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Evaluation of the sole and integrated application of nano-graphene oxide, zeolite, and chitosan on gas exchanges and silymarin content of milk thistle (*Silybum marianum* L.) under salinity stress

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Abstract

Identifying environmental factors, plant characteristics, and agronomic activities plays an essential role in medicinal plant production. Milk thistle (*Silybum marianum* L.) is a well-known medicinal plant with extensive use in diverse liver diseases and is economically a significant crop. This research was conducted to evaluate the effect of the sole and integrated applications of graphene oxide (GO), zeolite, and chitosan as modifying materials on gas exchange and the secondary metabolites of milk thistle under severe salinity stress. Seven sole and integrated combinations of nano-materials comprised of T1, T2, T3, T4, T5, T6, T7, and control (T8, no nano-materials application) and two levels of saline water (12 ds/m) and tap water (control, 0.8 ds/m) were applied to the soil of experimental plots based on a factorial design with three replications. The results showed that the highest photosynthesis rate was obtained with T7 treatment for both water treatments. The highest plant silymarin concentration was obtained from the T6 treatment under both saline and tap water conditions. This treatment increased the silymarin concentration by 15.9% compared to the T8. The highest plant silymarin yield (180 mg per plant) was recorded for the T7 under tap water (control) condition, and 130.3 mg/plant for T6 under salinity stress, respectively. The Transmission Electron Microscope technology indicated that GO at low concentration (0.01%) could be safely used to enhance milk thistle germination and growth under severe salinity stress conditions.

Keywords: Carbon nanotubes, Medicinal plant, Photosynthesis rate, TEM, Seed yield, Silymarin.
Abbreviation: GNM_Graphene nano-material; GO_Graphene oxide; T1_GO; T2_Zeolite; T3_Chitosan; T4_Graphene+zeolite; T5_ Graphene+chitosan; T6_Zeolite+ chitosan; T7_Graphene+ zeolite+ chitosan; T8_Control (no nano-materials application).

Introduction

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a promising new crop. Its seeds contain silymarin (complex flavonolignans), which is widely used in the pharmaceutical industry mainly to produce dietary supplements (Petrásková et al., 2020. Silymarin is the extract derived from the seeds of milk thistle. Besides its hepatoprotective action against several liver diseases, silymarin exhibits anticancer, anti-inflammatory, anti-fibrotic, and antioxidant properties (Arampatzis et al., 2019).
Salinity is detrimental to crops because of its adverse physiological, biochemical, and morphological effects, which result in reduced biomass production and yield (Tian et al., 2020). The reduction of plant growth due to salinity is mainly determined by the factors that contribute to the decline of photosynthetic activity (Tian et al., 2020). Moreover, Mosaffa and Sepaskhah (2019) showed that the effects of salinity stress on crops are extremely adverse as it led to leaf necrosis, altered phenology, and, finally, plant death.

Nanotechnology has increasingly been applied to medicines, alternative energy, electronics, environmental protection, and agriculture. The application of engineered nano-materials to agricultural sciences has been particularly active and exciting (Zhang et al., 2015). In the past few years, nanotechnology's beneficial potential in agriculture to improve crop productivity has been recognized. Recent studies on this subject can be found in the literature (Cheng et al., 2016; Ren et al., 2016; de la Rosa et al., 2017; Babaei et al., 2017). Graphene oxide (GO) is one of the most used Graphene nano-material (GNM) due to its hydrophilic nature and water dispersibility. Considering the potentially widespread use of GNMs, including their direct use in agricultural and environmental applications, the inevitable release and presence of graphene in the environment has raised environmental concerns. To date, the environmental fate and behavior of GNMs, particularly in soil, is poorly understood, and this urgently needs to be addressed (Navarro et al., 2020). The beneficial effects of GO application on crop growth and development have been reported by Anjum et al. (2014) on faba bean (*Vicia faba* L); Hu et al. (2014) on wheat; Zhang et al. (2015) on tomato; and Ren et al. (2016) on maize. GO have tremendous potentials for a wide variety of biomedical applications in drugs, tissue engineering, bio-detection, and bioimaging due to their biocompatibility. However, there is a growing need to develop environmentally friendly graphene synthesis processes that do not use toxic chemicals. Chitosan is a cationic polysaccharide, a derivative of chitin extracted from exoskeleton of shellfish such as shrimp, lobster, or crabs and fungi's cell walls. Chitosan emboldens its application in medicine, food technology, biomedical engineering, and agriculture (Ahmed et al., 2020). In arid and semi-arid environments, materials such as zeolite can increase soil's water holding capacity and buffer, nutrient absorption, and release to avoid the damage caused by stress to the photosynthetic apparatus (Sahebi et al., 2020). Zeolites have been incorporated as soil conditioners, remediation material for contaminated soil treatment, and slow-release fertilizers (Bhardwaj et al., 2019). Due to their high porosity, zeolites can improve plant growth by increasing the long-term water and nutrients holding capacity (Hazrati et al., 2017).

We hypothesized that the application of nano-materials such as graphene oxide, chitosan, and zeolite would mitigate the harmful effects of salinity stress on milk thistle. However, there are no research findings in the literature to explain the interactive effects of salinity stress and the nano-materials' integrated application. Therefore, this research aimed to evaluate the sole and integrated application of nanographene oxide, zeolite, and chitosan as modifying materials on gas exchange and silymarin in milk thistle under salinity stresses. The major goal of this experiment was to follow the terrace of nano-materials' accumulation in the leaf cells of milk thistle. This phenomenon is of major interest to the research community as this can be used to certify the security of nano-material application in medicinal plants for human consumption.

Results and Discussion

Plant height

Plant height was significantly inhibited by the application of nano-materials under salinity conditions and their interaction effects ($P < 0.01$) (Table 1). Under salinity stress, the highest plant height (20.3 cm) was recorded for the T7 treatment (Figure 3). These results showed clearly the deleterious effects of saline stress as well as the remarkable ability of T7 treatment to restore plant height affected by salinity. However, it should be noted that the height of stressed plants always remains significantly lower than those of unstressed plants even when treated with nanomaterials. According to Bahreininejad and Allahdadi (2020), the saline water irrigation significantly reduced the plant height of *Cynara cardunculus*. For example, the plant height of rice cultivars was significantly ($P<0.05$) reduced by salinity (Puvanitha and Mahendran, 2017). Stimulation of plant growth under chitosan + zeolite has also been reported by Kumari et al. (2020). They observed enhanced plant height over control where fenugreek plants (*Trigonella foenum-graceum* L) were treated with nano compounds (50 mg/L).

Physiological traits

Chlorophyll content (SPAD chlorophyll meter values)

Salinity stress plays a key role to the perturbation in photosynthesis and, therefore, reduces plant growth and production. We found a significant ($P < 0.05$) effect on chlorophyll content under salinity stress, season, and the interaction of the two (Table 1). Due to increased photosynthetic potential, higher chlorophyll content in plants has helped them grow better under undesired stress conditions (Kang et al., 2014). The study results showed that chlorophyll content was negatively affected by salinity stress, consistent with Vangelisti et al. (2019). They reported the adverse effect of elevated salinity levels on root biomass, leading to a parallel decrease in chlorophyll content. A higher SPAD value was obtained for tap water (control) irrigation (0.8 ds/m), and the lowest was recorded under salinity stress (Figure 4). Salinity reduces the chlorophyll content, and this reduction is dependent on the intensity of stress and the degree of plant tolerance. This reduction in chlorophyll contents could be related to the suppression of the enzymes responsible for chlorophyll synthesis (Bensidhoum and Nabti, 2021). The tested parameters, such as chlorophyll content and total carbohydrates, were reduced significantly at higher salinity levels (Ashour et al., 2021). This notable decrease in the chlorophyll content due to high sodium chloride concentration could be explained by the negative effect of salt on leaf area and photosynthesis. This observation was previously reported by Hatam et al. (2020) for cotton (*Gossypium hirsutum* L). Similar behavior was recorded for common fig (*Ficus carica* L.) where SPAD readings showed a dramatic decline when salinity levels were increased for all treatments (Sadder et al., 2021). Nonetheless, it is recommended to measure additional parameters for future work in studying salinity stress in common fig including $Na+$ and $K+$ levels and proline accumulations. Pigment content is considered to be a sensitive indicator of nanotoxicity. The effects of GO on pigment content vary with GO concentration and plant type (Song et al., 2021).

Photosynthesis rate (Pn)

Salinity stresses, nano-materials, and their interactions had a significant effect on the photosynthesis rate of milk thistle (Table 1). Salinity reduced the plant photosynthesis rate. The lowest photosynthesis rate was obtained from T8 treatment under salinity conditions. However, the application of nano-materials improved the plant photosynthesis rate. There was no significant difference among nano-material treatments for tap water (control) treatment (Table 2). On this issue, previous authors reported increases in plants' growth characteristics subjected to salt stresses as a result of chitosan treatment (Ashour et al., 2021). Early detection of stresses by monitoring physiological properties would help study morphological properties to manage stresses and prevent yield losses (Hazrati et al., 2017; Negrão et al., 2017). In this regard, the photosynthesis rate has been widely assessed for the detection of salt stresses. Further validation comes from the studies of Ors et al. (2021) and Ahmad et al. (2018) who observed increased photosynthetic pigments when oligo-chitosan was applied. Thus, chitosan can have a favorable effect on photosynthesis and other physiological and biochemical processes resulting in enhanced growth and increased photosynthetic pigments. Under the salinewater treatment of 12 dS/m, there were significant differences among different nano-material treatments. The highest rate of photosynthesis was obtained from the triple combination of nano-materials. However, no significant difference was observed between this treatment and the sole application of zeolite, and its integrated application in T4 treatment (Table 2). Hatam et al. (2020) reported that salinity significantly decreased dry weight, chlorophyll content index, photosynthesis rate, leaf to air vapor pressure, transpiration rate, stomatal conductance, and minimum fluorescence of cotton. Studies have shown that chitosan directly affects stimulating physiological processes, improves vegetative growth, and transfers photosynthetic materials from source to sink (Abu-Muriefah, 2013). Exogenous foliar application of GO significantly increased chlorophyll and fluorescence of Milk thistle by increasing the photosynthetic Index (PI). The Performance of the PI Index is an indicator of the sample vitality of photosynthesis (Safikhan et al., 2018b). Zhang et al. (2018) discovered that compared to the non-zeolite treatment, zeolite application increased Pn in riceThis means that the zeolite effect was more evident under water stress.

Stomatal conductance (Gs)

Stomatal conductance quantifies the rate of passage of CO_2 that enters or water vapor that exits the leaf through the stomata. Under non-limiting conditions of water availability and ambient temperatures below heat stress levels, increased stomatal conductance increases the rate of photosynthesis (Ahmad et al., 2019). From our previous experiments, we found that with the increase of salinity stress levels, the stomatal conductance declined gradually (Safikhan et al., 2018b). Elevated salinity levels were coupled with a dramatic decrease in stomatal conductance and net photosynthetic rates in common fig (Caruso et al., 2017). Li et al. (2021) reported that stomatal conductance and transpiration rate decreased under drought stress. Kafi et al. (2021) showed that salinity stress in gas exchange variables was suppressed in Potato (*Solanum tuberosum* L.). Reduction in net photosynthesis, stomatal conductance, and internal CO_2 concentration have been reported under salinity stress in different plant species (Kalaji et al., 2011; Huang et al., 2016; Penella et al., 2016). In the present study, nano-material treatments significantly ($P \leq 0.05$) increased stomatal conductance and improved photosynthetic exchanges compared to control (T8) (Figure 5). Our results are in agreement with the findings of Ahmad et al. (2019) who reported that with the application of chitosan, Gs increased in both peppermint and broad bean. By decreasing the stomatal resistance and increasing stomatal conductivities in plants, application of nano materials improved the growing conditions of the milk thistle under salinity stress. Our results showed that the highest stomatal conductance was obtained for T2 treatment, which was not significantly different compared to T3 and T7 treatments (Figure 5). Zeolite application increased Gs by 22.2% in 2014 and 25.0% in 2015, respectively, relative to the non-zeolite treatment (Zhang et al., 2018).

Thermal infrared data

The use of canopy thermography is an innovative method for salinity stress detection. We obtained high-resolution thermal infrared images and recorded significant changes in leaf temperature for the milk thistle under control (no salinity), salinity stress, and application of nano-materials (Figure 6). Salinity significantly ($P \leq 0.05$) increased milk thistle leaf temperature. However, no significant leaf temperature differences were observed due to different nano-materials at each water treatment. Any increase in leaf temperature could be due to stomatal closure, an unspecific plant defense mechanism against both abiotic and biotic stressors (Table 2). It has been reported that the loss of internal water and stomatal closure is associated with increased leaf temperature affecting salinity and drought stress in rice varieties (Muthurajan et al., 2021). It has also been informed that leaf temperature of barley (*Hordeum vulgare* L), rice (*Oryza sativa* L), and wheat (*Triticum aestivum* L) was increased proportionally to increasing salt concentration in soil (Pineda et al., 2021). The general pattern is that high soil salinity values correspond quite well with high canopy temperature values. In our study, for for tap water (control) application, the corresponding leaf temperatures in the infrared images were around 90.3 ℉ (32.38 ℃) and 90.7 ℉ (32.61 ℃), respectively. With the application of nano materials, the leaf temperatures gradually decreased. The average canopy temperatures for the milk thistle with tap water (control) irrigation while receiving integrated nano-materials (T7) treatment were 87.6 ℉ (30.88 ℃) and90.3 ℉, respectively. Under saline water (12ds/m) treatment, the temperatures were 84.2 ℉ (29 ℃), and 90.7 ℉, respectively (figure 6-d, 6-b, 6-c, 6-a). The canopy temperature differences under different salinity stress levels have suggested that soil salinity significantly increases crop leaf temperature (Tian et al., 2020). Recent studies have shown that chitosan induces mechanisms against abiotic (salinity, drought, heavy metal, and cold) stresses in plants and maintains barriers to enhancing plant productivity (Katiyar et al., 2015). Accumulating evidence demonstrates that combining and supplementing nanoparticles to plants can significantly alleviate the injurious effects caused by various harsh conditions, including salt stress, and hence, regulate adaptive mechanisms in plants (Zulfiqar and Muhammad, 2021).

Seed yield and components

Translocation of pre-anthesis assimilates to the seed is a crucial physiological process during the filling phase of Milk thistle seeds, especially under abiotic stress. Analysis of variance on seed weight showed that salinity stress and the application of nano-materials ($p \leq 0.001$) affected the 1000-seed weight (Table 3). A similar view has been put forth by Boonlertnirun et al., (2017) who suggest that application of chitosan by varying application methods did not affect 1,000-seed weight. Salinity stress (12 ds/m) led to a 96.7% reduction in the 1000-seed weight compared to control irrigation conditions (0.8 ds/m). Hammami et al., (2020) reported similar results that salinity stresses limited plant growth and seed yield, and adversely affected seed quality and vigor. Among the nano-material application treatments, the highest 1000-seed weight (31.47 g) was obtained from the T8 treatment, which was not significantly different compared to T3 and T7

Table 1. Analysis of variance of growth and physiological characteristics of milk thistle affected by salt stress and nano-materials in two repetitions of the experiment.

S.O.V.	D.F.	Plant height (cm)	SPAD values	photosynthesis rate (µmol m-2 s-1)	Leaf Temperature (°C)
Year	1	1.53^{ns}	77.41^{*}	0.75^{ns}	0.1560^{ns}
Year (repeat)	4	0.46^{ns}	15.66^{ns}	0.41^{ns}	1.0176^{*}
Salinity	1	2015.38^{**}	837.03^{**}	92.47^{**}	116.3140^{**}
Year × Salinity	1	2.00^{ns}	122.96^{**}	0.02^{ns}	0.07763^{ns}
Nanomaterials	7	39.18^{**}	25.01^{**}	5.47^{**}	4.16549^{**}
Year × Nanomaterials	7	0.40^{ns}	12.15^{ns}	0.27^{ns}	0.18309^{ns}
Salinity × Nanomaterials	7	5.83^{**}	6.25^{ns}	0.99^{*}	1.84189^{**}
Year× Salinity × Nanomaterials	7	0.28^{ns}	14.59^{ns}	0.51^{ns}	0.098190^{ns}
Error	60	1.80	14.57	0.39	0.36452
C.V.	-	5.81	9.34	7.92	1.91
*and **: Significant at 5% and 1% probability levels.					

Table 2. Growth and physiological characteristics of milk thistle affected by salt stress and application of nano-materials.

Treatments	Photosynthesis rate (µmol m-2 s-1)	Leaf temperature (°C)	Seed Yield (g per plant)
12 ds/m			
Chitosan	7.118 def	31.69 cd	4.033 ef
Zeolite	7.633 cd	31.93 cd	4.007 ef
GO	6.413 fg	32.26 bc	5.037 de
Zeolite+ Chitosan	6.25 gh	32.9 ab	5.837 cd
GO+ Zeolite	7.672 cd	32.8 ab	4.44 ef
GO+ Chitosan	6.748 efg	32.86 ab	3.717 f
GO+ Zeolite+ Chitosan	7.935 bc	32.7 ab	4.21 ef
Control (No nanomaterial)	5.638 h	33.4 a	2.16 g
Control (0.8 ds/m)			
Chitosan	9.367 a	30.23 fg	6.033 cd
Zeolite	9.427 a	29.6 gh	6.733 c
GO	8.638 ab	31.1 de	5.8 cd
Zeolite+ Chitosan	9.317 a	30.57 ef	9 ab
GO+ Zeolite	8.965 a	30.58 ef	7.967 b
GO+ Chitosan	8.725 a	29.97 fg	6.667 c
GO+ Zeolite+ Chitosan	9.233 a	29.13 h	9.717 a
Control (No nanomaterial)	7.44 cde	31.73 cd	5.867 cd
Different letters in each column for each factor indicate a significant difference at P≤0.05. by Duncan test			

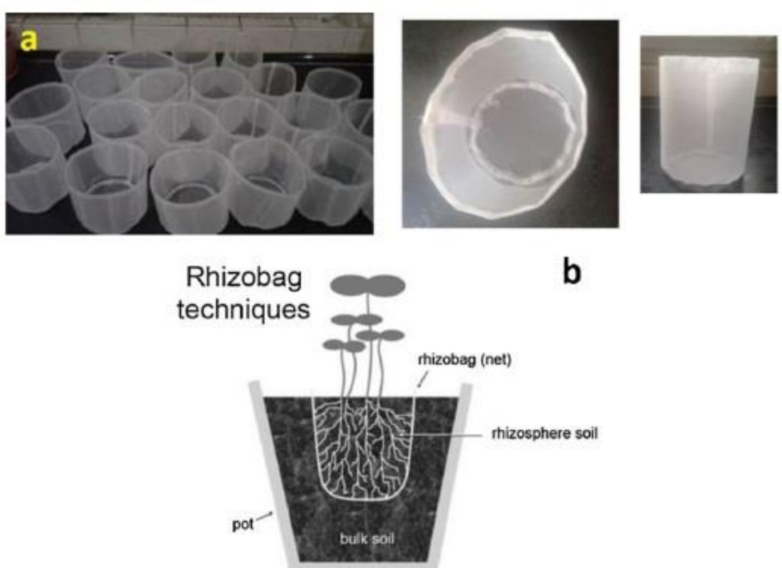

Fig 1. (a) Rhizobag structure, (b) Vertically set into a pot.

Table 3. growth and physiological characteristics of milk thistle affected by salt stress and the application of nano-materials.

S.O.V.	D.F.	Number of capitulum	1000-seed weight (g)	Seed Yield (g/plant)	Silymarin Yield (mg g^{-1} seed)	Silymarin content (mg/g seed)
Block	2	0.39 ^{ns}	3.34 ^{ns}	0.16 ^{ns}	1.98 ^{ns}	
Salt stress	1	1.02 ^{ns}	66.29**	111.11**	17277.12**	146.86**
nanomaterials	7	7.16**	29.79**	7.32**	4215.17**	10.34**
Salinity× nanomaterials	7	0.50^{ns}	7.44 ^{ns}	2.82**	1006.87**	0.63 ^{ns}
Error	30	0.28	6.11	0.39	130.73	0.33
C.V.	-	15.15	8.72	10.94	10.93	3.10

*and **: Significant at 5% and 1% probability levels.

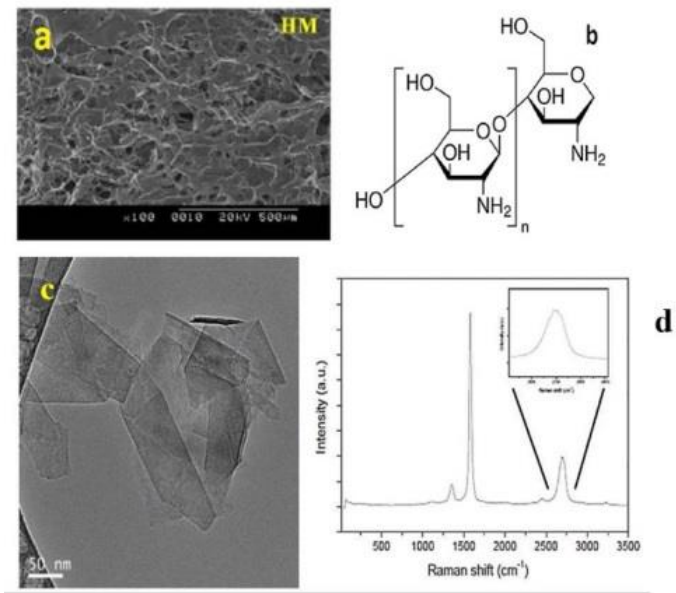

Fig 2. SEM image of HM (high molecular) Chitosan (a) and Chemical formula of chitosan (b) and SEM image of Graphene Oxide and (c) Raman of GO (d)

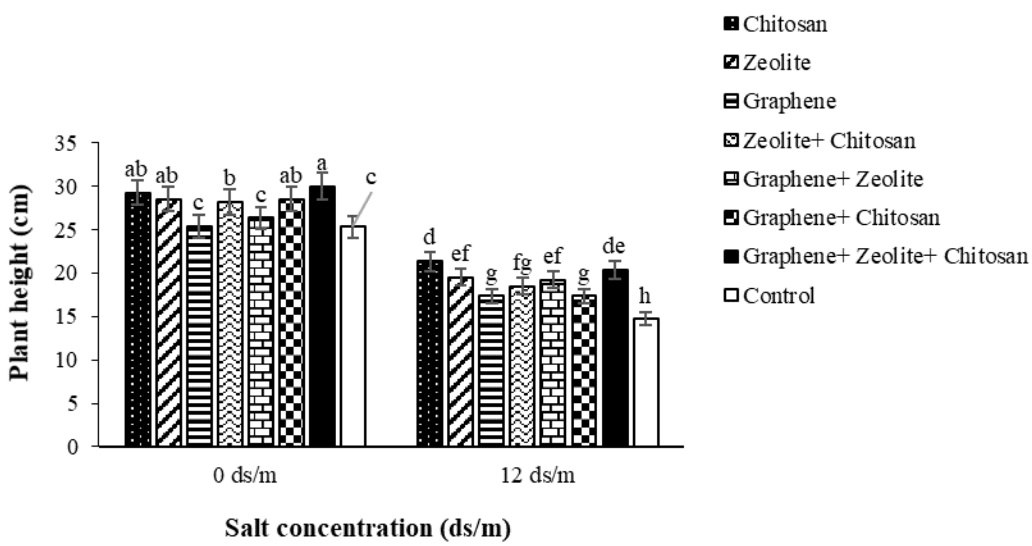

Fig 3. Effect of salt stress and application of nano-materials on plant height of milk thistle. Different letters in each column for each factor indicate a significant difference at P≤0.05

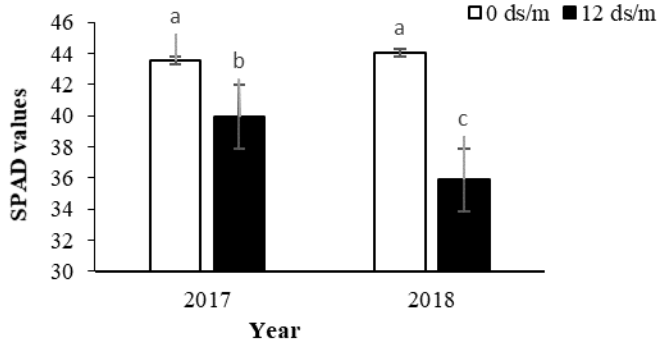

Fig 4. Effect of salt stress and year on SPAD values of milk thistle. Different letters in each column for each factor indicate a significant difference at P≤0.05

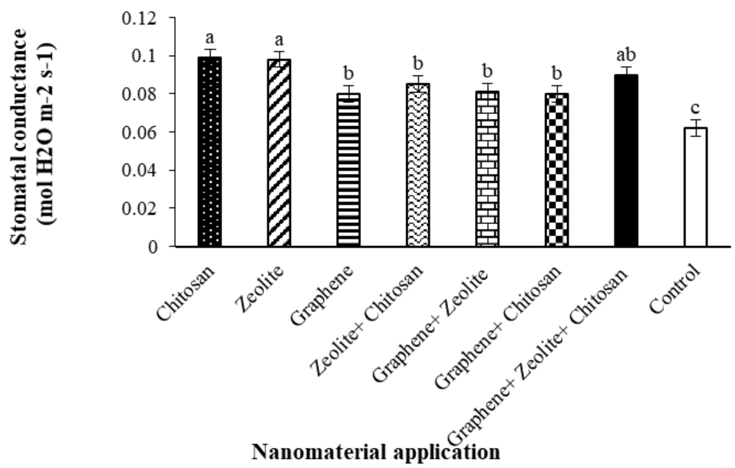

Fig 5. Effect of application of nano-materials on the stomatal conductance of milk thistle. Different letters in each column for each factor indicate a significant difference at P≤0.05.

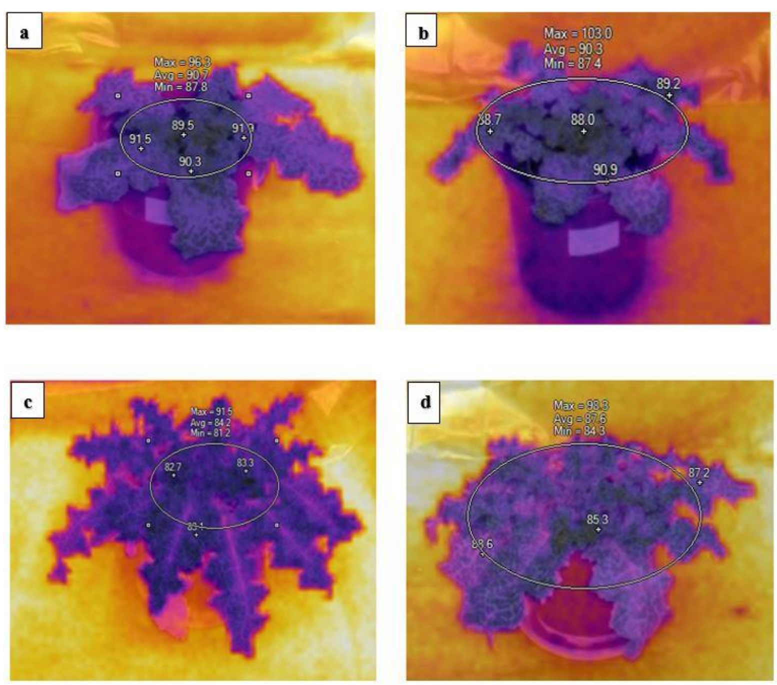

Fig 6. Infrared images of the milk thistle show the temperature of the leaves in different treatments; **a**- Non-application of nanomaterials under 12 ds/m, **b**- Non-application of nanomaterials in control (0.8ds/m), **c**- Integrated effect of nanomaterials (triple) under 12 ds/m, **d**- Integrated effect of nanomaterials (triple) in control (0.8 ds/m).

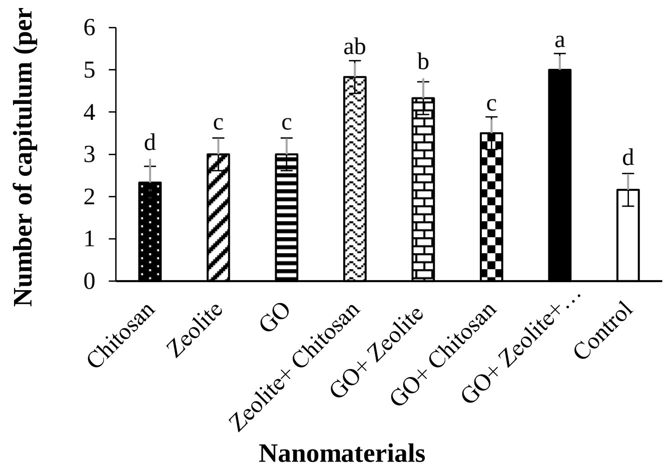

Fig 7. Effect of Nano-materials application on the Number of capitulum of milk thistle.

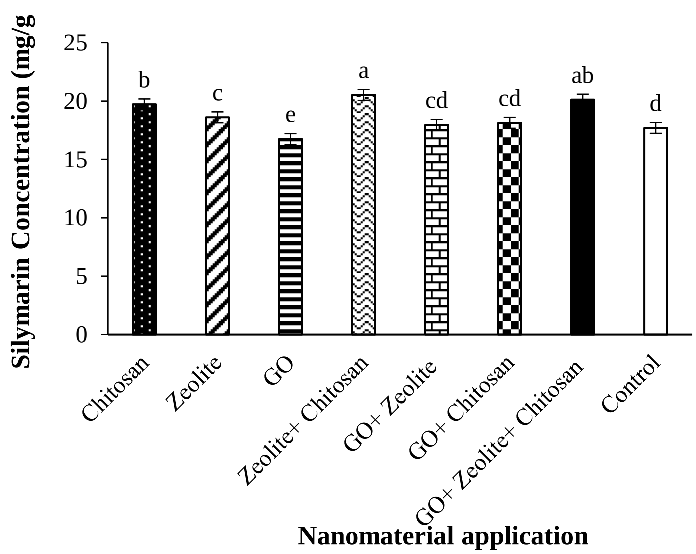

Fig 8. Effect of Nano-materials application on the Silymarin Content of milk thistle.

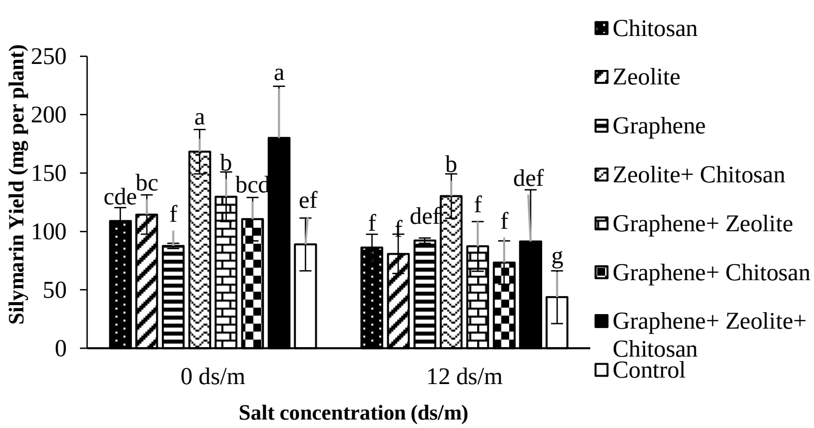

Fig 9. Effect of salt stress and application of nano-materials on Silymarin Yield of milk thistle. Different letters in each column for each factor indicate a significant difference at P≤0.05

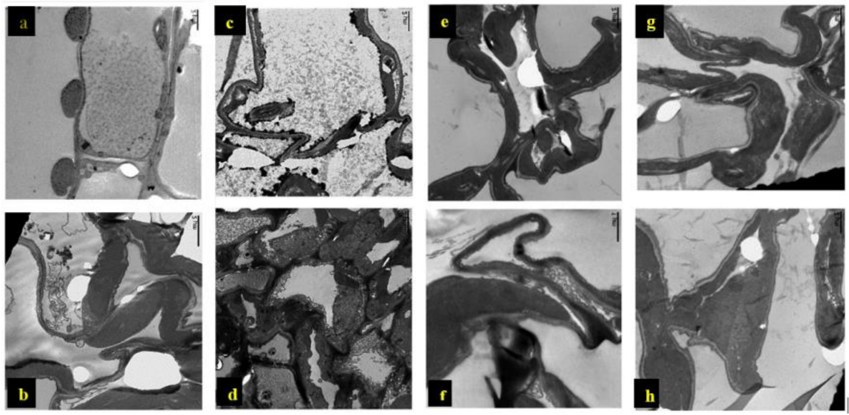

Fig 10. Transmission Electron Microscope (TEM) images of milk thistle leave cells treated by nanomaterials and control. **a:** Chitosan application in control (0.8 ds/m). **b:** Chitosan application under salinity stress (12 ds/m). **c:** Zeolite application in control (0.8 ds/m). **d:** Zeolite application under salinity stress (12 ds/m). **e:** GO application in control (0.8 ds/m). **f:** GO application under salinity stress (12 ds/m). **g** and **h**: contents of the milk thistle leaf cell in no-nanomaterial application treatments in both salinity stress (12ds/m) and control (0.8 ds/m).

treatments (Table 2). Our studies can be corroborated with the studies by Suthar et al. (2018) on Guar (*Cyamopsis tetragonoloba* L.) genotypes. The authors showed that 1000-seed weight and seed yield decreased with increasing irrigation water salinity level.

Seed yield was significantly ($p \leq 0.001$) affected by salinity stress, nano-materials application, and their interaction (Table 3). Under normal and salinity stresses, the highest seed yield of 9.71 and 5.83 g/plant were obtained from T6 treatment, respectively. In both irrigation treatments (salinity stress and tap water), the lowest seed yield was obtained from T8 treatment (Table 2). Van Phu et al. (2017) presented the increase of soybean seed yield by chitosan application compared with the control. Yeilaghi et al. (2012) reported a significant reduction in safflower seed yield in safflower genotypes following salinity treatment. Araus et al. (2021) on *Salicornia europaea* and Dadrasan et al. (2015) and Baghbani-Arani et al. (2017) on *Trigonella foenum-graecum* reported that seed yield decreased with increasing water deficit stress severity, particularly at the reproductive stage and without zeolite application. Baghbani-Arani et al. (2017) showed that under salinity stress, reduced sink capacity (yield components such as floret number per capitulum) and shorter growing period lead to lower seed yield in safflower.

The number of flower heads (capitulum)

High accumulation of salts in saline soils reduced soil water potential, which impairs the plants to extract water and nutrients from the soil, and ultimately experience "osmotic stress." Salinity impacts the various physiological processes (decreased respiration, low water uptake, and decreased photosynthesis), and ultimately crop yield (Hussain et al., 2016). Our result showed the Number of capitulum was significantly ($p \leq 0.001$) affected by the salinity and application of nano-materials (Table 3), and agrees with other similar studies Exposure to greater than 9 ds/m salt stress reduced plant height, number of leaves per plant, capitula number per plant, and the main shoot capitulum's diameter in milk thistle (Banerjee and Roychoudhury, 2017). Irrigation with saline water during all growth stages in general and at flowering and capitula development stage, in particular, was mainly responsible for lower grain yield. Hussain and Al-Dakheel (2018) reported that the number of capitulum in safflower (*Carthamus tinctorius* L) decreased from 24 to 44% at 7 and 14 ds/m, respectively. Further validation comes from studies by Hussain et al. (2018) who observed a significant reduction in the number of flowers and seeds per capitulum in sunflower (*Helianthus annuus* L) under drought stress. However, Shim et al. (2020) reported that milk thistle growth parameters such as the number of capitula per plant, main shoot capitulum's diameter, seed yield, and yield components per plant were reduced under severe salinity stress. In the present study, the T7 treatment led to the maximum number of the capitulum (5 per plant) while the minimum number of 2.16 per plant was obtained from the T8 treatment (Figure 7). Chitosan application at 0.28 g/l markedly improved the capitulum diameter by 25% for the reproductive stage (Shehzad et al., 2020). Thus, it may be that chitosan can have a favorable effect on photosynthesis and other physiological processes resulting in enhanced growth. The positive effect of the integrated nano compounds (zeolite + chitosan) on seed germination can be explained based on the role of NPs in regulating aquaporins, the water channels, which regulate the permeability of water in the seeds and enhance the rate of seed germination and plant growth (Chaudhary et al., 2021).

Silymarin content and yield

Soil salinization and alkalization affect the soil productivity and quality of crop plants in arid and semi-arid areas. Plants synthesize a multitude of secondary metabolites derived from central or primary metabolism, fixation of CO_2 in dark reaction and content of primary metabolites, which are closely associated with essential oil (secondary metabolite) accumulation (Ahmed et al., 2020). In our study, salinity stress resulted in a 20.67% increase in plant silymarin content compared to the control (tap water treatment). The highest silymarin content (20.51 mg/g) was observed in T6, which was15.94% higher compared to the T8. Also, the lowest silymarin content (16.74 mg/g grain) was obtained from T1 (Figure 8). Ebrahimian et al. (2019) attributed the increase in oil content to the plant's ability to induce a specific defense mechanism through fatty acids synthesis as osmotic regulation metabolites. As a substitute for traditional methods for enhancement in secondary metabolite production, plant physiologists are now exploring new options. One way is elicitation, a practical approach towards attaining improved synthesis of bioactive compounds (Hussain et al., 2012). Chitosan is one such elicitor employed in medicinal and aromatic plants, which could perk up their secondary metabolites content (Wiktorowska et al., 2010). There was a significant effect of interaction betweensalinity and application of nano-materials on silymarin yield (Table 3). Results indicated that the silymarin yield decreased under salinity stress, but the application of nano-materials improved it. The highest silymarin yield (180 mg per plant) was recorded for T7 treatment at no salinity stress, while the lowest silymarin yield (43.69 mg per plant) was recorded for control (no nano-material application) under salinity stress (Figure 9). Our results agree with the work of Banerjee and Roychoudhury, (2017) who reported the lower oil yield of *Ricinus communis* exposed to salt stress.

Nano-material (Graphene oxide, Zeolite, and Chitosan) absorption by the Milk thistle

The milk thistle seedlings were analyzed by scanning Transmission Electron Microscope (TEM) to determine whether GO, zeolite, and chitosan accumulated in plant cells. TEM images showed that chitosan nano-material in salinity treatment was mainly seen as scattered fine particles in the cell space complex with a higher concentration in cell vacuoles (Figure 10-b). These results were consistent with the studies by Wang et al. (2017) based on chitosan penetration into the plant cells.

Microscopic imaging of the plant cells under zeolite treatment showed that its particles, unlike the chitosan, were placed in a thin layer on the internal cell wall (Figure 10-c). On the other hand, zeolite application images under salinity stress (12 ds/m) showed that more zeolite was observed densely and uniformly on the edge and sides of the cell wall and less frequently inside the vacuoles (Figure 10-d). Other researches have reported that nano-zeolite has been used for penetrating the cell wall and transferred to the mitochondrial organ inside the cell for chemical studies (Deng et al., 2017). In the images of GO application in control (0.8 ds/m, Figure 10-e) and salinity stress conditions (12 ds/m, Figure 10-f), no trace of graphene nanosheets were found inside the cell. According to the TEM images, GO sheets are absent in the leaves of the milk thistle. These results confirmed that GO in the rhizosphere was not absorbed by milk thistle. Currently, the biosafety of nano-materials is still controversial with positive (Gao et al., 2008; Khodakovskaya et al., 2012), negative (Stampoulis et al., 2009; Ghosh et al., 2010), or no significant impacts (Lin and Xing, 2007; Stampoulis et al., 2009) being reported (Shim et al., 2020). The GO sheets had a flake structure of micrometer size that did not allow them to pass through the nanopores on the cytoderm of the plant, and the soil environment is not conducive to the dissolution and movement of GO. This prevented adsorption of GO by plants (He et al., 2018). A previous study also suggested that SEM, TEM, and Raman spectroscopy showed no GO found in *P. ostii* roots, stems, and leave cells. So, the GO improved the drought resistance of *P. ostii* and did not accumulate in the plants (Zhao et al., 2020). Presumably, the soil environment may also limit the movement of GO sheets. Also, some plants' roots can secrete plenty of mucilage, which carries abundant negative charges and coats the root surface (He et al., 2018). Ghorbanpour et al. (2018) examined the application of nano-graphene oxide on the growth and physiological characteristics of the broadleaf plantain (*Planta major* L) under drought stress and reported no trace of GO in microscopic images. However, a large amount of GO accumulated in the callus cells of P. *major,* grown in half-strength Murashige and Skoog (MS) medium, was found in tomato grown in Hoagland medium (Begum et al., 2011). This result could be explained because the soil environment was not conducive to the dissolution and movement of GO, which prevented the plants from absorbing GO. Graphene oxide has beneficial effects on some plant's growth parameters. Because of the stable and robust interaction between GO and the soil surface particles, the GO's dissipation could be prevented (He et al., 2018). Figures 10 (g) and (h) show the milk thistle leaf cell contents in control (no nano-material application) treatments in both salinity (12ds/m) and control (0.8 ds/m) water treatments. He et al. (2018) reported no GO detected either on the surface or inside the cells of plants; this finding suggested that roots were able to secrete large amounts of mucilage, which induced electrostatic repulsion that might prevent GO from touching the root surface. Therefore, GO may serve as a promising nontoxic additive to increase plant yield.

Materials and methods

Experimental design

This experiment was conducted in the Research Greenhouse of Huntley College of Agriculture, California State Polytechnic University, Pomona, CA, during 2017- 2018. The experimental treatments were arranged factorially (8×2) based on a completely randomized design (CRD) with 3 replications. The first factor comprised of 7 combinations of sole and integrated applications of nano-materials including T1 (Graphene Oxide), T2 (Zeolite), T3 (Chitosan), T4 (graphene+zeolite), T5 (graphene+chitosan), T6 (zeolite+ chitosan), T7 (graphene+ zeolite+ chitosan), and T8 (no nano-materials application) and two levels of saline water (12 ds/m) and tap water (control, 0.8 ds/m) as water treatments. The most effective concentration (DW/DW soil) of each material applied to pot soil was 0% as control and 0.01% (DW/DW) for graphene oxide, 0.05% (DW/DW) for zeolite, and 0.05% (DW/DW) for chitosan (Safikhani, *et.al.,* 2018a). The water salinity level (12ds/m) was created by NaCl application to tap water. Saline irrigation water treatment was applied all through the growing period (planting seeds to plant physiological maturity). To maintain a constant soil EC at 12ds/m salinity level, sufficient saline water was applied to reach soil saturation and free leaching at each irrigation. Constant soil EC produced by the saline irrigation water level was confirmed by measuring soil EC following each irrigation in additional pots treated with saline irrigation water. All pots were seeded on Oct. 10, and the experimental period continued to Dec. 20, 2017. During the experimental period, all the pots were kept inside a glass greenhouse under natural light. The greenhouse's minimum and maximum temperatures were maintained at 20 and 25 °C in day and night, respectively.

The RH was maintained at ∼50%. This experiment was repeated in the same time period in 2018.

Material characterization

Graphene oxide (0.01%/dry soil), zeolite (0.05%/dry soil), and chitosan (0.05%/dry soil) concentrations were created by applying various amounts based on dry soil weight in pots. To separate the rhizosphere soil from the bulk soil, a cylindrical rhizobag (13 cm diameter, 11 cm height, and 60 mesh (0.25 mm) pores) was utilized in each pot (Figure 1a, b). The rhizobag methodology was adopted from Wenzel et al. (2001). Each rhizobag was filled with 1000 g sieved soil and fixed vertically into the pots. Rhizobags did not prevent nutrient absorption by the roots (Ohta et al., 2004). Graphene oxide (with Product Number 806641, Batch Number: MKBW6542V, Appearance color; Grey to Black, powder form), zeolite (with CAS Number 1318-02-1, Product Number 96096-100G, beige to white color, powder form, particle size<45 µm) and chitosan (with CAS Number 9012-76-4 and MDL number MFCD00161512, physicochemical properties were beige to orange color, powder form, viscosity 800–2000 cps and high molecular weight) were purchased from Sigma-Aldrich, USA. The scanning electron microscopy (SEM) examination (a) as well as the chemical formula of each of nano-materials (b) are presented in Figure 2.

Soil characterization

Each pot was filled with Garden plus topsoil (#92432, Lowes Inc., North Wilkesboro, NC). Soil moisture percentage, field capacity (F.C.), and permanent wilting point (P.W.P.) were determined based on the Klute 1986 method.

Plant materials and plantation

Milk thistle (*Silybum marifigure figanu*m (L.) Gaertn.) seeds were purchased from Eden Brothers Seed Company, Arden, NC, USA. Fifteen milk thistle seeds were sown 2 cm deep in each plastic pot (23 cm diameter × 24 cm height) containing 8.0 kg of soil. Following seeding, tap (0.8 dS/m) and prepared saline water (12 dS/m) were added to each pot according to the corresponding treatments to reach soil saturation and drain to field capacity. After germination, when plants reached the 4-leaf stage, the seedlings were thinned to five plants per pot, and in a couple of weeks, they were thinned to three plants per pot. During the growing period, to prevent excessive salt accumulation in the rhizosphere, pots were irrigated with distilled water on two occasions. To prevent any drought stress incidence in tap and saline water treatments, the soil moisture in pots was kept at 80% of the F.C. all through the experimental period.

Studied traits

Root and shoot growth

After 72 days of planting, plants with consistent growth were selected to continue the experiment. The plant root and shoot were cut at the base and weighed to determine the dry root and shoot weight. Samples were dried at 60 °C for 48 h, and their mean root and shoot dry weight were recorded for each treatment and replicate.

Measurements at Harvest

The number of flower heads (capitulum) per plant was recorded at the main and sub-stems. To reduce seed dispersal, the harvest was performed when at least 60% of the heads had reached the phenological growth stage 88 of the BBCH scale. After the harvest, the 1000-seed weight was calculated by randomly weighing 3 × 100 seeds from each block.

Determination of gas exchange parameters

The photosynthetic parameters were measured before the sample harvest. In each treatment, the second last fully expanded leaves on new branches were collected. Net photosynthetic rate (*Pn*), stomatal conductance (*Gs*), transpiration rate (*Tr*), and intercellular CO_2 concentration (*Ci*) were measured using a Li-6400 photosynthesis system (Licor Corporation, US) under the photon flux density of 1000 µmol m-2 s -1 and CO_2 concentration of 390–410 µl L-1 (Schiattone et al., 2018). The chlorophyll content (SPAD values) was estimated by the SPAD-502 chlorophyll content meter (Konica Minolta Optics, Osaka, Japan).

Silymarin concentration and yield

For silymarin concentration (mg/g seed) in milk thistle seed and yield (mg/plant Dw) determination based on Arampatzis et al., (2019) method, calibration curves were prepared using the silybin in standard solutions in methanol (Sigma-Aldrich, St. Louis, MS, USA). Each component of silymarin was calculated using their peak area and the calibration curve established for silybin A.

Thermal infrared data

Canopy temperature was measured mainly by the thermal infrared imager (Fluke IR Flex Cam TiX620, Fluke Crop., USA; pixels: 640 by 480, sensitivity: 0.05 °C, accuracy: ± 2 °C, and measuring wave-length: 7.5–14 µm). For the thermal infrared imager system calibration and methodology, the work byGómez-Bellot et al. (2015) was adopted. Combined with the supervised classification methods of ENVI 4.8 software, it was possible to classify the crop and soil, and obtain data on milk thistle coverage.

Preparation of samples for transmission electron microscopy (TEM) analyses

For the TEM (Hitachi H-7650B) analysis, the seedling leaves were first cut into one mm³ cube with a razor blade, and the cubes were then fixed, washed, dehydrated, and embedded in paraffin. Thin slices were then cut off from the samples with a diamond knife of an ultramicrotome (Leica EM UC6) and stained in the laboratory of the University of California, Riverside, CA, USA.

Statistical analysis

A two-way ANOVA statistical test was used to evaluate the data with two factors and their levels (Factor A: nano-materials with 8 levels; Factor B: Irrigation water salinity with 2 levels-control (tap water) and 12 ds/m). All the data were statistically analyzed using SAS 9.1 software. The means of each trait were compared according to the Duncan multiple ranges at p≤0.05. All graphs were created using Excel.

Conclusion

The results showed that salinity stresses across all nano-material treatments led to a decrease in biological performances compared to non-stress conditions. The highest plant silymarin concentration was obtained from the T6 treatment for both saline and tap water conditions. The highest plant silymarin yield (180 mg/plant) was recorded for the T7 treatment under tap water (control, 0.8 ds/m), and 130.3 mg/plant for T6 treatment under saline water conditions, respectively. The lowest yield of silymarin (43.7 mg/plant) was obtained from T8 treatment (control, no nano-material application) under salinity (12ds/m) treatment. The TEM showed that chitosan is mainly observed as scattered and fine particles inside the leaf cell and is mainly concentrated in vacuoles. Unlike chitosan, zeolite particles were observed as thin layers in the inner wall of the cell wall. However, in the imaging treatment of graphene oxide application under both salinity stress and control treatments, no trace of graphene nanoparticles was found inside the cell. These results from this study showed that graphene oxide, chitosan, and zeolite can be introduced to soil as modifiers of the destructive effects of salinity stress in milk thistle growth and development. The results found that GO at low concentrations could be a safe, healthy, and effective substance for germination and growth stimulation under salinity stresses. The integrated application of these nano-materials (T7) in appropriate concentrations leads to a quantitative and qualitative increase in the milk thistle's secondary metabolites production. This research could help find ways to develop this medicinal plant in arid and saline areas.

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