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ORIGINAL ARTICLE

The Impact of Organic and Inorganic Fertilizers on Some Phytochemical and Antioxidant Properties in *Zingiber Officinale* Rosc.

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KEYWORDS

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ABSTRACT: Ginger is used as a medicinal plant in traditional medicine to treat many diseases. In the research greenhouse of Damghan University, a factorial experiment with three replications and a completely randomized design was carried out to determine the impact of organic and mineral fertilizers on the biochemical characteristics of ginger. The experimental treatments included: 1) livestock manure factor at 5 levels: control, poultry manure (5 t ha^{-1}), 50% poultry manure (2.5 t ha^{-1}), vermicompost (10 t ha^{-1}), 50% vermicompost (5 t ha^{-1}). 2) Chemical fertilizer factor at 3 levels: control, NPK (100 kg ha^{-1}) and 50% NPK (50 kg ha^{-1}). The outcomes demonstrated that the application of 50% chicken manure produced the maximum level of ginger protein. After treatment with vermicompost, the guaiacol peroxidase enzyme exhibited its maximum activity. The highest catalase enzyme activity was observed in the presence of poultry manure treatment with a 77% increase in comparison to the control. Additionally, the content of soluble sugars of ginger in the treatments of 50% organic and inorganic fertilizers decreased significantly. The highest amounts of proline and H_2O_2 were obtained in 50% vermicompost and NPK treatments. The free radical scavenging power (DPPH) decreased in the presence of different fertilizer treatments (organic and inorganic). All fertilizer treatments were able to increase the total phenol content of ginger. The results showed that the biochemical characteristics of ginger plants showed different reactions to different fertilizer treatments.

INTRODUCTION

Edible ginger with the scientific name *Zingiber officinale* Rosc. is a genus of monocotyledonous plants and the ginger family (Zingiberaceae). This species is an herbaceous perennial that is often planted once a year to produce a strong rhizome. It is native to Southeast Asia, but now it is cultivated in other tropical areas such as Africa and India, and one of the earliest oriental spices known to Europe [1-3]. Ginger has been used as a spice and herbal medicine for a long time. Also, it is used as an herbal medicine, an antiseptic, a digestive tract stimulant, and an anti-irritant. It has been reported that the rhizome can have diuretic and anti-inflammatory effects. Gingerol oral extract contained in the extract extracted from ginger rhizome can inhibit the growth of Helicobacter pylori [4]. Ginger is additionally extensively used as a spice in the shape of fresh ginger, dried complete or powdered ginger, and preserved ginger.

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Natural origin drugs are nowadays used in the treatment of diseases. Compounds such as flavonoids, phenolic materials, and antioxidants are found in medicinal plants abundantly. Ginger phytochemical compounds include basic oils, compounds of phenolic, carbohydrates, proteins, alkaloids, glycosides, steroids, terpenoids, saponins, and tannins, which play a necessary position in plant therapeutic properties. In addition, carbohydrates, fats, minerals, vitamins, and waxes are also found in it.

In general, the amount of antioxidant properties of medicinal plants is associated with their variety, environmental conditions, weather, seasonal variations, area of geographical, stage of maturity, growth, and many different elements at some stage in planting and harvesting [5]. The ginger plant requires balanced fertilizer consumption. In the event of a deficiency of some elements, the active ingredients in the rhizome will be affected and reduce protein and essential oils. High fertilizer concentrations will prevent the roots from absorbing nutrients due to their high ionic concentration as well as low water potential. Similar to other root and tuber goods, ginger has a high nutrient demand in soils [6]. Hence, plant nutrition management is one of the important issues in the production of herbal medicinal products. All available studies recommend the incorporation of organic and inorganic fertilizers to enhance the growth, yield, and ultimately profitability of ginger manufacturing [7- 9].

The lack of access to nutrients for plants can be solved by fertilizing, as inorganic fertilizers can quickly provide macronutrients. However, excessive chemical fertilizer use has resulted in several detrimental ecological and environmental effects, including soil compaction, acidification, and deterioration of soil fertility, all of which seriously impede the long-term growth of green agriculture [10]. Fertilizers can have value in many ways. The first obvious one is the direct nutritional value. The value depends on site-specific soil nutrient concentrations and fertilizer nutrient concentrations. Depending on the source of the fertilizer, its nitrogen can be in varying amounts in a slowly released form, which is very beneficial to the environment [11].

Fertilization causes plant growth, provides nutritious food for soil organisms, develops the genetic and functional diversity of the soil environment, and can ultimately enhance the chemical and physical features of the soil. Nevertheless, it can also cause pollution by adding toxic elements (e.g. heavy metals, antibiotics, and pathogens) and promotes nutrient loss. Soil organisms play an essential function in turning manure into soil and breaking down potential toxic substances; however, soil biodiversity is often neglected in fertilizer management practices [12]. The results have shown that fertilizer quality is more important than fertilizer quantity for soil biodiversity, and therefore agricultural practices that protect and boost soil biodiversity, using appropriate high-quality fertilizer or bio-stimulant products, can accelerate the transition to a more sustainable food production system [12]. Optimizing the use of nutritious fertilizers needs a whole series of activities, from feed through animals and fertilizers to soil and crops. A literature review was carried out on the relationship between animal diet and manure quality. It targeted the quantitative and qualitative factors of carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) in feed and fertilizer [13].

Considering the special ecological surroundings generated by using greenhouse cultivation, the effect of organic amendments on the microbial and enzymatic activity of greenhouse soils is turning a hot subject of attentiveness and investigation [14]. Vermicompost treatment is an attractive alternative to traditional chemical fertilizers. Vermicomposting is a non-thermophilic process that turns organic waste into valuable fertilizer through the interaction of worms and mesophilic microbes [15]. Vermicompost products have been shown to have a wide range of effects on abiotic and biotic stress, including improving soil salinity [16], mitigating drought stress [17], and controlling insects and pathogens [18]. As an excellent soil conditioner, vermicompost can augment soil organic matter, enhance acidic soil, and strengthen soil water and fertilizer preservation and plant growth [19, 20]. Vermicompost, as the final product of organic waste decomposition by earthworms, in addition to having a large specific surface area, a suitable agglomeration structure, and optimal absorption and ion exchange capacity, contains many suitable nutrients and useful microorganisms that can also effectively improve the physico-chemical properties of the soil and ultimately cause better growth of plants [21]. The vermicompost pH is close to neutral, which is acceptable for the growth of microorganisms and thus has a positive impact on microorganisms [22].

On the other hand, poultry manure is both affordable and environmentally friendly to reduce environmental threats. Poultry manure is a rich source of keratin proteins and amino acids that can turn into nitrogen-rich organic fertilizers [23]. Considering the importance of organic fertilizers especially poultry manure and vermicompost, a review is required to evaluate the effect of different kinds and doses of both types of organic fertilizers on the growth and yield of ginger. Poultry manure is a suitable organic fertilizer that can be used to improve soil fertility and increase plant productivity. Because poultry manure is a rich origin of nutrients, it can increase agricultural production by improving soil fertility. In addition, these fertilizers are a rich source of various macroelements such as nitrogen (N), potassium (K), and phosphorus (P) [24]. Additionally, poultry manure has an appropriate carbon/nitrogen ratio, which assists the activity of microorganisms: this can eventually improve soil characteristics and increase the productivity of plants [25]. Additionally, the addition of poultry manure augments the cation exchange capacity of the soil [26]. Maintaining a sufficient quantity of macro and micronutrients in organic fertilizers [27] enhances the physical, chemical, and biological properties of soil [28, 29]. Globally, agricultural production is being raised through the application of chemical fertilizers. Excessive use of chemical fertilizers causes these chemical elements to enter the underground water table through rainwater and eventually cause water pollution. Therefore, for the optimal management of organic waste, composting can be a low-cost and convenient way to use [23].

Therefore, the current study, considering the global trend of production and propagation of medicinal plants in sustainable and low-input agricultural systems, as well as the lack of sufficient studies regarding the reaction of different sources of fertilizers (poultry manure, vermicompost, and NPK) on the phytochemical and antioxidant properties of the plant Ginger was done. The results of this study can provide more knowledge and understanding about the effect of the application of vermicompost and poultry fertilizers on the production and bioactive plant compounds of the products, especially in the ginger plant.

MATERIALS AND METHODS

In the research greenhouse of Damghan University, this experiment was conducted in 2021 using ginger plants in a randomized full-block design with three replications. The rhizome sets have two or more viable buds and weigh between 10 and 20 grams. The planting depth of the rhizomes was 5 cm. The ginger plants in the pots were randomly selected from as many as three plants and used as samples.

The experimental treatments included the following: the first factor of livestock manure at five levels: control (without organic fertilizer), poultry manure at the rate of 5 tons per hectare, vermicompost at the rate of 10 tons per hectare, poultry manure at the rate of 2.5 tons per hectare, vermicompost Compost 5 tons per hectare. The second factor of chemical fertilizer at three levels: control (no use of chemical fertilizer), NPK chemical fertilizer at the rate of 100 kg per hectare, and NPK chemical fertilizer based on 50% of the initial amount. The rhizomes of the collected samples were transferred to the research greenhouse of Damghan University for cultivation. The recommended amounts of nitrogen, phosphorus, and potassium chemical fertilizers were determined based on the soil test results; which are added to the respective pots and mixed with the soil before planting, according to the plan and treatments. After 3 months of fertilizer application, the plants were removed from the culture medium and the necessary measurements including biochemical and antioxidant tests were performed on them.

Biochemical measures

Hydrogen peroxide content

The H_2O_2 concentration was determined using the Alexieva et al. [30] technique. Using this procedure, 3 ml of 1% trichloroacetic acid was mixed with fresh plant samples (0.2 g) in an ice-cold mortar. Following filtering and centrifuging the homogenate at 10,000 g for 15 minutes, 0.5 ml of potassium phosphate buffer (pH 7 with 10 Mm) and 1 ml of potassium iodide were combined with the supernatant. Next, a spectrophotometer was used to measure the absorbance of hydrogen peroxide at a wavelength of nm 390.

Malondialdehyde (MDA) content

The thiobarbituric acid (TBA) reaction was used to measure the MDA concentration. In a nutshell, 3 ml of 5% TCA was used to homogenize a 0.2 g tissue sample. For five minutes, the homogenate was centrifuged at 4000 × g. Four milliliters of 20% TCA with 0.5% TBA were added to one milliliter of the supernatant aliquot. After 15 minutes of heating at 95 degrees Celsius, the mixture cooled down well. At 532 nm, the absorbance was measured. Subtraction was made from the value of the non-specific absorption at 600 nm. The extinction coefficient of 155 MCm-1 was used to express the MDA concentration [31].

Peroxidase (POD) activity

Based on the amount of tetra guaiacol absorbed following production by oxidation of guaiacol catalyzed by this enzyme in 1 min at a wavelength of 420 nm, peroxidase (POD) activity was measured by Plewa et al. [32] technique.

Total Phenolic Content (TPC)

The Folin-Ciocalteau method [33] was used to determine the total phenolic content (TPC) in Zingiber officinale leaves. To summarize, 100 L of extract (100 µg) was added to 0.5 mL of Folin-Ciocalteau phenol reagent and 1 mL of sodium carbonate (7.5% w/v) to a final volume of 2 mL.

The contents were mixed and allowed to stand for 30 minutes. The absorbance at 765 nm was measured. The TPC is expressed in terms of gallic acid (GA) (equivalents of GA per gram of extract). The calibration curve is the basis for the following equation, which is:

$$Y = 7.5397X - 0.0291, R2 = 0.9993,$$

where X is the absorbance and Y is the mgGA g^{-1}.

Each sample was examined in quadruplicate.

The activity of DPPH Free Radical Scavenging

Using a modified procedure, the antioxidant properties of the Zingiber officinale leaves and standard were evaluated based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) - a stable free radical activity - radical scavenging action [34]. The test extracts' working solutions were made by serially preparing concentrations ranging from 15.6 to 250 µg mL^{-1}. Then, each extract was added in the same amount to a DPPH methanolic solution (0.1 mM). Both the standard solution and the 37.5–150 µg mL^{-1} solution individually included quercetin as a standard. A spectrophotometer was used to measure the optical density of these solution combinations at 517 nm after they had been left in the dark for 30 minutes. The blank was a 1 mL solution of DPPH (0.1 mM) in methanol. After recording the absorbance, the following equation was used to get the percent inhibition:

$$\text{\% inhibition of DPPH activity} = 100 - (\text{Abs sample} - \text{Abs blank} / \text{Abs control}) \times 100$$

Where the Abs sample is the absorbance of the DPPH plus sample extract/standard, and the Abs control is the absorbance of the DPPH plus methanol.

Statistical analysis

Following the statistical evaluation of the data by Duncan's multiple range test, a one-way analysis of variance (ANOVA) was performed with a significance threshold of 0.05 for the analyses of the data. The data means were then employed for the tests. The statistical program SPSS 23 was used for all statistical analyses.

RESULTS AND DISCUSSION

Because the soils of agricultural areas have low organic content and are deficient in nutrients, different agricultural operations are needed to improve the growth rate, yield, and nutrient quality of ginger. Hence, most crops respond positively to soil amendment with different crops [35, 36]. Poultry manure and vermicompost contain essential plant nutrients such as nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, copper, zinc, chlorine, boron, iron, and molybdenum [37]. Therefore, poultry manure can be used as a suitable organic fertilizer to supply all or part of the nutritional needs of crops. In addition, vermicompost fertilizer is a type of organic fertilizer such as peat with high nutrient content, aeration, porosity, and water-holding capacity. This fertilizer is the result of the action of earthworms and microbes. As a result, poultry manure and vermicompost are known as effective stimulants of plant growth [38].

The amount of protein

The results of the analysis of variance showed that the highest amount of ginger protein was obtained in the presence of 50% poultry manure applied (2.5 t ha^{-1}), which was significant compared to the control (Figure 1). The results showed that the amount of ginger plant protein decreased significantly in the presence of poultry manure treatment compared to the control. Khan et al. [39] showed that the significant increase in the chickpea protein amount was greatly influenced by chemical treatments and organic fertilizers. In the present study, the highest amount was obtained in the presence of 50% poultry manure (2.5 t ha^{-1}). In an experiment on spinach, it was shown that vermicompost could increase the amount of soluble protein [40]. Several reports have been observed regarding the positive effect of vermicompost on the improvement of plant protein [41, 42]. Rasoul et al. [43] reported that in the two cultivars of maize tested, the amount of protein in the presence of poultry manure treatments was lower than that of the control. In the present study, only 50% of poultry manure could show a significant increase compared to the control.

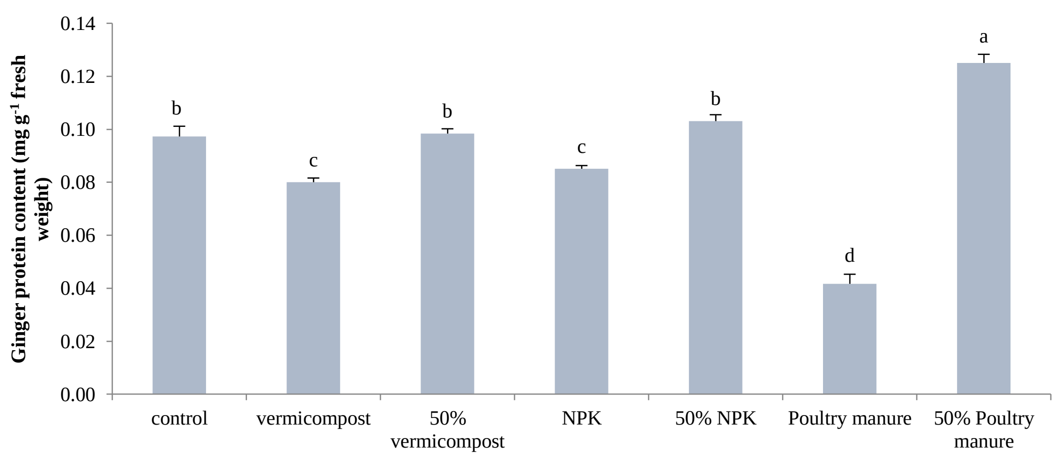

Figure 1. Effect of different organic and inorganic fertilizer treatments on ginger plant protein content (mg g^{-1} fresh weight). Values are the mean of three replicates ± SD. Different letters represent a significant difference between treatments ($P < 0.05$).

The amount of guaiacol peroxidase enzyme (GPX)

The results showed that the highest activity of the guaiacol peroxidase (GPX) enzyme was obtained in vermicompost treatment (7.44 units per milligram of protein). Only vermicompost different treatments were significantly different from the control (Figure 2). No difference was observed between the other applied treatments. The enzyme guaiacol peroxidase is active in the cytosol and applies glutathione as a cofactor [11]. During the decomposition process, the guageal peroxidase enzyme converts hydrogen peroxide into water. By increasing the activity of this enzyme and other defense mechanisms in the cytosol, stability in this part is improved [2]. Recently, several studies have been conducted regarding the role of vermicompost organic fertilizer on the activity of antioxidant enzymes [44, 45]. It appears that different treatments of vermicompost can increase the antioxidant enzyme activity [45]. In the present experiment, vermicompost treatment was able to improve the activity of the GPX antioxidant enzyme on ginger.

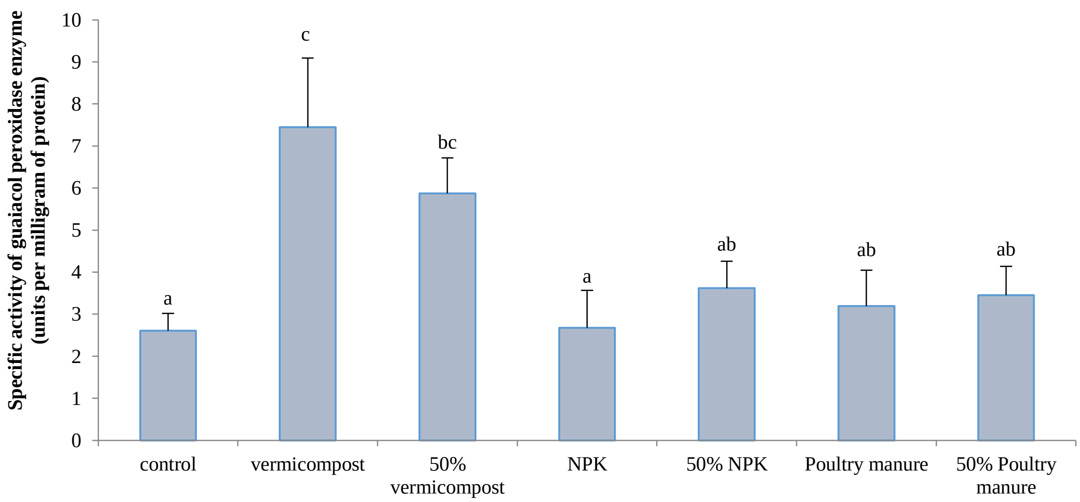

Figure 2. Effect of different organic and inorganic fertilizer treatments on activity of guaiacol peroxidase (GPX) of the ginger plant (units per milligram of protein). Values are the mean of three replicates ± SD. Different letters represent a significant difference between treatments ($P < 0.05$).

The amount of catalase enzyme (CAT)

The results of the data analysis showed that the activity of the catalase enzyme significantly decreased in the treatment of inorganic fertilizers except poultry manure. The highest enzyme activity was obtained in the presence of poultry manure treatment (5 t ha^{-1}), which showed a 77% increase compared to the control (Figure 3). Catalase enzyme is responsible for scavenging superoxide radicals and converting them into water and oxygen. Muhammad et al. [46] have proved the positive effect of nitrogen fertilizer application on the activity of the catalase enzyme. Wang et al. [47] reported that increased consumption and application of nitrogen cause the activity of antioxidant enzymes such as catalase increases in the plant. Hence, if there is not enough available nitrogen, the enzyme activity decreases, so the speed of electron transfer can be affected. On the other hand, Logan et al. [48] reported that nitrogen could have a great effect on the enzyme activities involved in photosynthesis. In the present study, the amount of catalase enzyme decreased in the presence of different vermicompost treatments. Different vermicompost treatments showed complex effects on the activities of both enzymes (CAT and GPX) of plants under growth [49]. Despite the biographical studies that we have done, no specific research work has been done regarding the effect of vermicompost and poultry fertilizers on antioxidant enzymes.

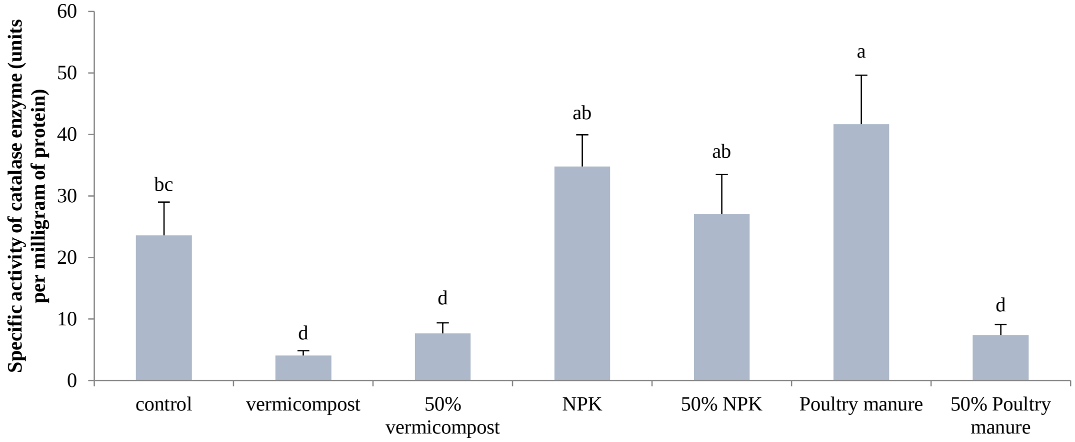

Figure 3. Effect of different organic and inorganic fertilizer treatments on activity of catalase enzyme of the ginger plant (units per milligram of protein). Values are the mean of three replicates ± SD. Different letters represent a significant difference between treatments ($P < 0.05$).

The amount of proline, soluble sugars, H_2O_2, and total phenols

The variance analysis of the data showed that the amount of soluble sugars in ginger plants decreased significantly in the presence of most of the fertilizer treatments (Table 1). However, only the poultry manure treatment significantly increased compared to the control. Soluble sugars are metabolic substrates that act a basic duty in the control of several processes during various phases of plant development. Soluble sugars act as nutrients, osmoprotective signaling molecules, and metabolites and are involved in various responses to abiotic stress [50]. A comparison of average data showed that the amount of proline increased in the presence of different fertilizer treatments. The highest amount of proline was obtained in the presence of 50% vermicompost (5 t ha^{-1}) and NPK (100 t ha^{-1}) treatments (Table 1). Proline abundance may result from a synthesis of de novo, protein hydrolysis, lowered utilization, or degradation. Proline, an amino acid, plays a very beneficial role in plants exposed to various stress conditions. Additionally, the interaction of proline with other osmotic protectors and signaling molecules, e.g. abscisic acid, nitric oxide, hydrogen sulfide, Glycine betaine, and soluble sugars, assist in creating robust defense mechanisms in stressful environments [51]. Hafez et al. [44] reported that the application of vermicompost in soil could reduce the amount of proline, which is consistent with the results of this research regarding the complete treatment of vermicompost. There are similar reports in this regard [52, 20]. The results of the data analysis showed that the value of H_2O_2 increased significantly in the presence of 50% vermicompost treatment (11.34 nmol g^{-1} WW). However, in the complete vermicompost treatment, the amount of H_2O_2 decreased compared to the control. In a study, it was shown that the application of vermicompost reduces the H_2O_2 content of corn plants in an environment with high salinity [53]. Nevertheless, the lowest amount of H_2O_2 was obtained in the treatment of 50% poultry manure (Table 1). The impact of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) on plant performance has received a lot of attention lately. Normally, in biological systems, H_2O_2 is more stable than its common precursor, superoxide ($O_2^{\cdot-}$). So, it can function as a signaling molecule and substrate in a process that is reasonably controlled. As a result, the synthesis of $O_2^{\cdot-}$ and H_2O_2 is linked, and the generation and interactions of nitric oxide ($NO^{\cdot}$), a molecule that signals free radicals, further complicate the issue [54].

Table 1. Changes of soluble sugars, proline, H_2O_2, and total phenols in ginger plant under different fertilizer treatments.

Treatment	Soluble sugars (mg g DW^{-1})	Proline (mg gFW^{-1})	H_2O_2 (nmol g^{-1} WW)	Total phenols (mg gFW^{-1})
control	1.43^{b}	0.00^{c}	1.37^{e}	0.0031^{e}
vermicompost	1.06^{d}	0.01^{c}	0.83^{ef}	0.0044^{dc}
50% vermicompost	0.59^{f}	0.35^{a}	11.34^{a}	0.0062^{b}
Poultry manure	2.00^{a}	0.11^{bc}	2.48^{d}	0.0094^{a}
50% Poultry manure	1.02^{d}	0.18^{b}	0.10^{f}	0.0049^{c}
NPK	1.24^{c}	0.38^{a}	7.75^{b}	0.0055^{bc}
50% NPK	0.85^{e}	0.12^{bc}	4.32^{c}	0.0033^{de}

Values are the average of three replicates ± SD. Different letters represent a significant difference between treatments ($P < 0.05$).

According to statistical analysis, the application of vermicompost and poultry affects the total phenols of the plants, significantly (Table 1). The most effective dose of organic fertilizer in increasing the total phenol of ginger was poultry manure treatment. The results obtained from this study indicate that the phenolic synthesis in ginger can be increased by using organic and inorganic treatments. Plants mainly produce phenolic compounds for growth, development, and protection. These aromatic compounds with benzene rings are essential during plant biotic and abiotic stress interactions. They play a fundamental function in various physiological and mechanical activities and are an important part of the secondary metabolites of the plant [55]. Researchers have investigated the effect of using biofertilizers on quality and quantity performance in some plants and reported that these fertilizers have increased the phenolic content in these plants [56]. To our knowledge, there is no data on the effect of poultry manure on the measured indicators (soluble sugars, proline, H_2O_2, and phenol) of ginger plants in the literature.

The antioxidant activities of the extract by the DPPH method

The results of investigating the antioxidant capacity of the extracts obtained from the aerial part of the ginger plant treated with biological and inorganic fertilizers are shown in Figure 4. The analysis of variance demonstrated that by applying different fertilizer treatments (organic and inorganic), free radical scavenging power (DPPH) decreased compared to the control, significantly. No differences were observed between the fertilizer treatments except the vermicompost treatment in terms of DPPH (Figure 4). Among the tested samples, only vermicompost showed a better effect in DPPH radical removal. Other treatments exhibited very poor scavenging activity. Antioxidants can donate hydrogen, which has an impact on DPPH scavenging [57]. The current study's findings demonstrated that the ginger extracts' capacity to scavenge DPPH radicals was higher in the control group. In addition, organic and non-organic fertilizer treatments had less effect on the DPPH radical scavenging abilities.

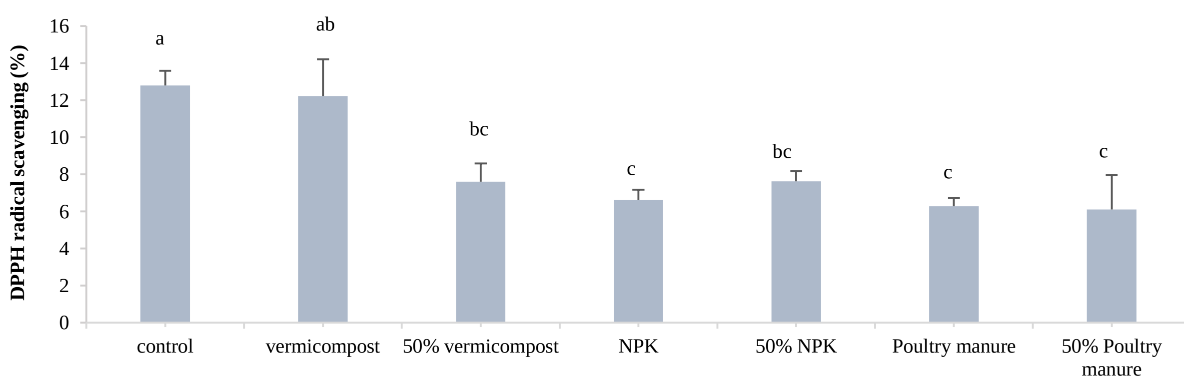

Figure 4. DPPH radical scavenging activity of different stem extracts of the ginger. Values are the mean of three replicates ± SD. Different letters represent a significant difference between treatments ($P < 0.05$).

Antioxidant compounds are substances that protect cells against free radicals. This action is done by supplying damaged cells with electrons from antioxidants. Antioxidants can also remove free radicals by turning them into waste by-products from plants [58, 59]. It has been reported that Antioxidants could affect DPPH due to their ability to donate hydrogen [60]. The property of antioxidants in inhibiting DPPH free radicals can be used to screen the antioxidant activities of plant extracts [58]. It was shown that the effect of biological fertilizers on quality indicators of Allium species, for example, onion, increases the antioxidant activity [61, 62]. On the other hand, the researchers showed that the treatment of *Allium cepa* L. with biological fertilizers could lead to an increment in antioxidant activity compared to the control [63]. In a research, it was observed that organic fertilizers and the combination of organic and biological fertilizers could increase the phenol content and antioxidant activity of the saffron plant (*Crocus sativus* L.) [64]. Based on the DPPH assay, it has been determined that the fruits of plants treated with chemical fertilizer and vermicompost had similar 50% inhibitory concentration values. They also showed significant DPPH radical scavenging activities compared to the control [65]. Therefore, it is suggested that future research could include studying and evaluating the efficacy of vermicompost and poultry manure on the stability of bioactive compounds in ginger, especially during product transportation, fruit storage, and fruit processing. This information can be useful for both producers and consumers, as it can lead to a better understanding and attention regarding the significance of organic fertilizers and sustainable agriculture.

CONCLUSIONS

Despite the different results obtained, it seems that organic fertilizers have positive impacts on the biochemical properties of ginger plants. Thus, it can be stated that vermicompost and poultry fertilizers may not be able to completely replace chemical fertilizers to produce products with a high content of *phytocompounds*, but they can be used as additional supplements to the new fertilization method to reduce environmental pollution and ensure the sustainability of agriculture. As far as has been investigated, there is not much data about the effects of chicken manure on the measured indicators, and more research is needed in this field. The benefits that plants receive from the application of vermicompost depend on their ability to extract the fertilization substrate and the materials necessary for their growth and development. However, the extensive benefits of vermicomposting and poultry as an environmentally friendly alternative to chemical fertilizers have been noted.

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Conflict of interests

The authors declare that there is no conflict of interest.

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