ISSN NO: 2771-8840
Date of Publication: 24-11-2022

Effect of Growth Regulators, Chitosan, and Silver Nanoparticles on Callus Induction and Stimulating the Myricetin Production in Moringa (*Moringa Oleifera* Lam.) Tree

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Abstract
The study was carried out in the laboratory of Fadak Company for Tissue Culture in Abi Al-Khassib district, and the Laboratories of the College of Pharmacy, University of Basrah, Iraq. The nodule stem segments were used as explants that were taken from five-year-old Moringa trees. These explants were cultured on an MS medium supplemented with benzyl adenine at different concentrations (1, 2, 3, and 4 mg L^{-1}) for callus induction. The 50 mg weight of induced callus was cultured on MS medium supplemented with different concentrations of chitosan (0, 5, and 10 mg L^{-1}) and silver nanoparticles (0, 10, 20, and 30 mg L^{-1}) for stimulating the myricetin production. The treatment of 4 mg L^{-1} benzyl adenine showed significant superiority in the percentage of explant response to callus induction compared to the other treatments, which amounted to 66.0%. But the treatment of 1 mg L^{-1} benzyl adenine was recorded the lowest response of explants to callus induction, which amounted to 13.77%. The results also showed that the treatment of 5 mg L^{-1} chitosan was significantly superior in the content of callus of the myricetin compound compared to other treatments, which amounted to 79.572 moles L^{-1}. The treatment of 10 mg L^{-1} silver nanoparticles, which recorded the callus content of myricetin compound, amounted to 26.387 moles L^{-1}. The leaves of Moringa trees recorded 4.714 moles L^{-1} myricetin compound. While the treatment of silver nanoparticles at 30 mg L^{-1} recorded the lowest callus content of myricetin, which was 0.668 moles L^{-1}.

Keywords: Bioactive compound, callus, explant, HPLC device, *in vitro*, nodule stem

Introduction

The *Moringa oleifera* or miracle tree is one of the plants that people have been interested in since ancient times. It belongs to the family Moringaceae. This tree is planted as an ornamental tree for its beauty and shade, and it is also used as a fence or windbreak plant (Poteet, 2006). Also, all parts of the plant are considered important from a nutritional and medical point of view and are considered as an integrated food for many regions such as Asia and Africa. Its leaves are widely used as nutritional supplements due to their large amounts of antioxidants, vitamins, amino acids, carbohydrates and important elements such as iron, potassium, phosphorous, calcium, zinc and selenium, as well as containing beta-carotene (Farooq et al., 2012; Yadav and Srivastava, 2016).

The Moringa plant is also rich in many compounds that are of medical importance, such as myricetin. These compounds are among the compounds belonging to the group of flavonoids that are beneficial to plants and humans in general. It is one of the compounds that reduce the risk of cancerous diseases, as well as some heart and circulatory diseases. It is also effective as an antioxidant and antibiotic against bacteria, fungi, and viruses. It has been proven that it can have anti-Covid19 properties (Maconnen et al., 1997; Abdulkarim et al., 2005; Colunga Biancatelli et al., 2020). The Moringa trees have been called the miracle tree due to their medical, industrial and environmental importance (Koul and Chase, 2015; Saini et al., 2016). The leaves of this plant are the most widely used parts of it because they contain a high percentage of minerals, proteins, and carbohydrates and contain bioactive compounds such as vitamins, carotenoids, polyphenols, alkaloids, tannins, saponins (Oladeji et al.,2017), as well as glycosides and flavonoids, including myricetin and phytosterol (Yadav et al., 2017).

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Although myricetin is present in all plants belonging to the plant kingdom, it is mainly produced by members of the families Myricaceae, Anacardiaceae, Polygonaceae, Pinaceae and Primulaceae (Chua et al., 2011, Abd El-kader et al., 2013). This phenolic compound is very common in various plants that have both free and bound structures of glycosides. Its history as myricetin extends back to a hundred years ago, which was first extracted in the late eighteenth century from the *Myrica nagi* plant in India (Perkin et al., 1896). Myricetin is poorly soluble in water, but it dissolves quickly in some organic solvents such as dimethylformamide, dimethylacetamide and tetrahydrofuran. Myricetin is more stable at pH = 2. Myricetin is structurally related to many phenolic compounds such as quercetin, murine, kaempferol, and fisetin, and the compounds sometimes referred to as quercetin hydroxide. Myrcetin also has valuable properties in antioxidant activities and a range of pharmacological activities including anti-inflammatory, analgesic, antitumor, hepatoprotective and antidiabetic (Lin et al., 2012; Yao et al., 2014). Myricetin showed an anti-aging effect by removing free radicals that cause skin aging. Topical application of myricetin has been found to inhibit wrinkles caused by ultraviolet-B (UVB) ultraviolet rays (Jung et al., 2010).

Chitosan is one of the compounds with bioactivities, as it acts as an antimicrobial, antioxidant, and stimulant of plant growth. Chitosan is a natural carbohydrate polymer derived from the deacetylation of the acetyl group of chitin, which consists of N-acetyl-D-glucosamine and D-glucosamine residues that are linked together by a ß-1,4-glycoside bond (Chibu and Shibayama, 2001). Chitosan is a powerful reducing agent that plays an important role in plant resistance to diseases and defense mechanism as an antioxidant and has an important and influential role in plant growth (Uthairatanakij et al., 2007).

Nanotechnology has opened a wide scope for innovative applications in various fields, including biotechnology and agricultural industries, and the reason is due to the fact that nanoparticles have unique properties such as the shape of the particles, the exact pore size, the high efficiency and the surface area. Nanoparticles also have unique and distinctive capabilities and physical properties that in turn enhance plant metabolism (Giraldo et al., 2014). It was also found that nanoparticles have the ability to enter into plant cells. It can also transfer nucleic acids, DNA, as well as chemicals, to plant cells. This field of research presented new possibilities in the field of plant biotechnologies (Galbraith, 2007; Torney et al., 2007). In a study conducted by Al-Obaidi (2016), on the use of different concentrations of silver nanoparticles to stimulate the production of some bioactive compounds in the hopbush (*Dodonia viscosa* L.) plant that cultured via in vitro. It was found that treatment with silver nanoparticles at a concentration of 2 mg L^{-1} led to a significant increase in the production of bioactive compounds such as quercein and luetolin. It was also found that treatment with a concentration of 0.5 mg L^{-1} silver nanoparticles recorded a significant superiority in the increase in the production of the bioactive compound apigenin.

This research aims to determine the best combination of growth regulators to induce and multiply callus, as well as the effect of different concentrations of chitosan and silver nanoparticles on stimulating the bioactive compound myricetin by in vitro callus tissue culture.

Material And Methods

The study was carried out in the laboratory of Fadak Company for Tissue Culture in Abi Al-Khasib district, and the laboratories of the College of Pharmacy, University of Basrah, Garmat Ali location, Basrah Governorate, Iraq. In this study, nodule stem segments were used as explants that were taken from five-year-old Moringa trees. The nodule stem segments were cultured after sterilizing their surfaces on an MS medium containing growth regulators at different concentrations to induce callus.

Surface sterilization of explants

Nodule stem segments were excised from the mother plant Moringa using a sharp scalpel (blade) with a length of 3 cm. Then they were washed with soap and water several times to get rid of dirt and suspended matter. Then it was washed with distilled water several times, after that the explants were cut and their lengths became 1 cm. Then they were preserved in glass beakers containing an antioxidant solution consisting of 150 mgL^{-1} of citric acid and 100 mgL^{-1} of ascorbic acid (Zaid and Tisserat, 1983). Then it was placed in the refrigerator at a temperature of 5 C for 24 hours until the surface sterilization process. Then, the surface sterilization process of the explants was carried out with 1.05% sodium hypochlorite solution at 20 minutes inside the laminar air-flow cabinet, which was previously sterilized with 70% ethanol and sodium hypochlorite diluted with distilled water. The explants were washed three times with sterile distilled water after the surface sterilization process (Ibrahim and Daraj, 2020).

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Medium Preparation

The MS medium formula was prepared by MS salts (Murashige and Skoog, 1962) with a weight of 4.43 g L^{-1} produced by the Indian company HIMEDIA. 30 g L^{-1} of sucrose and different concentrations of benzyl adenine (1, 2, 3, and 4 mg L^{-1}) and a constant concentration of naphthalene acetic acid (0.5 mg L^{-1}) were added to MS media for callus induction and multiplication. Then the pH of the MS medium was adjusted with 0.1N of NaOH or 0.1N of HCl. Then 8 g L^{-1} of agar agar was added to the MS medium after it had completed its volume to one liter. After the homogenization of the MS medium was distributed in glass jars. Then they were sterilized in an autoclave at 121°C and a pressure of 1.04 kg cm^{-2} for 20 minutes. The medium was then left to solidify at room temperature.

The initiation of callus cultures

Nodule stem segments were cultured on MS medium containing combinations of benzyl adenine (1, 2, 3, and 4 mg L^{-1}) and a constant concentration of 0.5 mg L^{-1} of naphthalene acetic acid. Then all the cultures were incubated at a temperature of 25 ± 2°C, and the duration of illumination was 8: 16 hours (light: dark) and the light intensity was 1000 lux. Each treatment was repeated ten times in the experiment. The data of the percentage of explant response to callus induction were recorded four weeks after culturing. From the results of the callus induction and initiation experiment, it was found that the MS medium supplemented with 4 mg L^{-1} benzyl adenine and 0.5 mg L^{-1} naphthalene acetic acid was the best treatment for callus induction. Therefore, the callus was multiplied by culturing it on this medium until the required quantity for the subsequent experiment was obtained.

Experiment of the effect of chitosan and silver nanoparticles on the induction of myricetin

50 mg weight of induced callus was taken from nodule stem segments and cultured on MS medium prepared with different concentrations of chitosan (0, 5 and 10 mg L^{-1}) and silver nanoparticles (0, 10, 20 and 30 mg L^{-1}). Then the cultures were incubated at a temperature of 25 ± 2°C, and the duration of illumination was 8: 16 hours (light: dark) and the light intensity was 1000 lux. Each treatment was repeated ten times. The myricetin content of the callus of Moringa trees was estimated after five weeks of culturing. Leaves were also collected from Moringa trees for the purpose of estimating the content of myricetin in them.

Estimation of myricetin using high-performance liquid chromatography (HPLC)

Samples were taken from the callus that was cultured on MS medium supplemented with different concentrations of chitosan and silver nanoparticles, as well as samples from the leaves of Moringa trees. Then the samples were placed in special flasks and lyophilized for 48 hours using the Labconco Freeze Dryer, a type of American origin. After completing the lyophilization process, the samples were finely ground to dissolve them with organic solvents for estimation of the myricetin in them by HPLC device. 5 mg of pure myricetin and plant samples were weighed and placed in 1.5 mg vials and 400 µL of the solvent of 70% ethanol with 30% acetonitrile was added. Then it was placed in an Ultrasonic machine for several minutes to ensure the plant particles were broken down and melted. Then it was placed in the Maxi mix vibrator for several minutes as well, to ensure that the particles were well mixed and dissolved, as the solution was 100%, then the solution was filtered with filter paper. Water and Acetonitrile were injected into the HPLC via the injection port of 30 µL to ensure that it was not contaminated with the injected solutions. Then, 10 microliters of myricetin standard solution were withdrawn and injected into the Angstrom 100LC-UV100 Plus HPLC high-performance liquid chromatography to determine the time retention and to find the area of the sample beam height for the standard solution (Al-Bahadli, 2020). The retention time was 1.80 minutes, while the peak area of the standard solution was 502288.1 (Figure 1). Then the plant samples were injected into the device individually sequentially using a Hamilton Syringe type syringe with a volume of 10 µL at the injection port (Figures 2-8). The stationary phase of the device was Nuceosil 100-S and the column used was Arcus type Column with dimensions of 4.6 x 250 mm and a column temperature of 30°C. The mobile phase was Water HPLC and Acetonitrile at 70:30 (V: V) ratio, a flow rate of 1 ml L^{-1}, and wavelength: 370nm.

The concentrations of the bioactive substance myricetin were quantitatively determined using the comparison equation between the standard model and the under of the same conditions using the following equation:

Sample concentration (mg g^{-1}) = area of sample / area of standard solution x number of dilutions

Experimental design and statistical analysis

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All experiments included in the study were carried out according to a completely randomized design (C.R.D.). The Data of results were analyzed using the analysis of variance based on the statistical program GenStat version 14. The significance was compared between the means of the treatments using the Least Significant Difference test to show the statistical differences between the treatments at the 1% probability level (Al-Sahoki and Waheeb, 1990).

Results And Discussion

The results from Figure 9 show that the treatments of benzyl adenine at 1, 2, 3, and 4 mg L^{-1} were recorded significant differences in the response of nodule stem segments to callus induction of Moringa trees after four weeks of culturing. The treatment of 4 mg L^{-1} benzyl adenine showed significant superiority in the percentage of explant response to callus induction compared to the other treatments, which amounted to 66.0%. The results also indicate that the response of the nodule stem segments to callus induction increased with the increase in the concentration of benzyl adenine that was added to the MS medium after four weeks of culturing. While the treatment of 1 mg L^{-1} benzyl adenine recorded the lowest response of explants to callus induction, which amounted to 13.77% (Figure 9).

The high response of nodule stem segments to the induction of callus with an increase in the concentration of benzyl adenine is because high concentrations of cytokinins lead to dedifferentiation of plant tissue and induction of cell division, which is positively reflected in the formation of callus tissue (Ibrahim, 2012; Ibrahim et al., 2012). The high response of explants to the high concentrations of cytokinins in callus induction is due to the balance between endogenous and exogenous hormones, which led to dedifferentiation in plant tissues that cultured via in vitro culture technique, which encouraged continous cell division and the formation of callus tissue (Gu and Zhang, 2005; Taiz and Zeiger, 2015).

The results from Figure 10 indicate the significant difference between the treatments of chitosan and silver nanoparticles in the callus content of the Moringa tree from the bioactive compound myricetin after five weeks of culturing. It is noted that the treatment of 5 mg L^{-1} chitosan was significantly superior in the content of callus tissues of the myricetin compound compared to other treatments, which amounted to 79.572 moles L^{-1}. It was followed by the treatment of 10 mg L^{-1} silver nanoparticles, which recorded the callus content of myricetin compound amounted to 26.387 moles L^{-1} compared to the callus content in the control treatment, which recorded 10,547 mg L^{-1} myricetin. The leaves of Moringa trees recorded the content of myricetin compound amounted to 4.714 moles L^{-1}, which did not differ significantly with the treatment of 10 mg L^{-1} chitosan in the callus content of the myricetin compound, which amounted to 4.895 moles L^{-1}. While the treatments of silver nanoparticles 20 and 30 mg L^{-1} recorded the lowest callus content from myricetin compound, which was 1.046 and 0.668 moles L^{-1}, respectively (Figure 10).

The results indicate an increase in myricetin production from callus tissue when chitosan and silver nanoparticles are added to the MS media as chemical stimulants. Perhaps the reason is that chitosan is a carbohydrate compound and that carbohydrates contribute to the production of plant secondary products. They are considered an important source of energy, and this is consistent with the results found by Ibrahim (2017). The decrease in the callus content of myricetin at a concentration of 10 mg L^{-1}-chitosan compared to the optimum concentration of 5 mg L^{-1} chitosan is because the increase in carbohydrates causes an increase in osmotic pressure than the normal level in cells that were cultured by in vitro, which reduces bioactivity processes and secondary metabolism activity within cells (Xu et al., 2003). The results of adding silver nanoparticles at a concentration of 10 mg L^{-1}, which stimulated an increase in the production of the myricetin compound, agree with the results obtained by Al-Obaidi (2016) when studying the hopbush (*Dodonaea viscosa* L) plant. The results of the decrease in the production of the secondary compound myricetin when increasing the concentration of silver nanoparticles from the optimum level is due to the increased stress on the cells in the callus tissues, which led to a decrease in the activity of the bioactivity processes of the cells. These results did not agree with the results of other studies. They found that there was a positive increase in the secondary compounds in the callus of the cucumber (*Cucumis sativus* L.) and bamboo (*Guadua angustifolia* Kunth.) plants with an increase in the concentrations of silver nanoparticles that were added to the nutrient medium (Vasudevan et al., 2004; Jimenez et al., 2006).

Conclusion

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The increase in the concentration of benzyl adenine that was added to the MS medium led to an increase in the response of the nodule stem segments to the induction of callus of the Moringa tree after four weeks of culturing. The treatments of chitosan and silver nanoparticles that were added to the MS media increased the stimulation of callus tissues to produce myricetin compound after five weeks of culturing.

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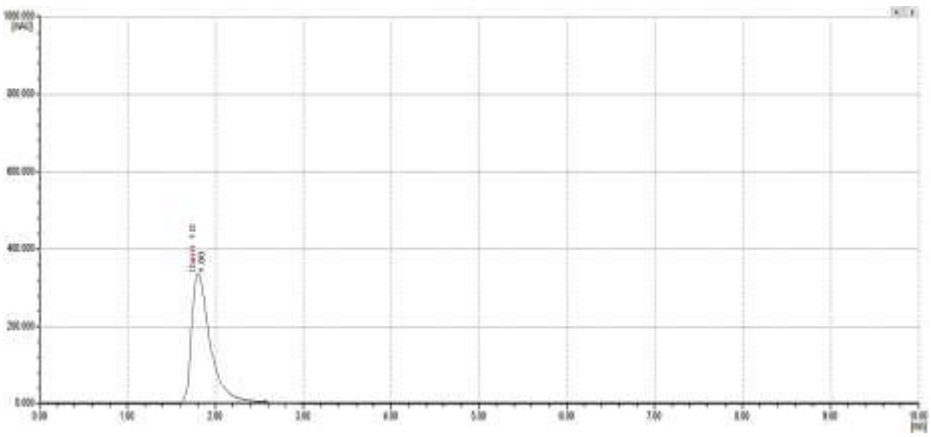

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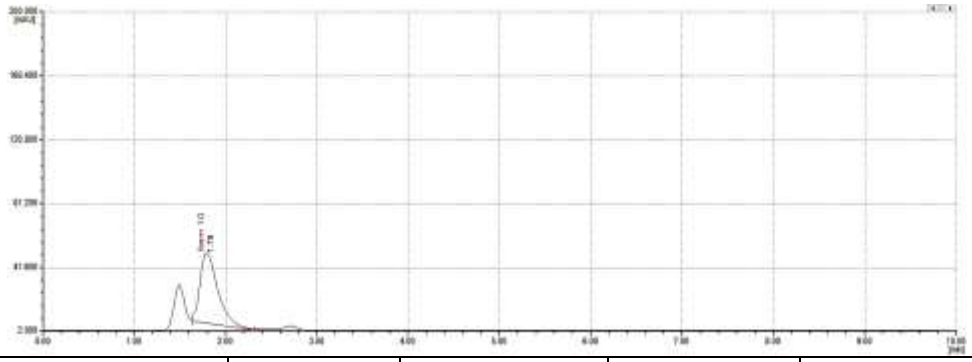
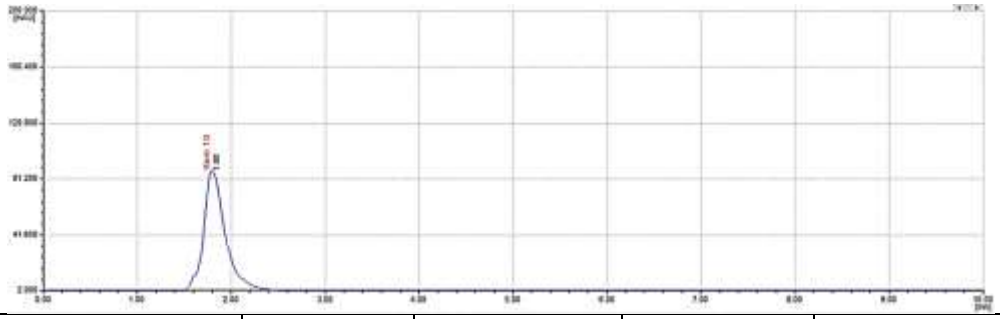
Treatment	Compound	Retention time	Peak area	Molecular weight
Myricetin standard	Myricetin	1.80	502288.1	318.23

Figure 1: Curve of the standard sample of the myricetin compound.



Treatment	Compound	Retention time	Peak area	Molecular weight
Leaves of moringa tree	Myricetin	1.79	60374.2	318.23

Figure 2: Curve of the leaves sample of the Moringa tree.



Treatment	Compound	Retention time	Peak area	Molecular weight
Control treatment	Myricetin	1.80	135057.1	318.23

Figure 3: Curve of the control treatment of the callus.

Treatment	Compound	Retention time	Peak area	Molecular weight
5 mg L^{-1} chitosan	Myricetin	1.85	1018910.5	318.23

Figure 4: Curve of the 5 mg L^{-1} chitosan treatment of the callus.

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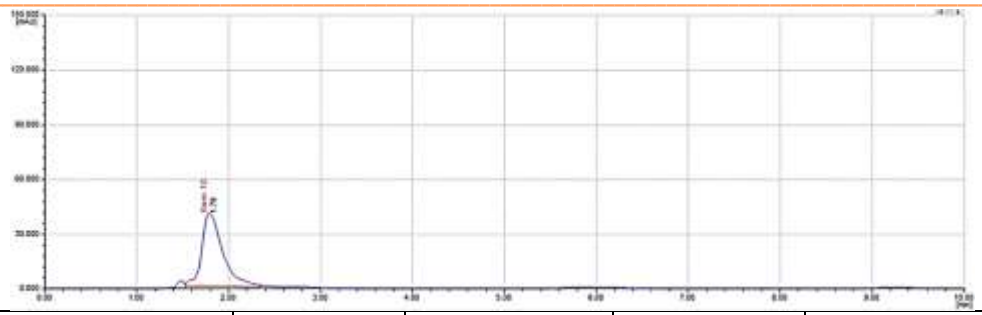
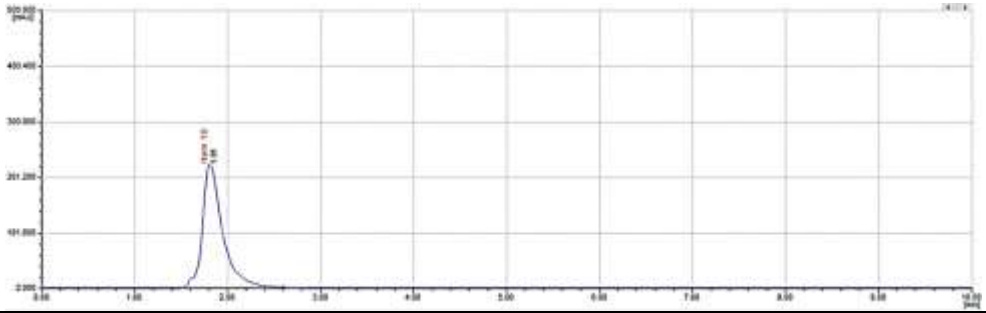


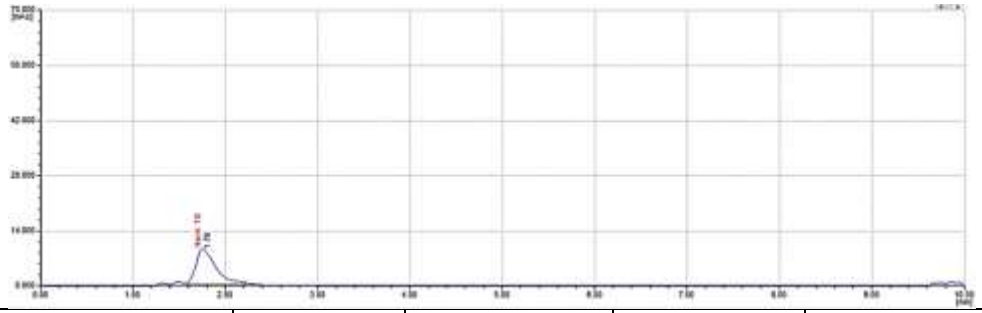
Treatment	Compound	Retention time	Peak area	Molecular weight
10 mg L^{-1} chitosan	Myricetin	1.79	62691.3	318.23

Figure 5: Curve of the 10 mg L^{-1} chitosan treatment of the callus.



Treatment	Compound	Retention time	Peak area	Molecular weight
10 mg L^{-1} silver NPs	Myricetin	1.81	337889.5	318.23

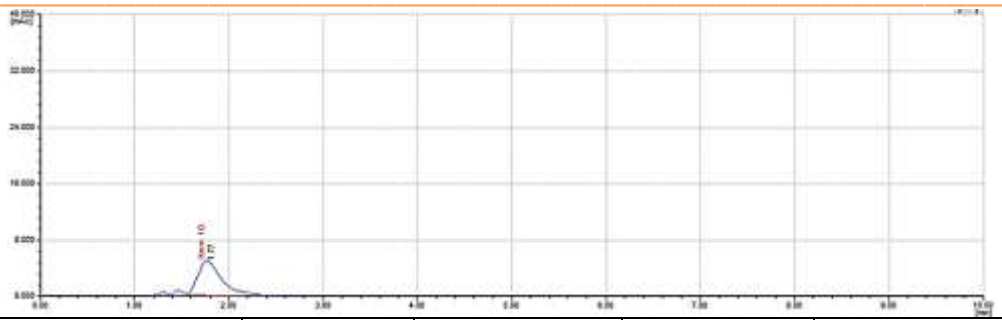
Figure 6: Curve of the 10 mg L^{-1} silver NPs treatment of the callus.



Treatment	Compound	Retention time	Peak area	Molecular weight
20 mg L^{-1} silver NPs	Myricetin	1.76	13409.6	318.23

Figure 7: Curve of the 20 mg L^{-1} silver NPs treatment of the callus.

ISSN NO: 2771-8840
Date of Publication: 24-11-2022



Treatment	Compound	Retention time	Peak area	Molecular weight
30 mg L^{-1} silver NPs	Myricetin	1.77	8557.7	318.23

Figure 8: Curve of the 30 mg L^{-1} silver NPs treatment of the callus.

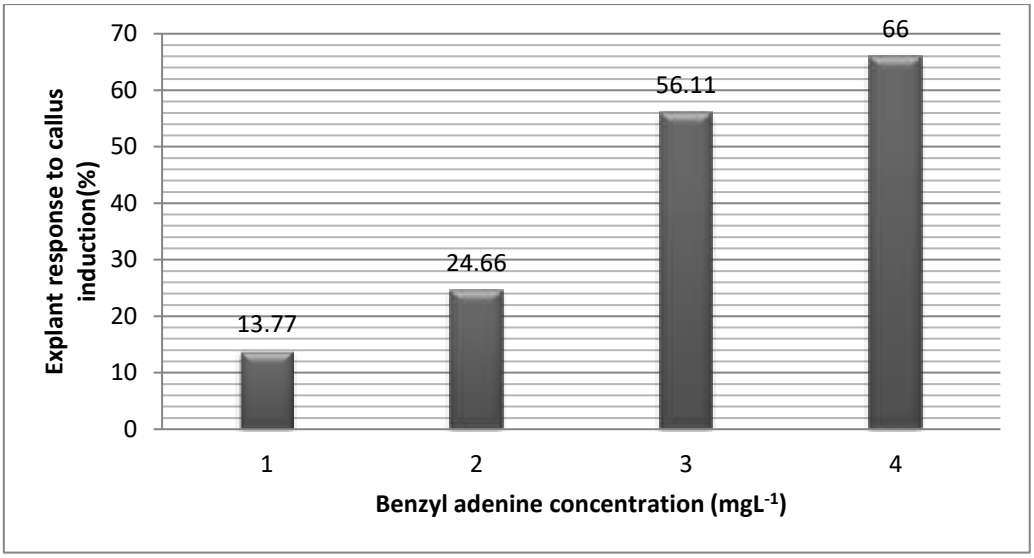


Figure 9: Effect of different concentrations of benzyl adenine on explant response to callus induction in moringa tissue (LSD p≥0.01 = 8.791).

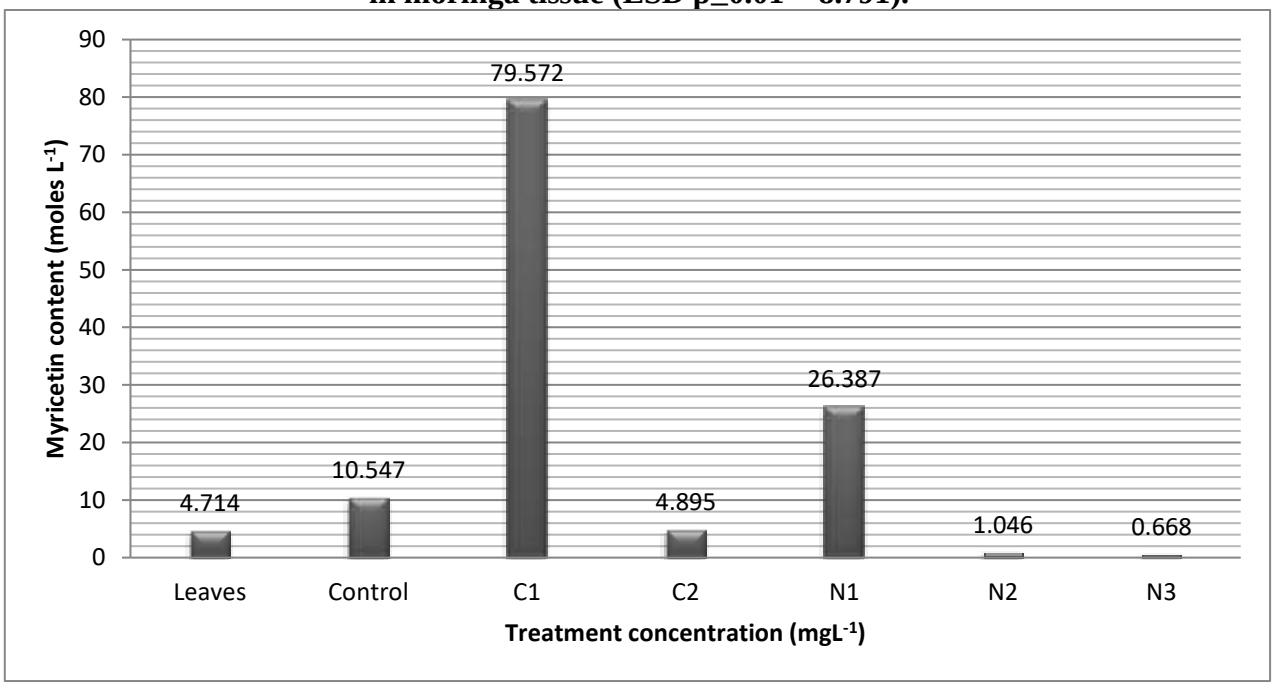


Figure 10: Effect of different concentrations of chitosan or silver NPs on myricetin content in callus and leaf of moringa tree (LSD p≥0.01 = 0.091).

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C1: 5 mg L^{-1} Chitosan; C2: 10 mg L^{-1} Chitosan; N1: 10 mg L^{-1} Silver NPs; N2: 20 mg L^{-1} Silver NPs; N3: 30 mg L^{-1} Silver NPs.