Feasibility Study of Copper Nanoparticle Biosynthesis using Leaf Extract as well as Green and Red Hull of Pistachios

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Information

Article Type:
Original Article

Article History:

Received: 05.07.2021
Accepted: 25.09.2021

Doi: 10.22123/PHJ.2021.303376.1110

Keywords:
Biosynthesis
Copper nanoparticles
Hull
Leaf
Pistachio

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Abstract

Introduction: Green synthesis of nanoparticles is more simple, inexpensive, non-toxic, and environmentally friendly than traditional methods. Thus, given the presence of secondary metabolites, the use of plant extracts has attracted much attention in causing metal ion reduction reactions into nanoparticles.

Materials and Methods: In this study, sampling was conducted from pistachio trees of the same age and from branches of the same diameter with a height of 1.5 meters. Leaves and fruits with green and red hull samples were harvested at a specified time. Besides, biochemical properties of the samples were examined for phenolic compounds, anthocyanins, and flavonoids. Copper nanoparticles were synthesized using 1mM copper sulfate salt at 55 and 80 °C. In addition, the absorption spectrum of the synthesized nanoparticles was analyzed through measuring the UV-Vis spectrum.

Results: The content of phenolic compounds in the leaves showed a significant increase compared to the fruit hull. In addition, red hull fruits had less phenols than green hull ones. Besides, anthocyanin and flavonoid contents were higher in the leaves than in the fruit hull. Unlike the phenolic compounds, the anthocyanin content increased upon fruit development. Nanoparticles were synthesized upon observing the change in the color of the plant extract and the copper sulfate from pale yellow to dark brown. In addition, the peak in the wavelength range of 400-430 nm confirmed the formation of copper nanoparticles.

Conclusion: The results of this study showed that the phenolic compounds, anthocyanins, and flavonoids exiting in the aqueous extract of the pistachio leaves and hull acted as copper-reducing agents and chemical stabilizers of copper metal ions into copper nanoparticles.

►Please cite this article as follows:
Dahajipour Heidarabadi M, Nozari S. Feasibility of copper nanoparticles biosynthesis using leaf, green and red hull extracts of pistachio. Pistachio and Health Journal. 2021; 4 (3): 41-50.

1. Introduction

The development of nanotechnology has made researchers become interested in synthesizing nanoparticles for their biological applications. Among the major uses of this technology in medicine, one could mention gene and drug transfer, biological analysis, medical diagnosis, as well as treatment of cancer, infections, allergies, diabetes, and inflammation [1]. Biosynthesis of metal nanoparticles, being one of the new topics in nano-biotechnology research, which is performed by biological systems, has provided materials sized 1-100 nm for biomedical and pharmaceutical purposes. Green nanoparticle synthesis is more simple, economical, non-toxic, and environmentally friendly than traditional methods, such as physical and chemical ones [2]. Copper nanoparticles act as antimicrobial agents in various fields. Antifungal, antibacterial, and antimicrobial properties of copper nanoparticles turn them into useful materials to be used in medical fibres and textiles, such as sterile gauze pads, clothing used in contaminated areas, and sutures. Research shows that many nanoparticles, in addition to having strong antimicrobial properties, have distinct advantages, such as overcoming antibiotic resistance mechanisms and having fewer side effects than antibiotics [3]. Plant extracts, given their secondary compounds such as phenolic compounds, flavonoids and anthocyanins, are potential causes of chemical reduction reactions. Besides, aside from synthesizing nanoparticles by reducing metal ions, they play a major role in the growth and stabilization of nanoparticles [1].

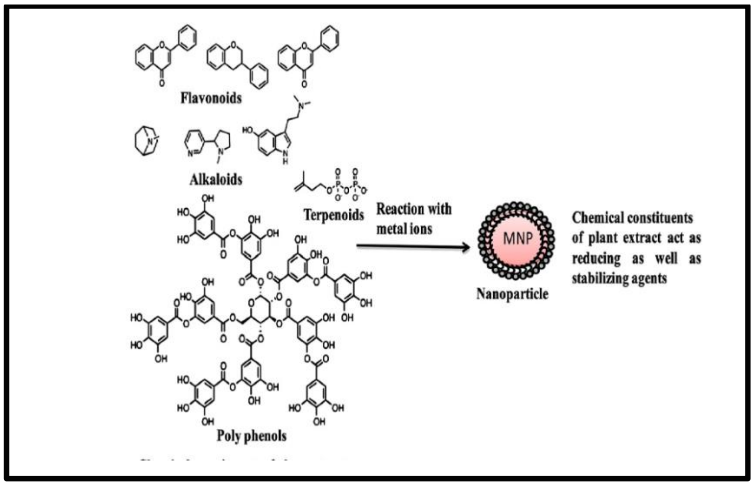

Fig. 1. Chemical compounds in plant extracts responsible for bioredution of metal ions [4]

Pistachio (*Pistacia vera* L.) is a plant of the Anacardiaceae family. Pistachio fruit has a soft shell on the outer surface (hull) and a hard bony shell surrounding the kernel [5]. Studies show that the leaves and hull of pistachios contain significant amounts of secondary metabolites with therapeutic properties and antimicrobial activity [6-8]. Few studies have been conducted on the antibacterial properties of soft pistachio hull and the use of pistachio trees in nanoparticle synthesis. Some studies have reported the synthesis of copper nanoparticles with the dimensions of 26-51nm using pistachio hulls, the synthesis of copper nanowires with a diameter of 10nm and a length of 500nm, and the synthesis of iron nanoparticles sized 5-12nm using pistachio leaf extracts [9,10].

This study aims to investigate the amount of phenolic compounds, anthocyanins, and flavonoids in pistachio leaves and hull as reducing compounds for the biosynthesis of copper nanoparticles. Accordingly, nanoparticle biosynthesis is investigated in this study using aqueous extracts of pistachio leaves, green hull, and red hull.

2. Materials and Methods

2.1. Plant materials

In this research, a pistachio orchard located in Vali-e-Asr University of Rafsanjan was utilized. Sampling was conducted from pistachio trees of the same age and branches with the same diameter with a height of 1.5 meters. Besides, leaf and fruit samples with green hull were harvested in July, and fruit samples with red hull were harvested in late September.

2.2. Measurement of total phenolic compounds

The amount of phenolic compounds was measured based on the Folin-Ciocalteu calorimetric method and according to the standard curve of Gallic acid at a wavelength of 765 nm [11]. An amount of 200 mg of the sample was ground with 3 ml of 80% methanol and placed in a hot water bath at 70 °C for 3 hours. Next, the mixture was centrifuged for 10 minutes at 9000 rpm, and the methanol extract was used to measure phenolic compounds. Besides, as much as 100 µl of the Folin-Ciocalteu reagent was added to 20 µl of the methanol extract. After 5 minutes, 300 µl of a 7.5% sodium carbonate solution was added to the resulting mixture and placed in the dark at room temperature for 1.5 hours. In the end, the absorbance of each sample was read at 765 nm with a spectrophotometer, and the amount of the total phenol was measured in the mg/g of the fresh weight.

2.3. Measurement of total flavonoids

An amount of 200 mg of the sample was ground in 3ml of acidic ethanol (ethanol and acetic acid in a ratio of 1:99) and centrifuged at 1500 rpm for 15 minutes. After filtration, the supernatant was placed in a warm water bath at 80 °C for 10 minutes. The absorption rate of the samples after cooling was read by a spectrophotometer at the three wavelengths of 270, 300, and 330 nm. In addition, to calculate the concentration of flavonoids, the extinction coefficient of 33000 $Cm^{-1}M^{-1}$ was applied [12].

2.4. Measurement of total anthocyanins

To measure anthocyanins, 200 mg of the plant sample was ground in 3 ml of acidic methanol, including methanol and hydrochloric acid in a volume ratio of 99:1. Next, the resulting extract was centrifuged at 10,000 rpm for 10 minutes. After filtering the supernatant solution, it was poured into test tubes with screw caps and placed in the dark at 25 °C for 24 hours, with its absorption read by a spectrophotometer at 550 nm. Besides, an extinction coefficient of 33000 $M^{-1}cm^{-1}$ was used to calculate the concentration of anthocyanins [13].

2.5. Preparation of the plant extract; synthesis and investigation of nanoparticles

An amount of 10 g of the plant samples (leaves, green hull, and red hull) was added to 100 ml of boiling distilled water after being ground using liquid nitrogen. After 5 minutes, the extract obtained was cooled at room temperature and centrifuged at 4500 rpm at 4 °C for the synthesis of copper nanoparticles using Whatman grade 1 filter paper. To biosynthesize copper nanoparticles, appropriate amounts of the extracts of leaves as well as green and red hull samples were added to the 1 mM solution of copper sulfate ($CuSO_4$, $5H_2O$) and mixed more using a shaker. Besides, it was stirred constantly. The copper nanoparticles were synthesized using an aqueous extract of the samples, not at room temperature. Therefore, the factor of temperature (temperatures 55 and 80 °C) was used to accelerate the reaction rate. After being mixed, the extract and the solution of copper sulfate were placed in a room for 24 h at 55 and 80 °C, respectively, with continuous stirring. Copper ion reduction reaction was observed by measuring the UV-Vis spectrum. After synthesizing the sample using a spectrophotometer (Avantes Avaspec-20488, USA), the amount of the light absorbed was measured at the wavelengths of 300 to 600 nm. In addition, based on the data obtained, the absorption curve was drawn in Excel. The absorption peak within the range of 400-430 nm indicated the reduction of copper ions and synthesis of copper nanoparticles in the solution.

2.6. Statistical analyses

Excel was used to determine the mean and standard deviation and to draw the graphs. Moreover, the one-way analysis of variance (ANOVA), based on the Duncan's test in SPSS V15.0, was used to determine differences at the significance level of $P \leq 0.05$.

3. Results

3.1. Investigation of plant biochemical compounds

As Table 1 shows, the levels of phenolic compounds, anthocyanins, and flavonoids in the leaf, green and red hull samples of pistachios, based on the type of the plant organ, were significant at a 1% level of probability.

Table 1. Analysis of the variance of effects of the plant sample type on phenolic compounds, anthocyanins, and flavonoids

Sources of Variation	Degree of freedom	Mean of squares		
		Phenolic compounds	Anthocyanins	Flavonoids
Plant sample	2	47.08**	30.78**	981507.51**
Error	24	0.16	0.15	1100.97
CV (%)		11.80	16.16	9.09

** Significant at the 1% level of probability

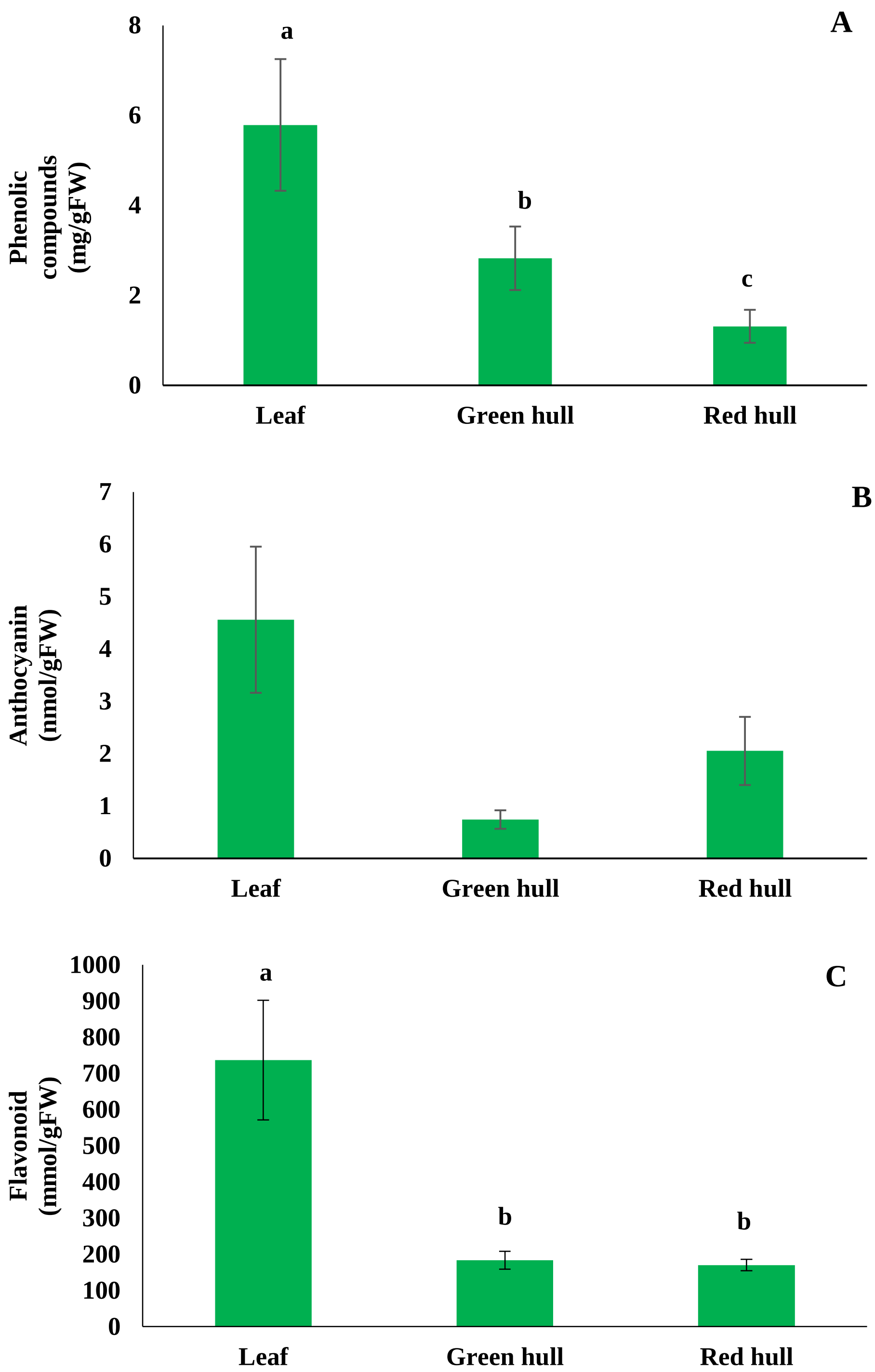

Fig. 2. The amounts of phenolic compounds (A), anthocyanins (B), and flavonoids (C) in leaves, green hull, and red hull of pistachios. The data are the mean of at least five independent replications ± standard deviation (vertical bars). Non-identical letters indicate a significant difference at the level of $P \leq 0.05$ based on the Duncan's test.

Phenolic compounds were the most abundant antioxidant compounds in the investigated samples. The comparison of the mean amount of phenolic compounds showed that the amount of phenolic compounds was higher in the leaves than in the fruit. Besides, the fruit with red hull had a lower amount of phenolic compounds than the fruit with green hull (Fig. A2). In addition, the content of anthocyanins showed a significant increase in the leaves compared with the fruit . Furthermore, the anthocyanin content was higher in the red hull than in the green hull. In other words, with an increase in the fruit growth, the level of anthocyanin increased in the hull (Fig. B2). Comparison of the mean content of flavonoids showed that it had a significant increase in the leaf samples compared with the fruit. However, the amount of flavonoids in the fruit samples with green and red hull no statistically significant difference was showed (Fig. C2).

3.1. Synthesis of copper nanoparticles

When the plant extract (B3) was combined with a solution of copper sulfate (A3), the resulting solution grew pale yellow (Fig. C3). However, over time, the color of the solution gradually grew dark brown (Figs. D3 and E3). The color change in the solution was a sign of the reduction of copper ions and synthesis of copper nanoparticles.

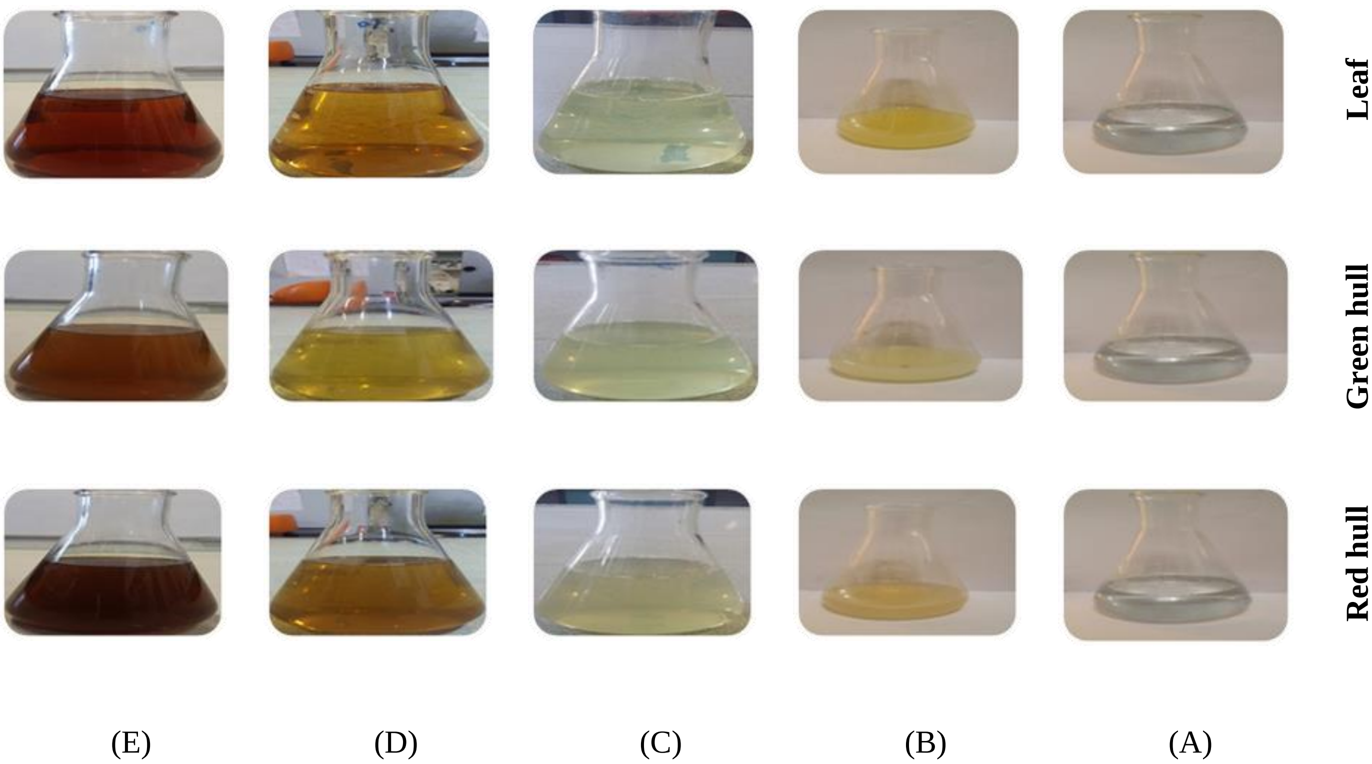

Fig. 3. Color change of the mixture of the copper sulfate solution and the leaf extract, green hull, and red hull at 80 °C. A) 1 mM of the copper sulfate solution B) The plant extract C) The mixture of the copper sulfate solution and the plant extract combined in the beginning D) The mixture of the copper sulfate solution and the plant extract after six hours E) The mixture of the copper sulfate solution and the plant extract after 24 hours

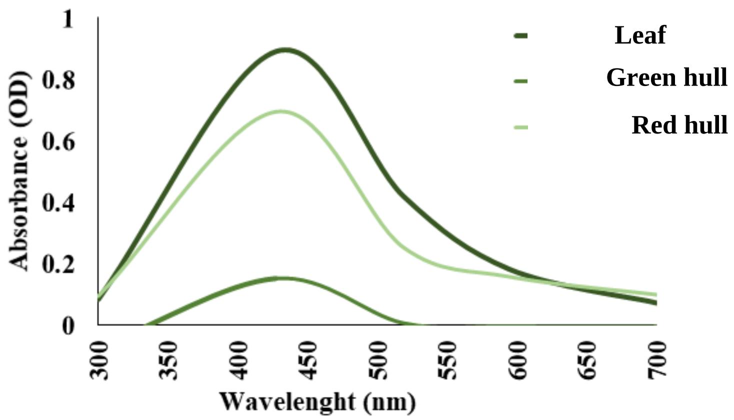

Fig. 4. Optical absorption of biosynthesized copper nanoparticles at 55 °C using a leaf extract, green hull, and red hull

Given the results obtained from the UV spectrum and plotting (Figs. 4 and 5), the light absorption of copper nanoparticles in the plant samples was determined to be 55 and 80 °C. According to the graph, at temperature 55 °C, the highest absorption within the wavelength range of 400-430 nm was related to the leaf sample, followed by red hull and green hull (Fig. 4).

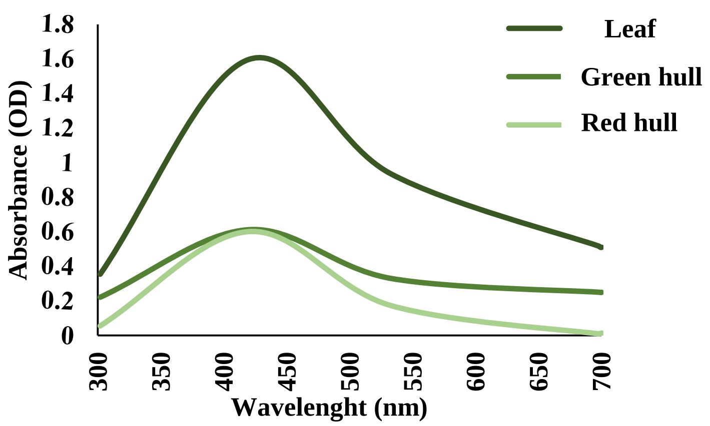

Fig. 5. Light absorption of biosynthesized copper nanoparticles at 80 °C using the leaf extract, green hull, and red hull

According to Fig. 5, i.e. the light absorption of biosynthesized copper nanoparticles at 80 °C, the highest absorption levels within the wavelength range of 400-430 nm were related to the leaves, green hull, and red hull.

4. Discussion

In recent years, using plant compounds as natural antioxidants has received increasing attention [14]. Secondary metabolites, such as phenolic compounds, flavonoids, and anthocyanins have necessary regenerative or antioxidant properties to cause reactions of chemical reductions [15]. Accordingly, this study primarily attempted to compare the amount of secondary metabolites in the aqueous extracts of the leaves, green hull, and red hull. According to the results, phenolic compounds were the most abundant chemical compounds in the leaves and hull of the fruit. Phenolic compounds are simple (gallic acid), complex (*gallotannins*), polar (flavonoids), quasi-polar (*gallotannins*), and amphiphilic (anacardic acids) [16]. In general, the results showed that the amount of antioxidant compounds in the leaves was more than that in the hull of pistachio fruit. These results were confirmed in the research conducted by Nadernejad *et al* as well (2013) [17]. Phenolic compounds are among the compounds undergoing many changes during fruit development [18, 19]. In the soft skin of pistachios (hull), the amount of phenolic compounds decreased with fruit ripening. Decreased phenolic compounds with fruit ripening have been reported in other studies as well [16]. According to research reports, the main difference between green hull and red hull in the pistachio fruit was the higher abundance of anthocyanins in red hull. In other words, the content of anthocyanins in the early stages of pistachio fruit development was low but increased with fruit ripening [20]. The results of the present study were consistent with past ones.

One of the features of nanoparticles is their unique optical properties that change according to the shape, size, concentration, and state of nanoparticle aggregation. Since metal nanoparticles have free electrons, these electrons show light absorption bands owing to the vibrations of free electrons in metal nanoparticles when exposed to light [21]. The UV spectrum was used to investigate the formation of copper nanoparticles (Figs. 4 and 5). The reduction of copper ions into copper nanoparticles confirms the surface Plasmon resonance of copper nanoparticles. In addition, their maximum absorption peak occurred within the wavelength range of 400-430 nm for copper nanoparticles, being consistent with the results of other studies [9, 22]. Based on the temperature graphs (Figs. 4 and 5), the leaf and green hull extracts showed an increase in light absorption or in the synthesis of copper nanoparticles upon an increase in the temperature. Similarly, the results of other studies on the synthesis of copper nanoparticles using a basil extract showed an increase in the synthesis of copper nanoparticles upon an increase in the temperature [23]. As Figs. 4 and 5 show, in the samples synthesized by the red hull extract, the increase in the temperature reduced the adsorption and synthesis levels of copper nanoparticles. The results of the study of Azizian Sharmeh *et al* (2016) showed that with the temperature exceeding 75 °C, the synthesis of gold nanoparticles using the extract of the *Sambucus ebulus* L. plant decreased [24]. Research shows that some compounds in plant extracts, which play the role of reducing and stabilizing nanoparticles, are decomposed upon an increase in the temperature. Thus, with an increase in the temperature, nanoparticle synthesis decreases [25]. Therefore, the decrease in the synthesis of nanoparticles by the red hull upon increasing the temperature was related to the degradation of active photochemical compounds involved in the synthesis of nanoparticles, including anthocyanins.

5. Conclusion

The amount of phenolic compounds, anthocyanins, and flavonoids in pistachio leaves showed a significant difference compared to the pistachio fruit. However, given the fact that the superficial skin of pistachios constitutes 40% of their weight, the leaf extract and soft skin of the pistachio fruit, having the abovementioned compounds as cheap and accessible sources, could be used for reducing metal salts and converting them into nanoparticles. Thus, these sources are recommended to replace chemical reducing and stabilizing compounds, such as organic solvents and surfactants, in other research fields.

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