be due to toxicity of nanoparticles and their effect on one of the above mediators and decreased gene expression (Siddiqui *et al.*, 2015) Stimulation of TPS gene expression by stimuli has also been observed in other studies (He *et al.*, 2018) TiO<sub>2</sub> nanoparticles increased the expression of key genes in the thymoquinone biosynthesis pathway in the plant (*Nigella sativa*) (Kahila *et al.*, 2018) In the study of Zhang *et al.* (2013), the effect of silver nanoparticles on increasing the production of secondary metabolites in the plant (*Artemisia annua*) increased artemisinin from 1.67 to 2.86 mg/g dry weight. Raei *et al.* (2014) by using titanium and silver dioxide nanoparticles reported an increase in

the amount of aloin in the cellular suspension of aloe vera. The amount of artemisinin production in artemisia plant increased due to the use of cobalt nanoparticles in artemisia cell culture medium (Ghasemi *et al.*, 2015)

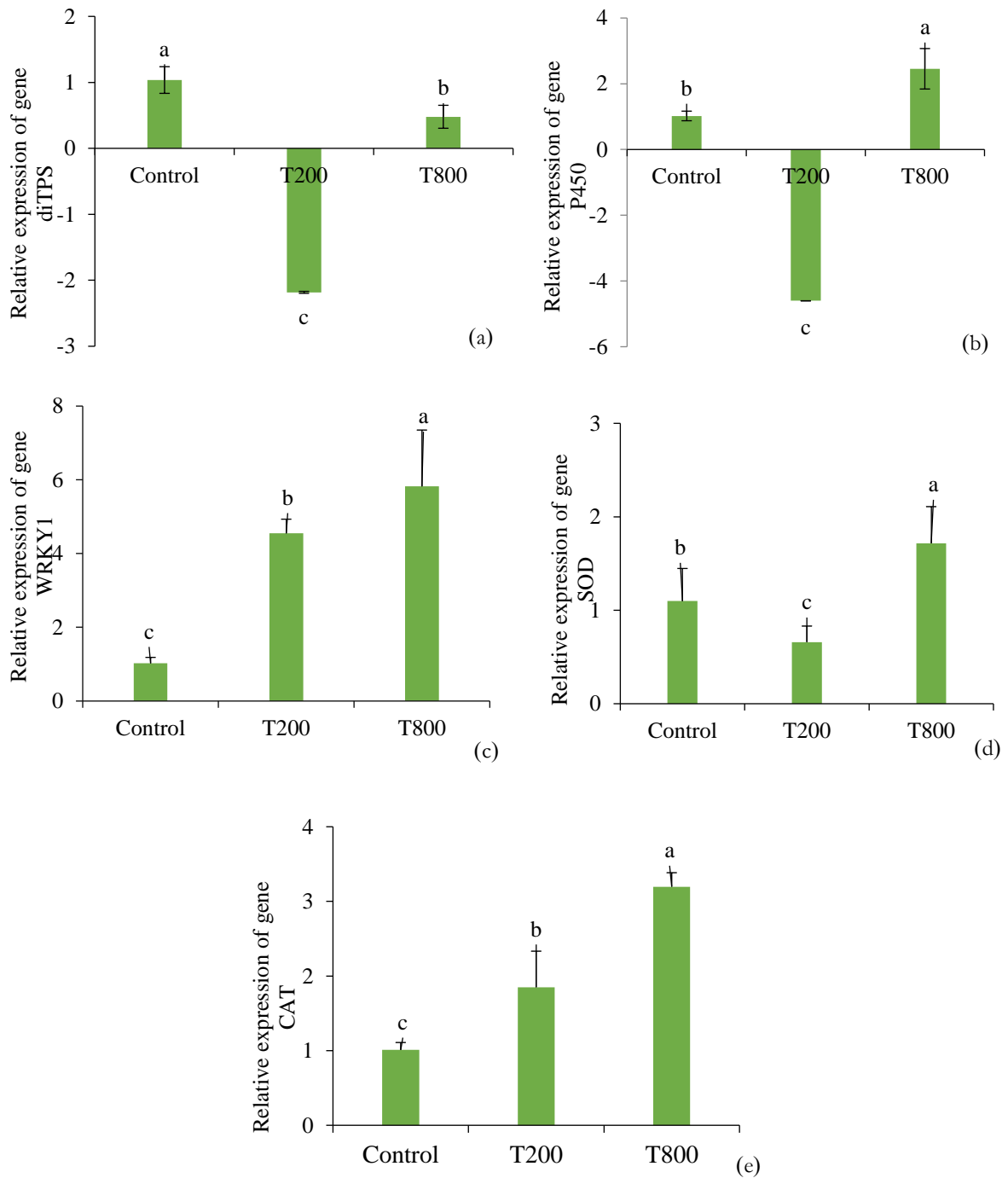
The relative expression of the P450 cytochrome gene decreased by 4.5-fold due to the application of 200  $\mu\text{g}/\text{TiO}_2$ , whereas at a concentration of 800  $\mu\text{g}/\text{ml}$ , a 2.4-fold increase in P450 gene expression was observed (Figure 4 b) The large cytochrome P450 enzymatic family plays a vital role in plant growth, development, and protection against stress through biosynthesis and detoxification pathways (Li *et al.*, 2012) There are many reports of the gene's important role in the biosynthesis of terpenes, some of which include triterpene in tobacco (Geisler *et al.*, 2013), homoterpenes in *Arabidopsis* (Lee *et al.*, 2010), sesquiterpene in cotton (Luo *et al.*, 2001) and Dieterpen in the Apiaceae family (Hamberger *et al.*, 2011)

The relative expression of the WRKY gene under treatment with concentrations of 200 and 800 micrograms per milliliter of  $\text{TiO}_2$  nanoparticles increased by 4.44 and 5.69 times, respectively (Figure 4 c) WRKY proteins belong to the large family of transcription regulators whose members are associated with defensive responses (Eulgem and Somssich, 2007; Rushton *et al.*, 2010) Both concentrations of nanoparticles caused a relatively large increase in the transcript of WRKY transcription factor compared to other genes studied in this experiment. Due to the regulatory role of transcription factors in the plant's signaling network, these genes induce a variety of physiological and biochemical responses at the bottom of their hands. Therefore, their effectiveness in applying the nanoparticle stimulus is significant, and the expression of the lower genes in the hand of this transcription factor should be more closely examined. In other studies, the expression of this family's genes has been influenced by stimuli (Naoumkina *et al.*, 2008) The expression of WRKY genes in transgenic tobacco shows that these transcription factors play a broad role in regulating metabolic responses that affect the production of secondary metabolites (phenol and lignin) and stress tolerance (Naoumkina *et al.*, 2008) It has also been reported that the induction of WRKY gene expression is correlated with the accumulation of phenolic compounds and isoflavones in cells (*M. truncatula*) (Naoumkina *et al.*, 2007) An increase in lignin accumulation in transgenic rice lines has also been reported in the above expression of the OsWRKY89 gene (Wang *et al.*, 2007) Increases in the biosynthesis of indole alkaloids (*Catharanthus roseus*) (Yang *et al.*, 2013) and sesquiterpene arboretum (Xu *et al.*, 2004) have been reported with increased WRKY expression.

The relative expression of the SOD gene at a concentration of 200  $\mu\text{g}/\text{ml}$   $\text{TiO}_2$  decreased by 0.6 times compared to the control, while in the treatment of 800  $\mu\text{g}/\text{ml}$   $\text{TiO}_2$ , the expression of SOD gene expression was increased by 1.56 times compared to the control (Figure 4 d) The use of  $\text{TiO}_2$  nanoparticles had an increasing effect on the relative expression of the CAT gene. According to (Figure 4 e), concentrations of 200 and 800 micrograms per milliliter increased the expression of CAT gene expression by 1.83 and 3.16 times, respectively. Application of nanoparticles induces oxidative stresses and damage biomolecules by releasing free oxygen radicles (Chomoucka *et al.*, 2010) Probably for this reason, in this study, an increase in the expression of antioxidant genes SOD and CAT due to the use of nanoparticles was observed. Other studies have reported an increase in the expression of stress-related genes in nanoparticle treatment (Khodakovskaya *et al.*, 2011)  $\text{TiO}_2$  nanoparticles (*Hyoscyamus niger* L.) activate the antioxidant enzymes and biosynthesis of hyoscyamine and scopolamine (Ghorbanpour *et al.*, 2015)

In general, as can be deduced from Figure 4, all the genes studied were stimulated in response to the nanoparticle stimulus (although the concentration of the stimulus in this case is considered an important and influential factor) In this study, a concentration of 800 micrograms per liter of titanium nanofibers increased the expression of CAT, P450, SOD and WRKY genes and decreased the expression of diTPS gene. In contrast, concentrations of 200 micrograms per liter only increased the expression of CAT and WRKY genes. The higher concentration of titanium nano-oxide (800 micrograms per liter) appears to increase the expression of the studied genes, with the exception of the diTPS gene. These differences in response to nanoparticle treatment may indicate that the expression of these genes does not have a common or similar regulatory mechanism. It is also necessary to study the expression of genes after applying the nanoparticle treatment at

different times, because the maximum expression of different genes may occur at different times after the induction of the stimulus.



**Figure 4.** Relative expression of gene expression a) diTPS, b) P450, c) WRKY, d) SOD, and e) CAT in the leaves of the *Vitex* plant under different concentrations of titanium nano-oxide

## Conclusions

The results of this study showed that the use of TiO<sub>2</sub> nanoparticles was a stimulus to alter the expression of the studied genes of the *Vitex* plant, and this nanoparticle could affect the expression of the genes of terpenoid biosynthesis pathways as well as antioxidant enzymes of the plant. However, due to the complexity of the biosynthetic pathways of metabolites, the study of gene expression of other enzymes and their interaction should be further studied. It should also be noted that the use of nanoparticles to increase or decrease gene expression depends on the species and tissue, the duration of treatment with the stimulus and the concentration and characteristics of the nanoparticle. According to the results of this study, the concentration of 800 micrograms per milliliter of titanium nanoparticles increased the expression of CAT, P450, SOD and WRKY genes and decreased the expression of diTPS gene. At concentrations of 200 micrograms per milliliter, only the expression of CAT and WRKY genes increased. Since there is not much information about the use of nanoparticles in plants, deciding on their function in plant cells requires further studies on the expression of genes after several hours or days of application of nanoparticles, because it is estimated that nanoparticles have a toxic effect on the mitotic index and genomic. The results of this study also show that a more detailed study of the effect of nanoparticles on plant transcription factors such as WRKY will be effective in expanding the application of nanoparticles in agriculture.

## Authors' Contributions

SMMF: Main Person of All activities: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review and editing; AI: Project administration, Supervision, Validation, review and editing; HM: Supervision, Validation; ME: Supervision; Validation.

All authors read and approved the final manuscript.

## Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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