Enhancing the production of active compounds in wild *Silybum marianum* L. using biofertilizers, conventional and nano-fertilizers: A comparative study under field cultivation conditions

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https://doi.org/10.59658/jkas.v13i1.5742

Received:
Oct. 21, 2025

Accepted:
Dec. 7, 2025

Published:
Mar. 15, 2026

Abstract

A field experiment was conducted on wild thistle (*Silybum marianum* L.) at the College of Agriculture, Al-Qasim Green University. This study employed a completely randomized design (CRD) with four treatments to investigate the effect of foliar fertilization with biostimulants, in addition to conventional and nano-enriched NPK fertilizers, on the synthesis of secondary metabolites and antioxidant activity during the winter of 2024. The initial treatment (T0) was water spraying. In treatment T1, a standard NPK fertilizer was sprayed at a concentration of 2 g/L. The third treatment (T2) involved spraying a nano-enriched NPK fertilizer at a concentration of 2 g/L. In the fourth treatment (T3), a biostimulant was sprayed at a concentration of 2 mL/L, in addition to the wild thistle treatment. High-performance liquid chromatography (HPLC) analysis was performed on the plants, and the data showed that wild thistle plants outperformed those sprayed with water alone. The T3 plants were found to outperform other produced plants and natural samples in all measured parameters, including total phenolic compounds, flavonoids, and alkaloids. T3 plants contained higher levels of glutathione (GSH), cysteine, methionine, gallic acid, apigenin, kaempferol, and ferulic acid (192.1, 59.9, and 86.9 ppm, 125.9, 80.9, 85.4, and 97.4 mg/g, respectively), indicating their antioxidant activity. This research reveals that biostimulants are essential for increasing crop productivity and plant selection in modern agriculture.

Keywords: Wild plant, *Silybum marianim*, biofertilizers, nano-fertilizers, NPK, secondary metabolites.

Introduction

An increasing number of researchers are looking into the potential of medicinal plants and biologically active compounds found in food to treat metabolic diseases [1]. A lot of the physiologically active chemicals used to treat metabolic diseases come from herbs and shrubs. One of these herbal varieties, milk thistle (*Silybum marianum* L.), has a long history of use as a medicine and a therapeutic herb [2].

Because of its abundance of secondary chemicals such as phenols, flavonoids, and alkaloids, *Silybum marianum* is a medicinal plant that is utilized extensively. Among its numerous pharmacological actions, these chemicals are associated with its antioxidant and liver-protective capabilities [3,4]. Southern Europe, North Africa, the Americas, Australia, and some regions of Asia are native habitats for this Asteraceae species. More than two thousand years of traditional medicine have made use of it [5].

According to research conducted by [6], *S. marianum* contains numerous phenolic compounds, including gallic acid, an agent that reduces inflammation and acts as an antioxidant. Additionally, it finds applications in both industry and medicine. Some of the components that aid the plant's biological activity are rutin, apigenin, and kaempferol; these serve to protect tissues, combat inflammation, and act as antioxidants [7,8,9]. Research shows the plant's medicinal value and the link between its secondary chemical concentration and treatment efficacy.

The biological value, productivity, and chemical content of the wild plant depend on the nutrients and conditions in which it grows. Recent developments in agricultural technology, such as biostimulants and nano-fertilizers, have shown promise in improving mineral uptake and triggering the biological processes that lead to secondary chemical synthesis [10, 11]. However, few studies compare wild and cultivated milk thistle in controlled environments, and even fewer investigate the effects of these fertilization methods on wild milk thistle.

Therefore, this study seeks to examine the impact of three foliar fertilization methods (conventional NPK, nano-NPK, and biostimulant) on the secondary metabolite composition and antioxidant activity of the *S.marianum* plant, while also comparing the findings with those of the wild plant specimen to identify the most effective treatment for enhancing its medicinal properties.

Materials and Methods

Agricultural Experiment Design

A research experiment was conducted at the College of Agriculture, Al-Qasim Green University, in the winter season of 2024-2025, where seeds of the *S.marianum* plant were planted at a rate of 5 seeds in plastic pots with dimensions of 20 cm height × 15 cm diameter and a capacity of 5 kg. The soil was filled with a soil mixture that included German peat moss. The soil mixture included river soil in a ratio of 2:1. The date of planting was 12/12/2024, and the seedlings emerged in their entirety on 22/12/2024. The process of thinning was initiated once four genuine leaves emerged. Afterwards, on January 19, 2025, fertilizer was sprayed on. There are four procedures involved in fertilization (Table 1). There was a total of six sprays administered, with five replicates for each treatment. The treatments were randomly dispersed, with a ten-day gap between each spray, and the last spray was on 2025/3/10. During the growth stage, watering was kept up in accordance with the plant's requirements, and weeding was done continuously. The necessary measurements were collected one week after the most recent spray.

Table (1): Type of Fertilization Treatments

Code	Type of Treatment	Concentration
T0	Comparison Treatment – Water Only Spray	-
T1	Balanced NPK (20:20:20 + Trace elements)	2 $g.L^{-1}$
T2	Nano NPK (Non-Nutrient) Fertilizer	2 $g.L^{-1}$
T3	Biostimulant (Tecamin Algae, seaweed extract – Spain)	2 $ml.L^{-1}$

Fertilization-Related Metabolites

For the purpose of comparing amino acids, flavonoids, and alkaloids in both wild and field-grown plants that were treated with various fertilizers, a clean glass beaker was used to deposit 1g of plant powder for each species and treatment. To validate solvent-plant material contact, the beaker had a seal after adding 10 ml of strong organic ethanol solvent. It was then left at room temperature for 24 to 48 hours, stirring occasionally. The active compounds were isolated after the extract was filtered using clean gauze and filter paper. Before use, the extract was preserved in opaque, sterile containers [12].

Detection of Active Chemical Compounds in Leaves

A. Detection of glycosides: as outlined by [13].
B. Detection of terpenes: as reported by [14].
C. Detection of tannins: as described by [13].
D. Detection of flavonoids: as described by [15].
E. Detection of alkaloids: as outlined by [12].
F. Detection of phenols: as described by [12].

Aluminum chloride colorimetry was employed to assess plant flavonoids for the determination of total flavonoid content

Ten ml of the extract were obtained by sequentially adding solutions of sodium nitrite, aluminum chloride, and sodium hydroxide. Measure the total flavonoid concentration by recording the absorbance at 510 nm after incubation and correlating it with a calibration curve. [16] indicate that rutin is quantified in milligrams per gram of dry weight.

Total Phenolic Content Estimation

After the phenolic content of the ethanolic extract has been measured using the Folin-Ciocalteau reagent, 10ml of sodium carbonate should be added, the volume should be adjusted to 10ml, the incubation time should be 10 min, and the absorbance should be measured at 765 nm. A gallic acid calibration curve was developed using the data by [17]. The curve reported mg gallic acid equivalents/g dry weight.

Estimation of Total Alkaloids

Twenty of plant material were pulverized and extracted in methanol using a Soxhlet apparatus in just 24hr at a temperature of 45°C. Redissolved in hydrochloric acid and filtered after dry residue from vacuum-induced evaporation, the extracts were taken.

Before the addition of phosphate buffer and bromocresol green, the solutions were neutralized. In volumetric flasks, chloroform mixtures were transferred. Using 470nm UV-Vis spectrophotometry, [18] calculated atropine equivalents/g dry weight.

Amino acid concentration

It was determined by dissolving 3 g of dry powder from each sample in 1ml of hydrochloric acid at 55 C° for 3 hr., drying the mixture, and then dissolving it again in a sodium citrate solution with a pH of 2.2. The resulting mixture was then filtered. Shake the samples after exposure to orthophthalaldehyde (OPA). The solution was subjected to an HPLC analysis by adding 100 µL to a C18-NH2 column (1.6 µm particle size, 2.1 × 150 mm), which allowed for a total run time of only 10 min, including equilibration with a 20:60:20 methanol:acetonitrile:5% formic acid carrier phase, all flowing at a rate of 1 mL/min. [19] found that glutathione, methionine, and cysteine were detected by a fluorescence detector operating at 445 and 465 nm.

Antioxidant Activity Test

For the A-DPPH approach (act as a positive control), mix 400 µg/mL DPPH in methanol with 5000 mg. L^{-1} vitamin C stock solution and separate samples at 30–500 mg. L^{-1}. Mixed the solutions, let them stand at room temperature for 30 minutes, and measured absorbance at 517 nm with a UV-VIS spectrometer [20].

Elimination implications (%) = (A0-A1)/A0 x 100 for DPPH free radical inhibition .

The dose-response curve, used to calculate IC_{50}, shows that lower absorbance suggests stronger antioxidant activity.

For the Hydrogen Peroxide (H_2O_2) Method: 100 µg.ml-1 samples were added to a 40 mM solution at pH 7.4, interrupted for 10 minutes, and compared to a control solution at 230 nm. The H_2O_2 elimination rate was determined using the following formula :

H_2O_2 removal % = (AC-AS)/AC x 100, where AS is sample absorbance and AC is control absorbance [21].

Statistical analysis

The Completely Randomized Design (CRD) was utilized to evaluate the experiment with four treatments and the wild *S.marianum* plant as a second chemical comparison treatment. The study evaluated antioxidants in both wild and cultivated plants using a two-factor factorial approach. Initially, a comparison was made between wild plants, vitamin C (V.C.), and four treatments (T0, T1, T2, and T3). The second part of the study involved measuring the concentration of free radicals at various parts per million (ppm): 30, 60, 120, 250, 500, and IC50. LSD at 0.05 was used to compare treatment averages in GenStat v.12.

Results and Discussion

After confirming the presence of flavonoids through indicative detection, the concentration of five flavonoid compounds (gallic acid, rutin, apigenin, kaempferol, and ferulic acid) was compared between the wild *S.marianum* plant and the field-treated plants under the influence of four treatments (T0, T1, T2, and T3) using the HPLC technique, as shown in Figure 1 .

Results indicated that flavonoid content ratios varied significantly. Before treatments T1 and T2, the control plants were not as effective as the wild plants at time zero

(T0). In contrast to the prior agricultural treatments and wild plants, treatment T3 demonstrated superior performance. In addition, Figure 2 shows that various flavonoid compounds reacted differently to field treatments. From treatment zero to treatment three, the rutin compound rose to levels higher than those in wild *S. marianum*.

The chemical apigenin showed a considerable rise in the T2 and T3 treatments compared to the others, with T3 concentrations approaching those of wild *S.marianum*. Gallic acid levels peaked in the T3 treatment, surpassing those found in wild *S.marianum*. Ferulic acid, kaempferol, and quercetin levels increased significantly with each treatment, peaking in T3 at levels higher than those seen in wild *S.marianum*.

These findings represent the different reactions of flavonoid compounds to field treatments, emphasizing the importance of the type of treatment in increasing the levels of specific compounds above those in wild plants. It was also discovered that the fertilization factor influenced the variance in flavonoid concentration, resulting in an increase in flavonoid accumulation. This data shows that the way the plants are grown or treated has a direct effect on their chemical makeup, which is crucial for biology. The fact that they are accumulating means that the plants are becoming more nutritious and useful for medicine. From this, it is clear that the studied field treatments contribute to increasing the flavonoid content in the *S.marianum* plant to levels that may exceed those of the wild plant, thus enhancing its nutritional and medicinal value and indicating the possibility of improving the plant's quality through careful field management.

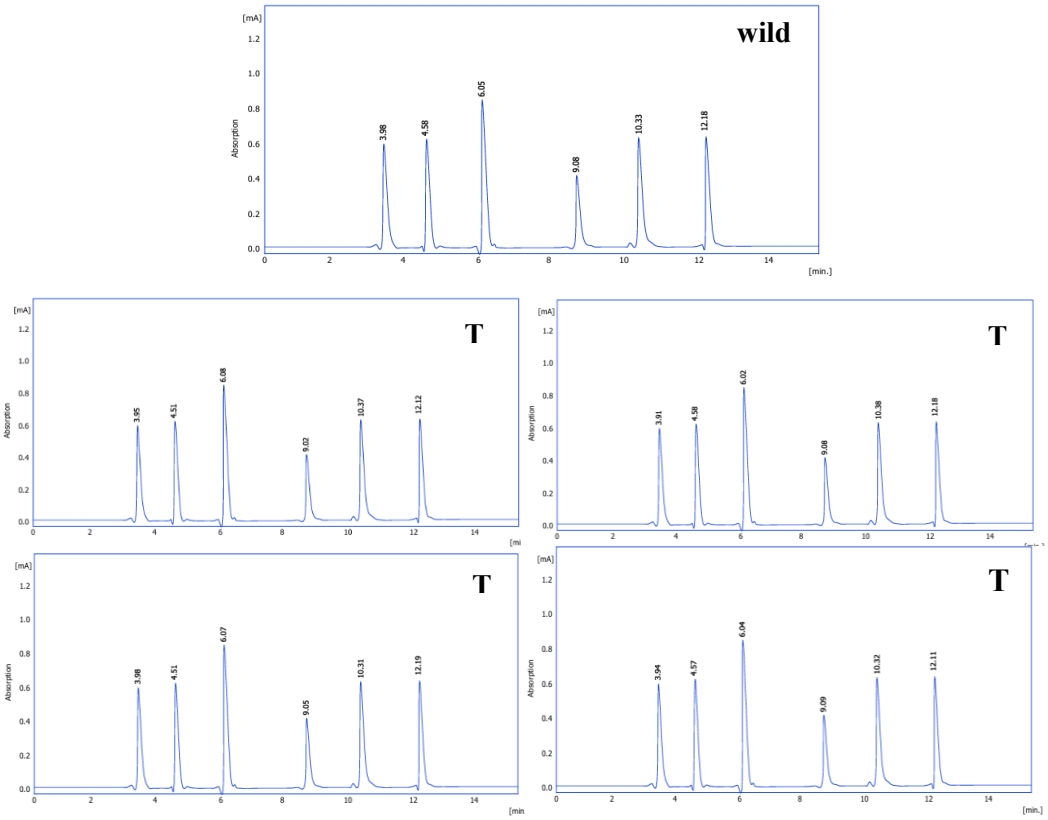

Figure (1): Content of flavonoid compounds in wild and cultivated *S.marianum* plants, Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, (T3) Biostimulant , retention time (Rt), and area (Ar).

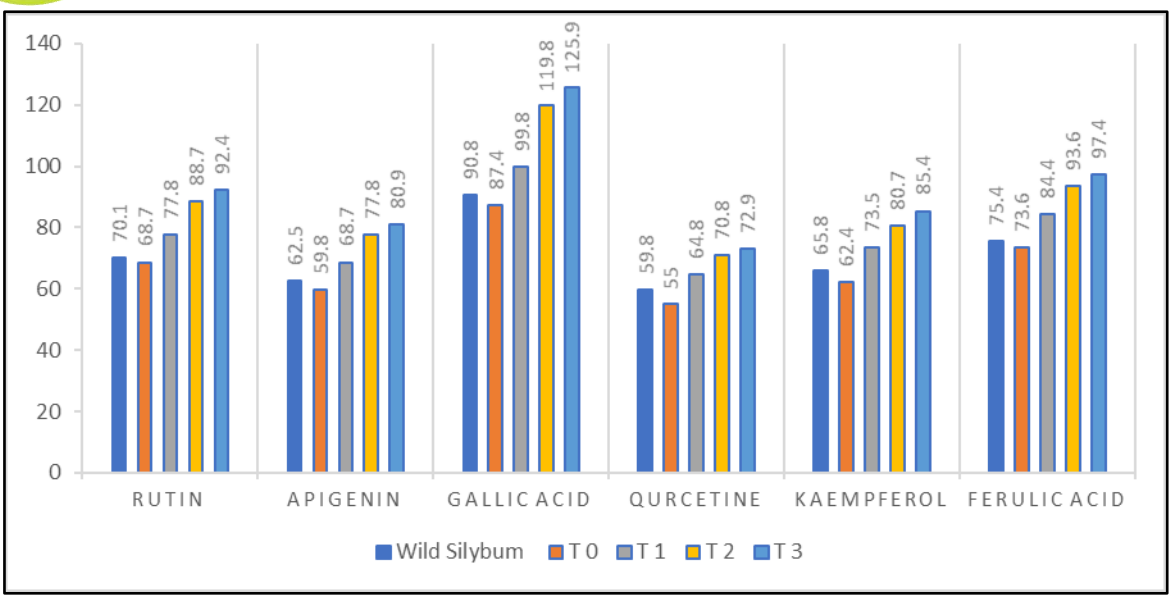

Figure (2): Comparison of flavonoid content in wild and field-treated chicory using HPLC. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (L. S. D. = Rutin = 1.18, Apigenin = 1.22, Gallic acid = 1.414, Quercetin = 0.99, Kaempferol =1.175, Ferulic acid = 1.113.

When comparing the total flavonoid content between the wild Silybum plant and the field-treated plants under four different treatments (T0, T1, T2, T3), the results indicate significant differences in flavonoid content. Treatment T0 had the lowest amount, at 33.6 mg/g, and treatment T1 had the next lowest amount, at 43.1 mg/g. As for treatment T2, it witnessed a significant increase in flavonoid content, reaching 61.8 mg/g, while treatment T3 recorded the highest value at 74.9 mg/g. This concentration is in contrast to the flavonoid content found in the wild plant, which was approximately 41.5 mg/g, as depicted in Fig. 3.

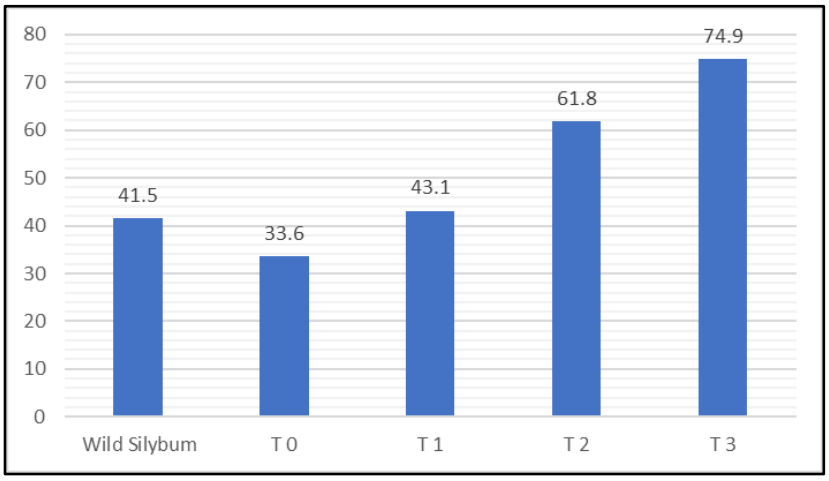

Figure (3): Comparison of total flavonoid content (mg/g) in wild *Silybum* and field treatments. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level = 1.45).

The analysis looked at the total phenolic content of wild *Silybum* plants as opposed to that of field-treated plants, which went through four different treatments (T0, T1, T2, and T3) and noted large differences. T0 had the lowest phenolic content at 165.8 mg/g, which in turn was followed by T1 at 173.8 mg/g. Treatment T2 had a significant increase in phenolic content, reaching 188.9 mg/g. Treatment T3 had the highest level, at 197.8 mg/g. This measurement contrasts with the wild plant, which had a phenolic content of 174.0 mg/g (Figure 4). The same is true for the alkaloids, as seen in Figure 5.

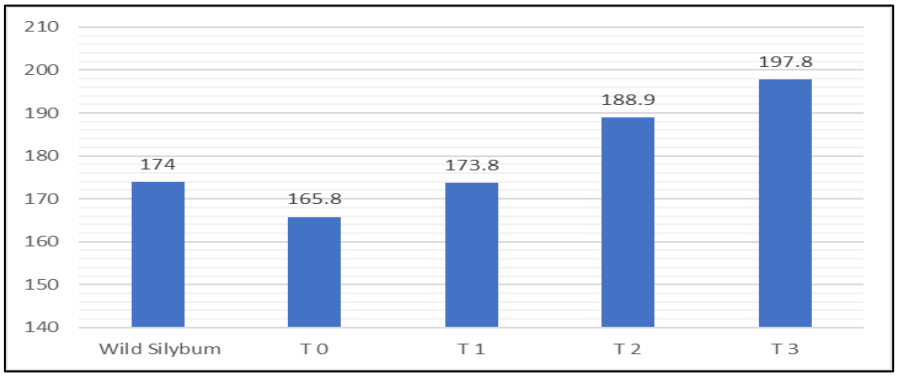

Figure (4): Comparison of total phenolic content (mg/g) in wild *Silybum* and field treatments. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level= 1.85).

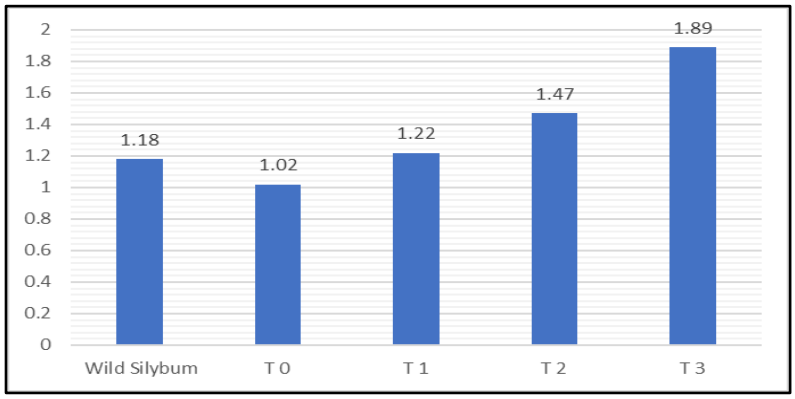

Figure (5): Comparison of the total alkaloid content as a percentage (%) in wild *Silybum* and field-treated. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level = 0.09).

The concentrations of two amino acids (methionine and cysteine) were compared, as well as the antioxidant glutathione (GSH), between the wild *Silybum* plant and the field-treated plants under the influence of four treatments by using the HPLC technique, as shown in Fig. 6. There was a significant difference in the concentration of the studied amino acids and antioxidants in the wild *Silybum* plant. Thus, the differences in the cultivated plants and field treatments were observed, as it was found that the amino acid methionine in wild *Silybum* has the highest concentration at approximately 62.5 ppm, while the amino acid cysteine recorded the lowest concentration at approx-

imately 22.5 ppm. The concentration of the GSH compound reached 141.5 ppm. But when comparing these values with the field-cultivated plant without the addition of any type of fertilizer (T0), we notice a clear decrease in those compounds. T0 records 62.0, 20.1, and 137.7 ppm in methionine, cysteine, and GSH, respectively. The T2 plant was recorded after the T1 plant, while the plant treated with organic fertilizers (T3) showed the highest concentration of all the active compounds identified in the study. Overall, all studied active compounds showed a gradual increase in content from the control treatment (T0), which used only water, to the biofertilizer-supplemented plant (T3), with the latter exhibiting the highest concentration of all compounds. The studied field treatments contribute to increasing the content of active compounds in the plant to levels that may exceed those of the wild plant, thereby enhancing its nutritional and medicinal value and indicating the possibility of improving the quality of the plant through the studied field management.

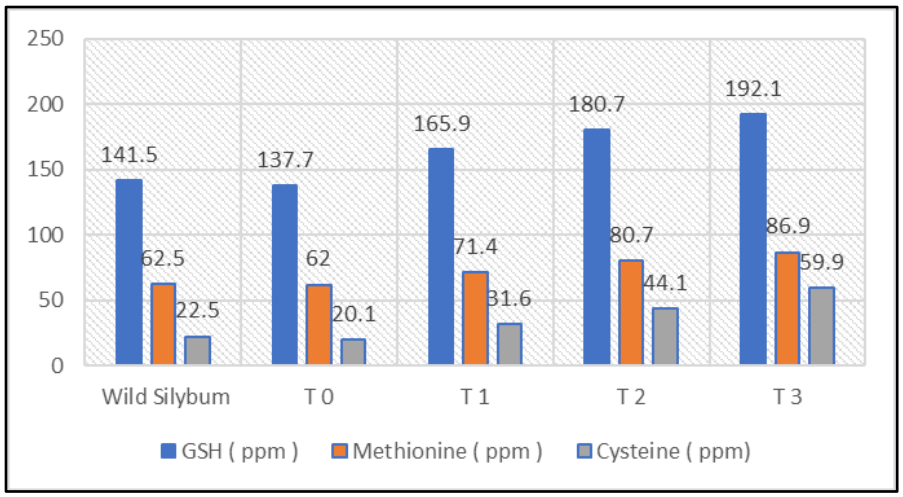

Figure (6): Comparison of the content of active compounds in wild and field-treated Silybum plants. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level: GSH=2.70; Methionine =2.59; Cysteine=1.198)

The study compared the antioxidant activity of medicinal plant extracts from species in the Asteraceae family with the synthetic antioxidant vitamin C using the DPPH and hydrogen peroxide (H_2O_2) methods. The comparison was made between the wild Silybum plant and the field-treated plants under the influence of four treatments (T0, T1, T2, T3). Figure 7 shows that there is a difference in antioxidant activity between the wild Silybum plant, the cultivated plants and Vitamin C.

In general, the observed antioxidant effects gradually increased from the plant grown with only water as a control treatment (T0) to the plant supplemented with biofertilizer (T3), which exhibited the highest inhibition of free radicals at all concentrations.

The concentrations of 30, 60, 120, 250, and 500 ppm recorded values of 27.00, 49.74, 66.44, 75.48, and 79.00%, respectively. They also showed a clear variation in

the response of the antioxidant effect of the field treatments compared to Vitamin C, which recorded the highest value.

For the antioxidant activity at a concentration of 500 ppm, it was 61.42%, while the lowest recorded value at a concentration of 30 ppm was 12.05%. The concentrations of 60, 120, and 250 ppm averaged those limits with values of 22.14, 42.65, and 55.08%, respectively. Through the results that demonstrate the antioxidant activity and the amount of active compounds in the plant extracts of the studied samples and Vitamin C, shown in Figure 7

Additionally, it was found that the DPPH free radical scavenging activity, measured as IC_{50}, was 140.6, 104.5, 120.0, 100, 94.6, and 90.9% for the samples Vit C., Wild Silybum, T0, T1, T2, and T3, respectively.

As for the examination of antioxidant activity using the hydrogen peroxide (H_2O_2) test, the results, as shown in Fig. 8, indicated a difference in antioxidant activity in the wild Silybum plant. Consequently, there was a difference between the cultivated and field-treated plants (T0, T1, T2, and T3) and vitamin C, which were 3.25, 3.00, 3.19, 3.66, 3.74, and 2.75%, respectively.

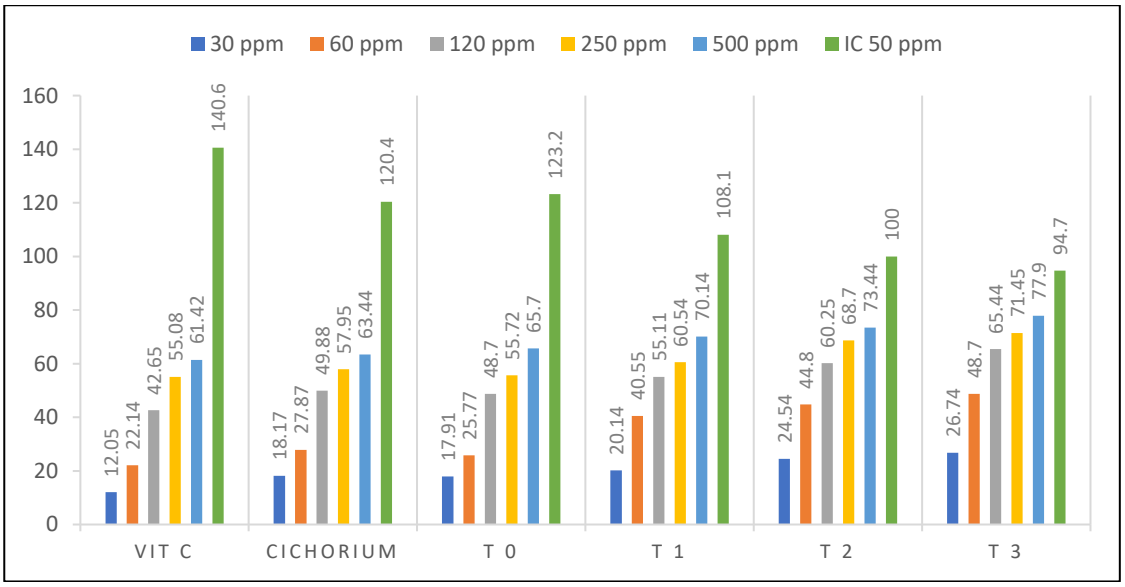

Figure (7): Comparison of antioxidant content using the DPPH% method in wild and field-treated Silybum plants. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level = 1.27).

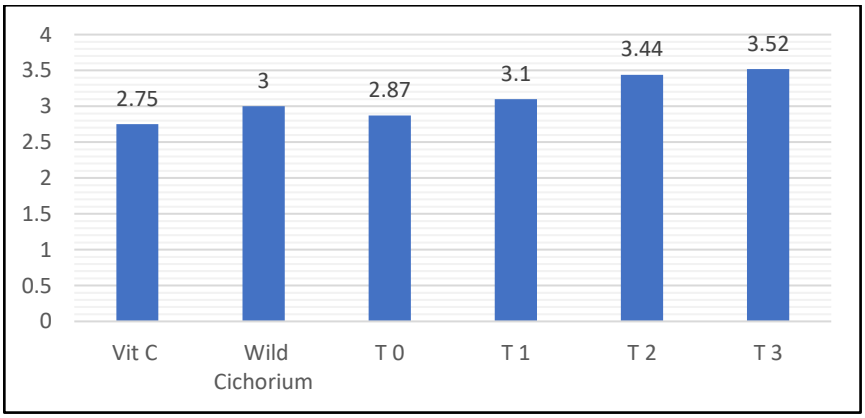

Figure (8): Comparison of antioxidant content by hydrogen peroxide reduction method (H_2O_2%) in wild and field-treated Silybum plants. Where (T0) Water Only Spray; (T1) Balanced NPK; (T2) Nano NPK, and (T3) Biostimulant; (LSD at 5% probability level = 0.079).

The findings indicated that fertilization treatments, particularly bio-treatment, resulted in a significant enhancement of secondary component levels, aligning with the research aim of augmenting the plant's medicinal value through field management.

The increasing popularity of medicinal plants necessitates the application of diverse fertilization methods and agricultural tactics to enhance nutrient absorption and optimize the efficiency of secondary chemical production in plants. [11, 22] are merely two of numerous studies that underscore the significance of tailored fertilization tactics for ensuring sustained production and enhancing the quality of medicinal plants. In accordance with the findings of [23], organic or biofertilization is advocated as a sustainable alternative to traditional chemical inputs, aimed at improving plant attributes.

Compared to the control treatment (T0), the findings of this study indicated that the use of biostimulants during fertilization (T3) markedly elevated the concentrations of secondary chemicals, including gallic acid, rutin, apigenin, kaempferol, and caffeic acid. The increase is primarily attributed to the correlation between essential nutrients, particularly nitrogen, phosphorus, and potassium, and fundamental biological processes such as photosynthesis, respiration, and the synthesis of proteins, enzymes, and plant hormones, as noted by [24, 25]. This therefore influences the augmentation of secondary chemicals.

The findings align with the conclusions of [26] about the role of phosphorus in activating biosynthetic pathways and enzymes responsible for the production of beneficial chemicals. The results corroborate [27] on phosphorus's role in augmenting the antioxidant system by increasing the activity of SOD, CAT, POD, and APX enzymes. An elevation in phosphorus results in enhanced biosynthetic chemical production, reduced oxidative stress, and improved electron transport [28, 29]. The synthesis of phenols and flavonoids involves phosphorus, which is essential for energy transmission inside plant cells [30].

Potassium's fundamental roles encompass sustaining ionic equilibrium, regulating osmotic pressure, activating enzymes, and augmenting the efficacy of photosynthesis. For therapeutic reasons, these minerals facilitate the plant's chemical synthesis [31, 32]. [33] contend that the synergistic effects of nitrogen, phosphorus, and potassium augment the metabolic processes of the plant .

The results of the nano-fertilizer impact (T2) align with prior studies demonstrating that nano-fertilizers enhance secondary compound accumulation by improving nutrient absorption due to their diminutive particle size, supporting the findings of [34 and 35]. This underscores the substantial advantage of nano-fertilizer treatment over traditional methods.

The study also found that the biostimulant (T3) significantly increased secondary component levels and antioxidant activity. According to [36 and 37], this is due to the fact that it contains plant hormones, amino acids, and macronutrients, all of which pro-

mote quicker plant growth and initiate biosynthetic pathways that produce secondary chemicals. Another reason it has this effect is that it stimulates the PAL enzyme, which is involved in the phenylpropanoid pathway and the production of phenols [38, 39]. There is a correlation between phenol concentration and antioxidant capacity, according to [40].

These findings align with multiple studies that have illustrated the role of biostimulants in enhancing the quality of medicinal plants, including research by [41, 42, 43, and 44] regarding the impact of organic fertilization on augmenting the active compounds in wild *Silybum* .

This study's results provide more evidence that natural stress caused by the wild plant's habitat increases concentrations of some secondary compounds. But these compounds were found to be much higher in biofertilized plants than in wild plants, showing that careful field management can improve a plant's medicinal value. This finding is supported by [45 and 46].

According to [10] research shows that using modern fertilization techniques, especially biostimulants, is a sustainable way to make plants more effective and have better medicinal capabilities.

This research has shown that agricultural methods, especially biofertilization, greatly improve Silybum marianum's chemical and medicinal properties. The study found that the biostimulant (T3) used in the experiments performed better than all other treatments. The results saw an increase in glutathione, cysteine, and methionine, and the levels of alkaloids, flavonoids, and phenols were the highest reported. Furthermore, this treatment, which supports the plant's defense system, did the best in our DPPH and H_2O_2 tests. We note that in some cases, the farmed plant did better than the wild in terms of medicinal and nutritional value. Biofertilization is a very large-scale solution for improving the quality and yield of medicinal plants.

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