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Article

Effect of Growth Regulators and $AgNO_3$ NPs on the Leaves Extract of *Stevia rebaudiana* in the Production of Bioactive Compounds in Vitro

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Abstract: The experiment was conducted in the graduate laboratory, Department of biology, College of Science, Al-Qadisiyah University, to study the effect of different concentrations of Clorx on sterilizing explanted on the growth response , and study of the effect of different levels of plant hormones and AgNO3 NPs on the production of active compounds. Used concentrations of (8, 10, and 12%) of Clorx in sterilization explanted where grown in MS medium at a concentration of 4.44 g with sucrose 30 g and agar 7 g and incubated in the growth room at a temperature of 25 °C. The results were recorded after 40 days. The results showed that the response rate of the growths was higher at a concentration of 10%, and this percentage was ideal for sterilization, as the response rate was recorded. 72%, while the contamination rate in the samples was 12%, while the chlorine concentration was 8%, it recorded a growth rate of 78%, but the contamination rate was high if it was recorded at 28%, while the concentration was 12%, the growth response rate was low, as it was recorded at 48% and the contamination rate was 10%. Plant Growth Regulators and AgNO3 nanoparticles were added to the medium at different concentrations. The results of the GC-MS analysis showed a group of compounds of medical importance, including n-Hexadecanoic acid, Octadecanoic acid, Cycloheptasiloxane, Diethyl Phthalate, Phytol, and Propanoic acid. The The compounds n-Hexadecanoic acid, Octadecanoic Acid, and Cycloheptasiloxane showed the highest ratio in the treatment (4 NAA, 2 Kin, 3 AgNO3 NPs), while the compound Diethyl Phthalate achieved the highest ratio in the treatment (2 NAA, 1 Kin, 6 AgNO3 NPs). The compound Phytol showed the highest ratio in the treatment (2 NAA, 1 Kin, 0 AgNO3 NPs), and the highest ratio for the compound Propanoic acid was observed in the treatment (4 NAA, 2 Kin, 6 AgNO3 NPs).

Keywords: Stevia Rebaudiana Bertoni, Agno3 Nps, In Vitro.

Introduction

Medicinal plants known as medicinal herbs have been used in traditional medicine since ancient times, as they produce various types of chemical compounds (plant substances) that have many biological roles that include defense against bacteria, fungi, viruses, insects, and infectious diseases. Mammalian herbivory [1]. These compounds have biological properties such as antioxidants,

antimicrobial activity, and preparation of food detoxification enzymes [2]. However, the amounts of these phytochemicals vary from plant to plant based on genetic, ontogenic, morphological and environmental factors [3].

The stevia belongs to the Compositae and is called (sugar bush), which means sugar tree. As for its scientific name, it is ***Stevia rebaudiana Bertoni*** [4]. Of the 230 species in the Stevia genus, only rebaudiana and phlebophylla species produce steviol glycosides [5].

Stevia is one of the medicinal plants that has many therapeutic benefits, including anti-hyperglycemic effects in the blood and anti-hypertensive effects [6]. also protects against obesity and tooth decay [7] , and has properties that prevent fatigue. Depression, and yeast infection. It dilates blood vessels, improves taste, is anti-fungal, anti-bacterial, and increases urination [7]. These positive effects highlighted the importance of the stevia plant[6] . Stevia shows therapeutic benefits as a common factor in chronic diseases including obesity, fatty liver, cardiac fibrosis, liver fibrosis, cirrhosis, inflammatory bowel disease, colitis, and some others [8] .

Stevia leaf extract contains many compounds that have different activities, as was shown by the detection conducted by [9] on the ethanolic extract of stevia leaves using gas chromatography and mass spectrometry (GC-MS). The results showed the presence of 40 compounds in it based on the retention time. And the relative surface area, the main metabolic by-products identified were Oleic acid, n-Hexadecanoic acid, Octadecadienoic acid, Phytol, Isosteviol, Hydroxydehydrostevic acid, 1-Heptatriacotanol, Vitamin E and Campesterol. also conducted detection of ethanol extract of Stevia to determine the chemical components using gas chromatography and mass spectrometry (GC-MS) [10]. The analysis led to the identification of a group of compounds present in ethanol extract, the most important are:

Benzene, 1,3-dimethyl, 1-Hexene, 2,4,6-tris-trimethylsilyl, Methyl farnesoate, Hexadecanoic acid methyl ester, Pentadecanoic acid methyl ester, Nonyl pentyl ether, 1,2-Benzene dicarboxylic acid, dibutyl ester, Tricosanol, Heptacosane, Nonacosane, and Octacosane.

Materials and Methods

2.1. *Prepare MS Media*

Prepare the medium and sterilize it MS plant growth medium was used (Murashige and Skoog 1962). Then add sucrose 30 g/l. The volume was completed to 1 liter by adding distilled water, then the pH was adjusted to 5.7 using NaOH or HCl, then 8 g/L of agar was added, the medium was placed on a Hotplate with a magnetic stirrer, then the medium was distributed at a rate of 10 ml into glass bottles. . The media was sterilized using an Autoclave device at a temperature of 121°C and a pressure of 15 kg/cm² for 20 minutes. The medium is then left at room temperature to cool and is ready for planting the explants.

The volume was completed to 1 liter by adding distilled water, then the pH was adjusted to 5.7 using NaOH or HCl, then 8 g/L of agar was added, the medium was placed on a Hotplate with a magnetic stirrer, then the medium was distributed at a rate of 10 ml into glass bottles.

2.2. *Sterilization Explant*

Leaves and apex and lateral buds of stevia seedlings were collected as plant parts for in vitro cultivation. The explants were washed well with running tap water for 15 minutes. After washing, the explants were soaked in 70% ethanol for 30 seconds. Surface sterilization was performed. Surface sterilized explants were cut into small pieces using a sharp sterile scalpel and plated. Whole explants were treated with different concentrations (8, 10, 12 %) of Clorox (15% NaOCl) for 20 min to avoid contamination. After surface sterilization, explants were washed four times with distilled water to properly eliminate Clorox within the laminar airflow. all laboratory tools, such as Petri dishes, forceps, scissors, scalpels, and conical flasks, were sterilized by autoclave at a temperature of (121) degrees Celsius for (20) minutes at a pressure of (15) psi. Square. All experimental experiments were carried out under a laminar air flow cabinet of a laminar air flow after sterilization using 70% ethanol as the sterilizing agent.[11].

2.3. Culture Explained

After the sterilization process, the plant parts were transferred to laminar airflow to be cut into small pieces inside sterile petri dishes, then planted inside the bottles containing the medium. After completing the cultivation of all the parts, they were transferred to the growth room to be incubated at temperature 25±2 °C and were distributed into two groups, one incubated under conditions of exposure to light and the other in the dark. After six weeks, the results were recorded and statistical analysis (CRD) [12]

Results and Discussion

1- Occupational ***Sterilization***

shows the table (1) the use of sodium hypochlorite at certain concentrations to sterilize the plant shoot due to the presence of an influence on the effect of In plant growth.

It shows that the growth rate of the organs was at a concentration of 10%, and this is the next percentage for sterilization, where the effect rate was recorded. 72%, after the contamination rate in the dimension is 12%, the concentration of sodium hypochlorate 8% actually recorded 78%, but the contamination rate is not high if it was recorded as 28%, while the concentration is 12%. %, especially for members of the low forum, where it was recorded at 48% and the pollution rate was 10%.

Table 1. Effect of using sodium hypochlorite on plant growth.

Dilution of sodium hypochlorite	Response	Contaminate
8	78%	28%
10	72%	16%
12	48%	10%

2- GC-Mass analysis

The results indicate that the treatment effect causes variations in all detected bioactive compounds (Table 2). GC-Mass analysis reveals qualitative and quantitative variations in the bioactive compounds of the alcohol extract from the shoot of *Stevia rebaudiana Bertoni* , with the shoot containing many of the most important bioactive substances (Figures 2-7).

Shown through analysis that the compound **Propanoic acid** achieved the highest relative area ratio to the treatment(4 NAA 2 Kin 6$AgNO_3$ NPs) , which was ratio (12.37 %) , also achieved a compound **Cycloheptasiloxane** highest percentage of treatment(4 NAA 2 Kin 3 $AgNO_3$ NPs) which was (3.32 %) , also compound **Diethyl Phthalate** reached its highest percentage in treatment (2 NAA 1 Kin 6 $AgNO_3$ NPs) It was (5.07 %) , also compound **Hexadecanoic acid** was highest ratio of treatment (4 NAA 2 Kin 3 $AgNO_3$ NPs) was (17.27 %) , as for the compound **Phytol** was highest level (2 NAA 1 Kin 0 $AgNO_3$ NPs) it reached the rate (2.81 %) ,

also achieved a compound **Octadecanoic Acid** of treatment (4 NAA 2 Kin 3 $AgNO_3$ NPs) it reached the rate (7.79 %).

Table 2. Relative area of bioactive compound of alcohol extract from the shoot of *Stevia rebaudiana Bertoni* from different combination treatments $AgNO_3$ NPs and (NAA, Kin) hormones (mg).

A : 0 NAA 0 Kin 0 $AgNO_3$ NPs	**B**: 2 NAA 1 Kin 0 $AgNO_3$ NPs	**C**: 4 NAA 2 Kin 0 $AgNO_3$ NPs
D: 0 NAA 0 Kin 3 $AgNO_3$ NPs	**E** : 2 NAA 1 Kin 3 $AgNO_3$ NPs	**F** : 4 NAA 2 Kin 3 $AgNO_3$ NPs
G : 0 NAA 0 Kin 6 $AgNO_3$ NPs	**H** : 2 NAA 1 Kin 6 $AgNO_3$ NPs	**I** : 4 NAA 2 Kin 6 $AgNO_3$ NPs

compounds	R T	Area %								
		A	B	C	D	E	F	G	H	I
Eugenol	**15.132**	1.915	0.394	2.651	4.837	2.147	2.170	2.062	3.037	8.016
Hexadecanoic acid	**21.982**	4.667	3.815	2.666	13.39	4.209	2.126	6.124	6.137	12.93
Stigmasterol	**32.225**	0.943	3.955	2.097	-	4.221	1.784	2.034	1.043	-
Diethyl Phthalate	**18.109**	1.704	1.561	2.459	2.771	0.212	-	2.812	1.666	1.723
Cinnamaldehyde	**13.954**	-	0.386	0.300	-	0.224	0.327	0.175	0.447	1.596
Anethole	**14.126**	0.680	0.833	0.849	1.258	0.985	0.890	0.939	0.956	1.973
Octadecanoic Acid	**23.592**	5.928	0.538	1.275	13.87	0.475	1.987	7.505	8.593	19.00
Pentadecanoic acid	**21.589**	1.366	1.294	1.565	1.848	1.203	1.856	1.813	0.414	2.219

Discussion

The results showed a clear effect in increasing the percentage of some active compounds as a result of treatment with plant hormones and NO_3Ag Nps . the treatment 4 NAA 2 Kin 6 $AgNO_3$ NPs had a significant effect on the rate of a large number of compounds.

These results are similar to those obtained in study [13] , It was shown that the use of silver nanoparticles (Ag NPs), either alone or in combination with naphthalene acetic acid (NAA), has stimulating effects on callus proliferation, growth, and the accumulation of fresh and dry biomass. Growth media supplemented with nanoparticles (NPs) resulted in 100% callus proliferation treatment to media supplemented with NAA, thus increasing the percentage of active compounds.

Some studies suggest that treating plants with nanomaterials has led to an increase in phenol production [14]. It has also been shown that nanoparticles can stimulate signaling through reactive oxygen species (ROS), which act as signaling molecules to enhance secondary metabolism in plants. Reactive oxygen species are considered to be molecules that help activate this secondary metabolism [15].

Regarding the addition of cytokinins and auxins and their effect on increasing the production of active compounds, as shown in the tables (2), growth regulators significantly influence the percentage of active compounds. This is due to the provision of optimal conditions for plant cell growth, which enhances the synthesis and accumulation of active substances [16], This result aligns with what researcher has [17].

They also promote cell division and growth, leading to increased production of amino acids and proteins, thereby boosting the biosynthesis of active compounds .[18]

Additionally, growth regulators play a role in enhancing enzymatic activity and contribute to increased cell size by promoting cell division .[19].

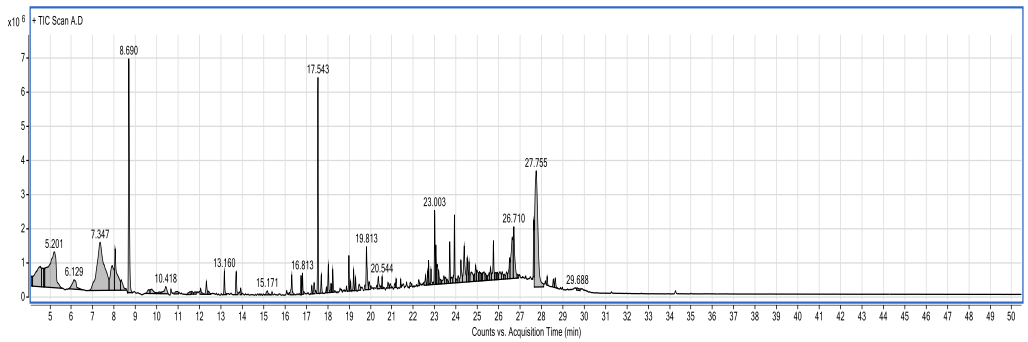


Figure 1. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 0 NAA 0 Kin 0 AgNO3 NPs.

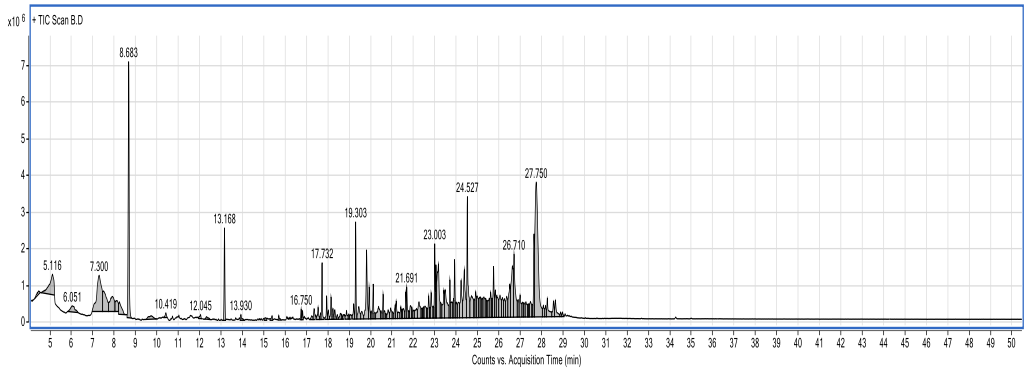


Figure 2. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 2 NAA 1 Kin 0 $AgNO_3$ NPs.

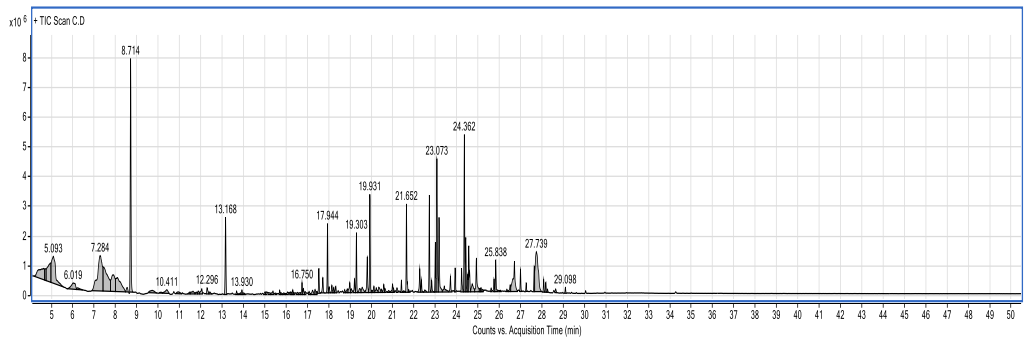
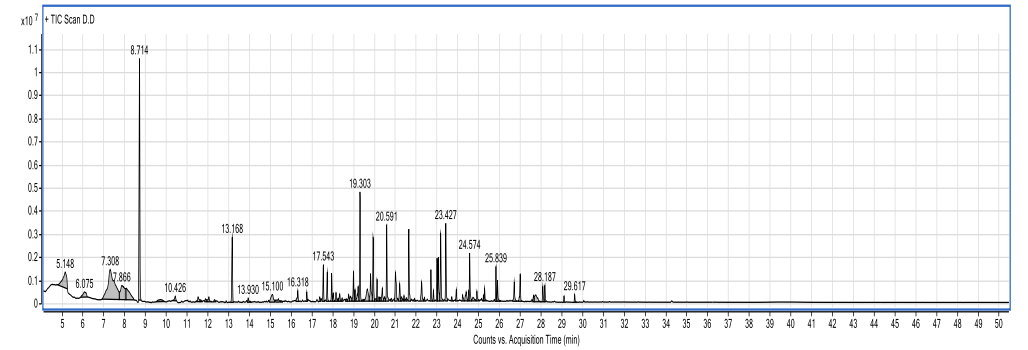


Figure 3. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 4 NAA 2 Kin 0 $AgNO_3$ NPs.



Figur 4. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 0 NAA 0 Kin 3 $AgNO_3$ NPs.

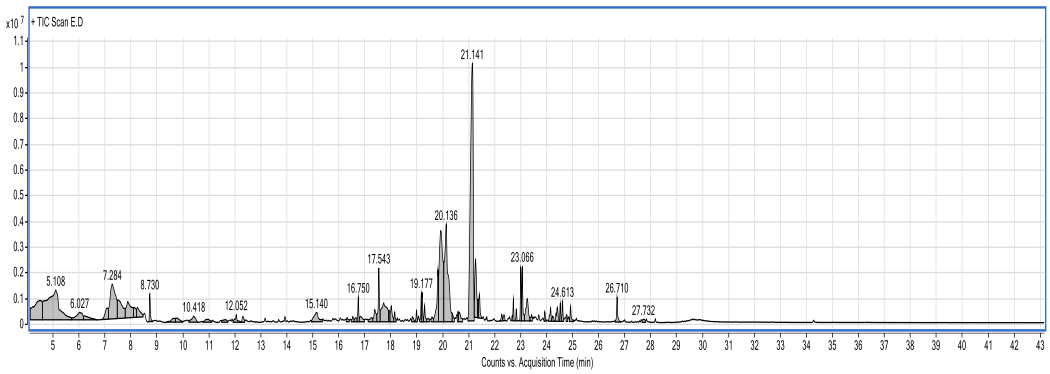


Figure 5. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 2 NAA 1 Kin 3 $AgNO_3$ NPs.

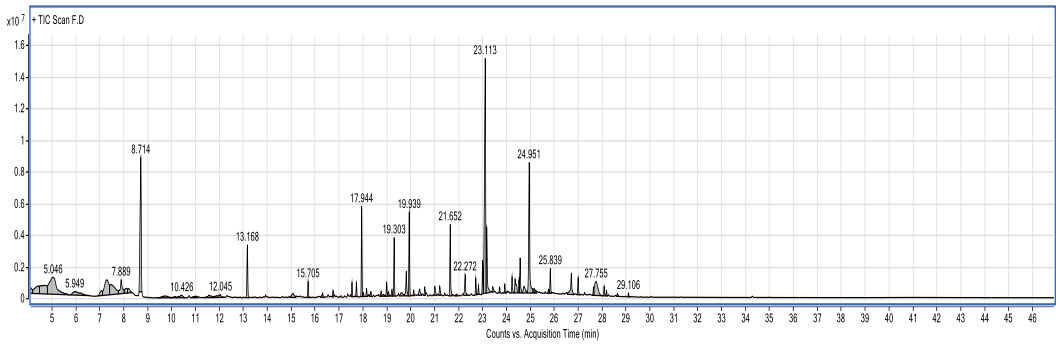


Figure 6. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 4 NAA 2 Kin 3 $AgNO_3$ NPs.

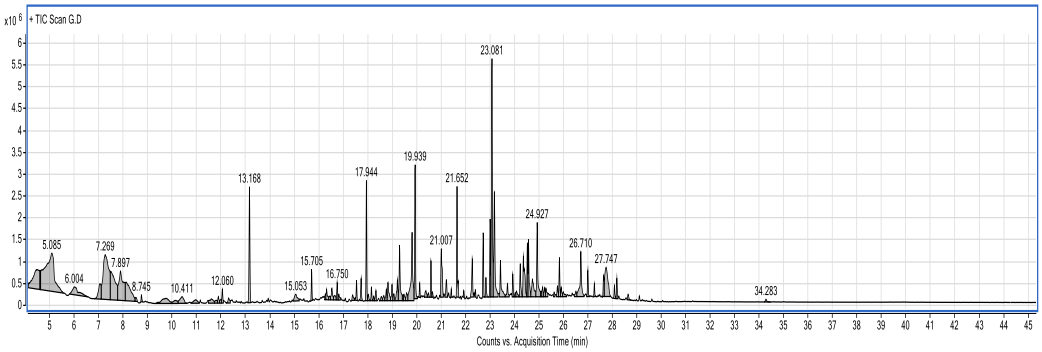


Figure 7. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 0 NAA0 Kin 6 $AgNO_3$ NPs.

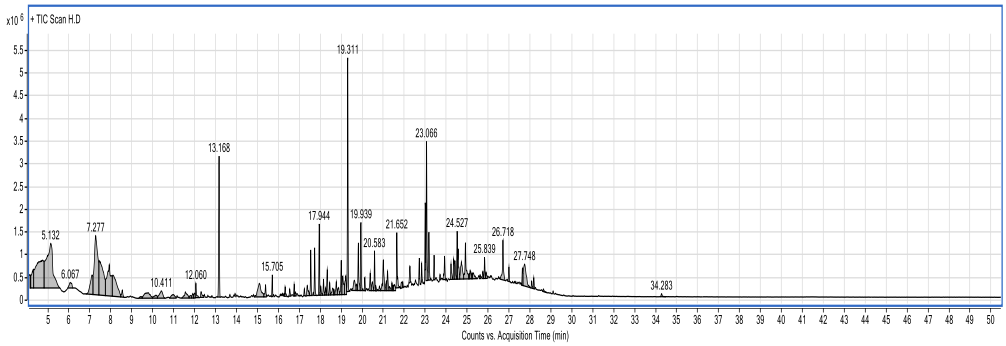


Figure 8. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 2 NAA 1 Kin 6 $AgNO_3$ NPs.

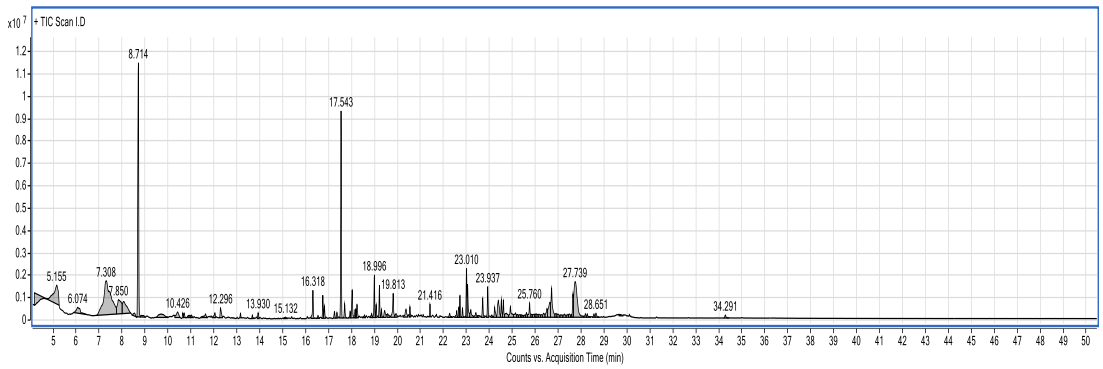


Figure 9. GC-MS chromatogram of alcohol extract from shoot of ***Stevia rebaudiana Bertoni*** from combination treatment of 4 NAA 2 Kin 6 $AgNO_3$ NPs.

Conclusion

This study explored the effect of growth regulators and $AgNO_3$ nanoparticles on the bioactive compound production in Stevia rebaudiana in vitro. The sterilization process, using different concentrations of Clorox, showed that a 10% concentration yielded the best growth response with minimal contamination. The addition of plant growth regulators (NAA and Kin) and $AgNO_3$ nanoparticles significantly influenced the production of key bioactive compounds, as confirmed by GC-MS analysis. Notably, treatments with 4 NAA, 2 Kin, and 3 $AgNO_3$ NPs exhibited the highest production of n-Hexadecanoic acid and Octadecanoic acid, which are compounds of medicinal importance. The study demonstrates that integrating nanomaterials with plant hormones can enhance secondary metabolite production, highlighting the potential of using these treatments in the agricultural and pharmaceutical industries to optimize the therapeutic benefits of Stevia. Future research should explore the underlying mechanisms and optimize these treatments for larger-scale production.

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