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Green biosynthesis of magnetite nanoparticles via neem extracts effect on molecular and biochemical *Stevia rebudiana* callus

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Abstract:
Background: *The present study describes the successful green biosynthesis of magnetite nanoparticles (Fe_3O_4 NPs) as a benign and eco-friendly material using a non-toxic leaf extract of Azadirachta indica as a reducing and stabilizing agent.*
Materials and Methods: *A study was held for comparing between aqueous neem plant extract, magnetite chemically nano-engineered (MC NPs) and green biosynthesized nanoparticles (MG NPs) impact on the stevia callus biomass and bioactive constituents.*
Results: *The highest mean value of callus fresh biomass and dry weight was enhanced by chemically magnetite NPs 4 mg/L followed by magnetite NPs 2 mg/L and the last magnetic treatment also possess highest glutathione content and lowest malondialdehyde pool when compared to control and green synthesized magnetite. The maximum antioxidant activity was determined by neem extract 4%. On contrary, GC-MS analysis revealed the presence of 54 active compounds in chloroform callus extract but the major compounds identified were 29 compounds and all treatments had a positive effect on the accumulation of active compounds and area percent. The maximum area percent was recorded at all treatments when stevia callus treated with green bioengineered magnetite NPs at 4 mg/L which is Phenol,2-[[5-(2-methyl-3-benzofuranyl)-1H-pyrrol-2-yl][5-(2-methyl-3-benzofuranyl)-2H-pyrrol-2-ylidene]methyl]-, acetate (ester). The HPLC separation of the sweet bioactive constituents (Stevioside and Rebaudioside-A) showed that, the bioengineered green magnetite NPs 4 mg/L enhanced the highest level of biosynthesis of both active ingredients. In the molecular level, we used five simple sequences repeats (SSR) to evaluate genetic action against the different NPs treatments using seven treatments included control showed 90% polymorphism with primer SSR4 and the highest number (50%) of genetic stability GTS showed in treatment with magnetite NPs biogenic 4 mg/L. On the other hand the polymorphism information content (PIC) was enabled to measure of allelic variability and evenness at a particular locus, PIC values was ranged from 0.467 to 0.712.*
Conclusion: *Green synthesis of magnetic NPs 4 mg/L succeeded in the increasing of bioaccumulation of bioactive constituents in stevia callus and enriched with antioxidants like glutathione with higher genomic template stability.*
Keywords: Stevia rebudiana, Green nanosynthesis, Bioactive compounds, GC-MS, Antioxidants, SSR-DNA, Genomic template stability.

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I. Introduction

The construction of nanoscale iron oxide NPs such as magnetite Fe_3O_4 with unique characteristics like biocompatibility, physiological environment stability, size-dependent magnetic properties, superparamagnetism, high coercively, high saturation magnetization, and low cytotoxicity **(Laurent et al. 2008, Chin et al. 2011)** opens up the window for vast array from applications especially for the secondary metabolites election in plant tissue culture field.Only magnetic NPs have been approved for clinical use by the Food and Drug Administration (FDA). The co-precipitation methods, micro-emulsion methods, sol-gel methods, solvo-thermal methods, thermal decomposition methods, and hydrothermal methods have all been used to produce iron oxide NPs using physical and chemical approaches **(Neuberger et al. 2005, Hasany et al. 2012, Wang et al. 2013, Wannoussa et al. 2015, Zhuang et al. 2015, Liu et al. 2019)**. The chemical synthesis of iron oxide NPs, on the

other hand, has drawbacks such as the use of hazardous chemicals, the formation of hazardous byproducts, and contamination from precursor chemicals (**Thakkar et al. 2010**) are highly reactive in nature and tend to aggregate, resulting in a loss of reactivity (**Song and Carraway, 2005**), and the resulting iron oxide NPs have limited application in biological systems due to low biocompatibility **(Khatami et al. 2018).** Furthermore, conventional methods for synthesizing iron oxide NPs, such as thermal decomposition and hydrothermal processes, needed high temperatures, high pressure, and significant volumes of hazardous and expensive chemical solvents (**Mahmoud et al. 2011)**. As a consequence, there is a significant need to develop nanoparticle manufacturing technologies that are clean, nontoxic, and ecologically benign. The research of eco-friendly and green synthesis for the production of NPs based on plant extracts has acquired considerable attention due to contemporary sustainability concerns. Green synthesis is getting prominence because: (1) it uses clean and environmentally friendly methods such as water; (2) it can scale up (3) it does not require high energy or high pressure, resulting in significant energy savings and (4) the overall synthesis process is cost-effective because the active biological component can act as a reducing and capping agent **(Klaus et al. 1999, Bansal et al. 2004, Senapati et al. 2005).**

Azadirachta indica which is commonly known as Neem is a member of the Meliaceae family and is widespread in Malaysia. It is well-known for its many uses, particularly its therapeutic properties. Terpenoids and flavanones are phytochemicals found in Neem that act as a reducing and capping agent, as well as , assisting in the stabilization of NPs. The primary constituents of *Azadirachta indica*, according to extensive literature, are nimbin, nimbidin, ninbidol, gedunin, sodium nimbinate , quercetin, salannin, and Azadirachtin **(Linton et al. 1997, Nisbet et al. 2001, Biswas et al. 2002, Gupta et al. 2017, Benelli et al. 2018, Chutulo and Chalannavar, 2018, Saleem et al. 2018).**We present a one-pot reaction, simple, safe, and environmentally friendly method based on *Azadiratcha indica* extract, a non-toxic and readily available substance, and sodium hydroxide (NaOH) as the alkaline medium. Transmission electron microscopy, zetasizer were used to characterize the produced magnetite NPs. By comparing chemically synthesized Fe_3O_4 NPs and green biosynthesized Fe_3O_4 NPs may have better properties, such as superior biocompatibility and biodegradability (**Mahdavi et al. 2013).**

Plant tissue culture is used to produce secondary metabolites due to its reliability and predictability, independence from geographical, seasonal and environmental factors, modification or elimination of unwanted taste, and production of high quality and standard product **(Abd El-Salam et al. 2015).** There are several strategies to improve the production of secondary metabolites in plant tissue and cell culture **(Bednarek et al. 2020).** One of these strategies is the use of elicitors. Elicitors are compounds that stimulate the production of secondary metabolites by biochemical changes in the plant **(Namdeo 2007).** Elicitors do this by activating the signal transduction cascades **(Karalija et al. 2020).** The production of secondary metabolites can be enhanced by using different elicitors, either biotic or abiotic, which act as stress agents and enhance the production of secondary metabolites in plant callus. From these elicitors are the green nanoengineered magnetite NPs from neem plant extract in stevia callus. There are many researches used magnetite nanoparticles **(Afshar *et al*., 2013, El-Saber et al. 2021a and El-Saber et al. 2021b)** on plant species**.** However, the production of active constituents in tissue culture (like *Stevia rebaudiana* L.) using nanotechnology technique takes center late (or limited) in the uses list of this science.

Stevia (*Stevia rebaudiana*) is a kind of Bertoni and a perennial herbaceous plant belonging to the Asteraceae family **(Yadav et al. 2011)**. Stevia's natural range includes the southwestern United States and the Brazilian highlands **(Soejarto et al. 1983).** There are around 300 species in the stevia genus **(Goyal et al. 2010).** Biologically active chemicals are primarily found in plants' leaves which used as a sweetener throughout Japan, Korea, China, and South America **(Tadhani et al. 2007).** It is considered to be a valuable medicinal plant native to Paraguay, where the climate is subtropical and rainfall averages 1500 mm per year **(Hajihashemi and Ehsanpour, 2013)**. Stevia is in growing demands in the food and beverage sector as a high-potency, low-calorie natural sweetener. Micropropagation of callus and boosting bioactive ingredients is a worldwide requirement nowadays days due to an increase in demand for Stevia's sweet herb. Steviol glycosides (the bioactive constituent of stevia) are sweet chemicals produced mostly in the leaves of *Stevia rebaudiana*. These compounds have been shown to have antioxidant action in addition to their sweet flavor **(Hajihashemi and Geuns 2013).**Stevia is a sugar substitute made from the leaves of the stevia plant. It's about 100 to 300 times sweeter than table sugar, but it has no carbohydrates, calories, or artificial ingredients. Stevia is natural, unlike other sugar substitutes. In South America and Asia, people have been using stevia leaves to sweeten drinks like tea for many years. The FDA approved only the purified form of stevia, called stevioside, as safe to use. Products considered safe contain words in their ingredient list such as stevia extract or *Stevia rebaudiana*. The FDA reported about the whole leaves extract that, they have no enough information about their potential impact on human being health, including kidney and cardiovascular problems **(https://www.webmd.com/food-recipes/what-is-stevia).**

Regarding genomic stability study, Microsatellite or simple sequence repeat (SSR) markers are very useful for plant breeding and genetic diversity studies for several reasons. SSR markers combine a number of

advantages for practical applications, as they are co-dominant and multi-allelic, stably inherited, amenable to automation and high-throughput analysis, highly variable, and detect the highest level of polymorphism per locus **(Röder et al., 2004).** They require only small amounts of DNA sample, are easy to amplify by polymerase chain reaction (PCR), are amenable to high-throughput analysis, and are largely co-dominantly inherited, multi-allelic, highly informative, and abundant in plant genomes **(Powell et al., 1996).** In the present investigation we used the SSR markers to investigate simple sequences repeats (SSR) to evaluate genetic action against the nanoparticle treatments using *Stevia* treatments samples to detect the polymorphic information content and levels of genetic stability value (GTS). On other hand, (PIC) for SSRs is an effective tool to measure of a marker's informativeness, different PIC values were obtained from marker studies using different genetic materials **(Khodayari et al. 2012).** This study was constructed to compare between the effect of neem extract with two concentrations, chemically synthesized magnetite and the biogenic nano-engineered form on enhancing growth, biochemical markers, eliciting the biosynthesis of secondary metabolites of *Stevia rebaudiana* callus. The behavior of the genetic material towards several sources of NPs was determined by the genomic template stability was also tested based on SSR PCR for DNA of callus.

II. Materials and methods

1. Preparation and characterization of magnetite NPs prepared by neem extract

Magnetite NPs were synthesized using neem extract as reducing and stabilizing agent Neem as previously reported in literature **(Zambri et al. 2019)** and figure (1), with some modifications. Briefly, neem extract 10% was heated at 70 ^{o}C and 0.16 g of $FeCl_3$ was added to the extract solution under stirring and left for about 1 h. To adjust the solution pH to basic environment, 5ml NaOH (1%) was added dropwise to the above solution. The mixture solution color turned from greenish to black color as indication of NPs of magnetite formation. The green magnetite NPs were evaluated using transmission electron microscope (TEM) and dynamic light scattering (DLS) and zeta potential to assess the particle size, shape, stability and surface charge.

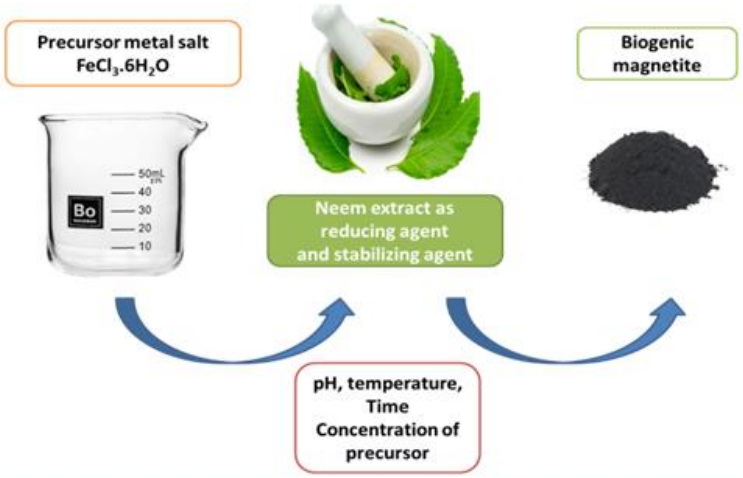


Fig.1. Green nano-synthesis of biogenic magnetite via neem extract

2. Preparation and characterization of magnetite NPs prepared by surfactant-free oxidation method:

The chemically fabricated magnetic NPs were according to **Nishio et al. (2007),** magnetite NPs were synthesized in an aqueous solution by a surfactant-free oxidation method with some modifications. In short, a sodium hydroxide solution (99.99%, Sigma-Aldrich, USA) about, 21mM in deionized water with intense stirring under the atmosphere of N_2. Sodium nitrate (99.99%, Sigma-Aldrich, USA) 8.80 mM was added as an oxidizing agent. Then, deaerated ferrous chloride aqueous solution (0.1 M) was added to the alkaline solution, which was kept at 60 °C for 24 h. The solution was then added to the alkaline solution. The magnetic NPs were separated and washed by centrifugation with deionized water. The resulting precipitates were dried under vacuum at 60°C. The chemically engineered magnetite NPs were evaluated using transmission electron microscope (TEM), dynamic light scattering (DLS) and zeta potential to assess the particle size, shape, stability and surface charge.

3. Plant material optimization

Stevia plants were obtained from Sugar Crop Institute, Agricultural Research Center, Giza, Egypt. The plants were maintained under greenhouse conditions of the Desert Research Center, Cairo, Egypt, for at least 30 days prior to removal of material for cultures. Leaves were removed from the branches and transferred immediately to the laboratory for sterilization. The leaves were washed for 15 mins in running tap water then rinsed in sterile distilled water and sterilized under aseptic conditions by immersion for 20 mins in 20% (v/v) commercial bleach (Clorox) followed by 3 mins in 0.1% (w/v) mercuric chloride solution then washed 6 times with sterile distilled water to remove the traces of mercuric chloride.

3.1. Callus initiation and proliferation

Leaves were excised and cut into small pieces (1cm), then cultured aseptically on solid Murashige and Skoog (MS) basal medium (Caisson, USA) **(Murashige and Skoog,1962)** supplemented with 100mg/L myo-inositol, 3% w/v sucrose and plant growth regulators were tested for callus induction. Naphthalene acetic acid (NAA) or 2,4- dichlorophenoxy acetic acid (2,4-D), at concentrations of 0.5,1.0, 1.5 and 2.0 mg/L, as auxins, and benzyl adenine (BA), at a concentration of 0.5mg/L as a cytokinin, in addition to MS medium without growth regulators. The pH of media was adjusted to 5.7-5.8 with 0.1 NaOH and 0.1N HCl prior to gelling with 2.8g/L phytagel.

Media were dispensed into large jars and sealed with autoclavable polypropylene caps and autoclaved for 15 min at 121^{o}C under 1.1 Kg/cm^{2} pressure, then left to cool and stored at room temperature till used. All cultures were incubated in an incubation room at a temperature 25+_ 2 ^{o}C under a 16/8-h (light/dark) photoperiod provided by cool-white fluorescent lamps (Toshiba) instead of tubes. Each treatment consisted of 10 replicates. Mean fresh and dry weights of callus and both color and texture of callus were recorded after eight weeks of culture.

3.2. Elicitation supplementation

After eight weeks callus was formed, then a piece of sub cultures callus (100mg) was placed on various concentrations of three elicitors Neem extract, Nano iron oxide and biogenic iron at a concentration 2, 4mg/L were added to medium. The control medium was made without elicitors. The samples of fresh stevia were collected after eight weeks from culture to determine growth parameters and some elicitors constituents.

3.3. Growth measurements:

Growth of callus was measured in terms percentage of callus percentage of callus induction (%), fresh and dry weight (mg). Fresh weights of callus were taken after removing the excess of moisture on the surface using blotting paper. Dry weight of callus was determined by drying in a hot air oven at 40^{o}C for 48hrs.

4. Biochemical markers

4.1. Total glutathione reduced

Glutathione reduced (GSH) is a tripeptide that interacts chemically with other free radicals in plant cells. GSH protects the membrane's structure by avoiding lipid peroxidation processes caused by acyl peroxides (maintains membrane integrity). As a substrate for glutathione-S-transferase enzyme and a precursor of phytochelatin, it aids in the detoxification of xenobiotics as a substrate for glutathione-S-transferase enzyme and a precursor of phytochelatines that function as heavy metals that bind peptides in plants. GSH is the main compound of non-protein thiol that is widely distributed in plants and animals **(Foyer et al. 1994)**. GSH was determined using the **Moron et al. (1979)** method, which involves reacting GSH with DTNB (5,5'-dithiobis nitro benzoic acid) to create a yellow product that absorbs at 412 nm.

4.2. Lipid peroxidation content

Reactive oxygen species degrade lipids, making them one of the most sensitive cellular components (by peroxidation of unsaturated fatty acids in biological membranes). The TBARS (Thiobarbituric Acid Reactive Substances) assay is a well-known method for determining lipid peroxidation. The level of lipid peroxidation in stevia callus samples was measured in terms of estimating the end product, MDA (malondialdehyde) **(Heath and Packer, 1968)** with some modifications. Stevia callus samples 0.5 g was homogenized in 2.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 14.000 rpm for 15 min. 5 % thiobarbituric acid (TBA, 2.0 mL) was mixed with 20% TCA solution and the mixture was added to 0.5 mL of the liquid supernatant of leaf sample. The mixture was heated at 95 ° C for 30 mins in a water bath and then incubated in an ice bath for 5 mins. After centrifugation the supernatant was read at 532 nm. The value for nonspecific turbidity of each sample at 600 nm was also recorded and subtracted from the absorbance recorded at 532 nm. The concentration of MDA-TBA adduct was calculated from MDA standard curve and converted to nmol g^{-1} fresh weight.

4.3. Antioxidant capacity

The following procedure was used to test the scavenging activity of stevia callus extract to synthetic radical DPPH (2,2-di-phenyl-1-picrylhydrazyl): in brief, 0.5mL of stevia fresh callus methanolic extract was combined with 2 mL of a 0.004 % solution of DPPH in methanol 80%. The reaction mixture was vortexed and left in the dark for 30 mins at room temperature. Spectrophotometry was used to determine the wavelength of mixture absorption, which was 517 nm according to (**Oktay et al., 2003)**. The ability to scavenge DPPH radical was calculated by the following formula:

$$RSC\ \%= (A_{blank} - A_{sample}) / (A_{blank}) \times 100$$

where (RSC %)= DPPH radical scavenging activity (%) , Abs $_{blank}$ is the absorbance of DPPH radical + methanol (2 mL DPPH and 0.5 mL methanol).

5. Identification of active constituents by GC-MS

After 30 days of culture, the samples of fresh *Stevia rebudiana* L. callus were collected to determine some growth parameters and active constituents by GC-MS **(Thomas et al., 2013).**

5.1. Extraction of active constituents

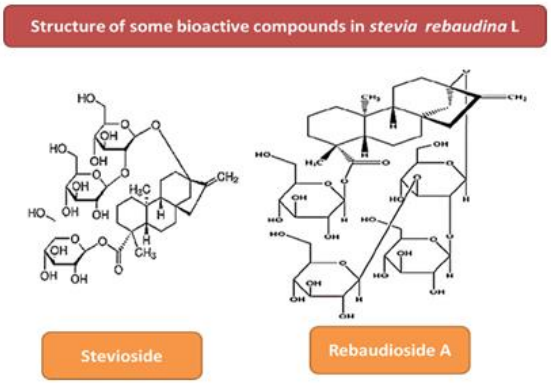
For gas chromatography- Mass Spectrometry (GC-MS) profiling, the collected callus was washed thoroughly with water to remove dirt. They were dried in the shade and ground into a powder using a grinder. The dried material (5 g) was extracted using chloroform (anhydrous, ≥99%, containing 0.5-1.0% ethanol as a stabilizer) as solvent. Then the extract was evaporated to dryness and finally weighed.

5.2. GC-MS Conditions

GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG -5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm path length). An electron ionization system with ionization energy of 70 eV was used for GC/MS detection. Helium gas with a constant flow rate of 1 mL/min was used as the carrier gas. The temperature of the injector and MS transfer line was set at 280 °C. The oven temperature was programmed from an initial temperature of 50 °C (hold for 2 min) to 150 °C at an increasing rate of 7 °C/min, then to 270 at an increasing rate of 5 °C/min (hold for 2 min), and finally to 310 as the final temperature at an increasing rate of 3.5 °C/min (hold for 10 min). Quantification of all identified components was investigated using percent relative peak area. Preliminary identification of the compounds was performed by comparing their relative retention time and mass spectra with those of the NIST WILLY library data from the GC/MS system.

6. Determination of stevioside and rebaudioside A by HPLC

Extraction of bioactive compounds was performed according to (**Brandle, 1998** and **Nikolai et al. 2001**) a known weight of fresh callus was homogenized in methanol, the homogenate was then filtered and filterate stored until HPLC analysis. The stevioside obtained by methanol extract analyzed by HPLC-DAD**.** The system Thermo (Ultimate 3000) consisted of: pump, automatic sample injector, and associated DELL-compatible computer supported with Chromeleon © Dionex version7 interpretation program. A diode array detector DAD-3000 was used. The Thermo-hypersil reversed phase C18 column 2.5× 30cm was operated at 25º C. Mobile phase consists of distilled water (solvent A) and methanol (solvent B) 37:63 isocratic and separation temperature was 25°C. The UV absorption spectra of the standards as well as the samples were recorded in the range of 219 nm. Injection volume was 10 µl from samples and standards solutions as well as the mobile phase were degassed and filtered through 0.45 µm membrane filter (Millipore). Identification of the compounds was done by comparison of their retention's time and UV absorption spectrum with those of the standards by computer software.



7. Molecular analysis

7.1. Simple sequence repeats (SSR technique) Microsatellite Markers, DNA extraction and PCR Amplification

Seven samples of stevia fresh callus among them control were used in this investigation.

Genomic DNA was isolated from callus treatments using the Gen-Elute Plant Genomic DNA Miniprep Kit. Five microsatellite primers were developed on the basis of the associated to treatments expression have been used for this study. They were on the average 18-24 bp in length. Primers' sequences are listed in Table (1), Genotyped markers were assigned using the Grain Genes data base (http://grain.jouy.inra.fr/cgibin/graingenes/browse.cgi) **(Kleinhofs and Graner, 2001).**

Table (1) showed the SSRs primers related to treatments and their sequences

No	Primers	PCR Sequances
1	SSR1	F:GTCGGGCTCCATTGCTCT R:CCGGTACCCAGTGACGAC
2	SSR2	F: CTCCCATCACACCATCTGTC R: GACATGGTTCCCTTCTTCTTC
3	SSR3	F': ATGGTAGAGGTCCCAACTG R :ATCACTGCTGTGCCTAGC
4	SSR4	F: CTAGCAACTTCCCAACCGAC R:ATGCCTGTGTGTGGACCAT
5	SSR5	F: AAGCTCTTTCTTGTATTCGTG R: GTCCATACTCTTTAACATCCG
Abbrev. F: forward R: Reverse		

7.2. PCR amplification and electrophoresis

PCR amplification was performed in a volume of 25 µl containing approximately 30 ng of template DNA, 1 µl of each forward and reverse primer, suitable quantity of dNTPs, $MgCl_2$ and Taq DNA Polymerase and PCR buffer. Reactions were conducted in Eppendorf PCR system (Germany) with initial denaturation step for 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 54~56°C for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 5 min. The PCR reaction products were evaluated for polymorphisms on 3% agarose gel. After staining with 8 µl Nancy (revelation dye) for 60 min, the gels were photographed by gel documentation system.

7.3. Data scoring and statistical analysis:

To ensure the absence of artifacts, bands were carefully selected from replicated amplifications (three times). Amplified bands designated by their primer code and their size in base pairs. Data recorded as discrete variables: 1 for the presence and 0 for the absence of a similar band. Only intense and reproducible bands appearing on the gel were scored. Band scoring was analyzed using Gene Tools-gel analysis software of SPSS ver. 16.

The Polymorphic Information Content value (PIC) refers to the value of a marker for detecting polymorphism within a population and depends on the number of detectable alleles and the distribution of their frequency. PIC was calculated using the equation:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij2}$$

Where, PICi is the polymorphic information content of a marker i; Pij is the frequency of the j the pattern for marker i and the summation extends over n patterns on other hand Genomic template stability (**GTS**) value calculated for each treatment, was calculated using the equation,

$$GTS\% = [(1- d/n) x100]$$

Where, d: Average number of bands detected in each treatment sample and n: number of total bands in control.

8. Statistical analysis

The data was subjected to one way ANOVA and the differences between means and standard error at the 0.05 probability level were determined using Duncan's multiple range tests. The SPSS software version 16 (Richmond, USA) was used as described by **Dytham (1999).**

III. Results and discussion

1. Characterization of magnetite NPs prepared by neem extract (MG NPs)

1.1. Transmission electron microscope (TEM)

Figure (2) showed the TEM of magnetite NPs prepared using neem extract at two different magnifications. It can be seen from the figure that there are fine dark spots indicating magnetite nanoparticle. The particles of the magnetite have spherical shape with very small size ranged from 3 to 15 nm. The figure indicated also that, there are many aggregations of magnetite NPs affected by plant extract. The plant aqueous extract containing many polar organic compounds having different reactive functional groups such as hydroxyl, carboxyl, and amine groups that involved in reduction and capping of iron slat into magnetite NPs.

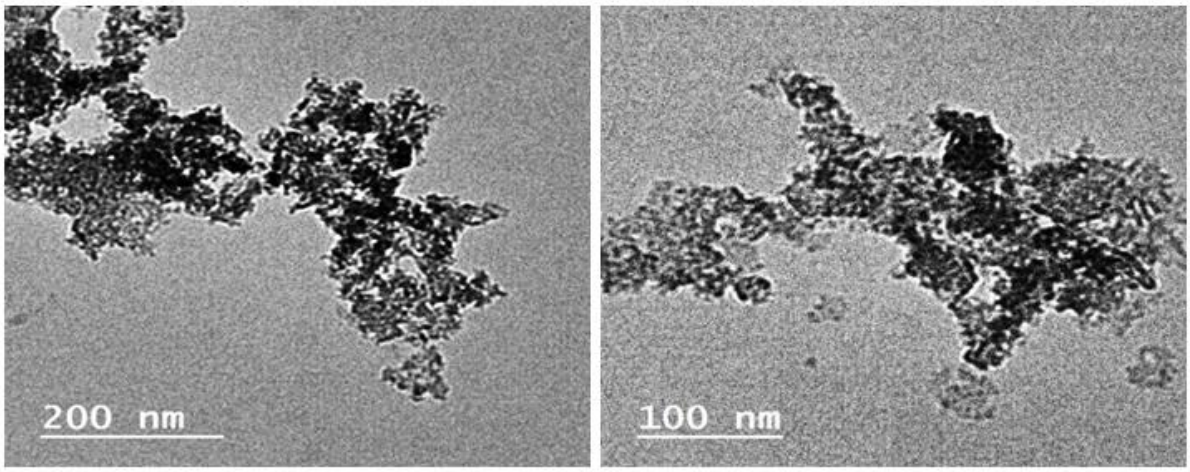
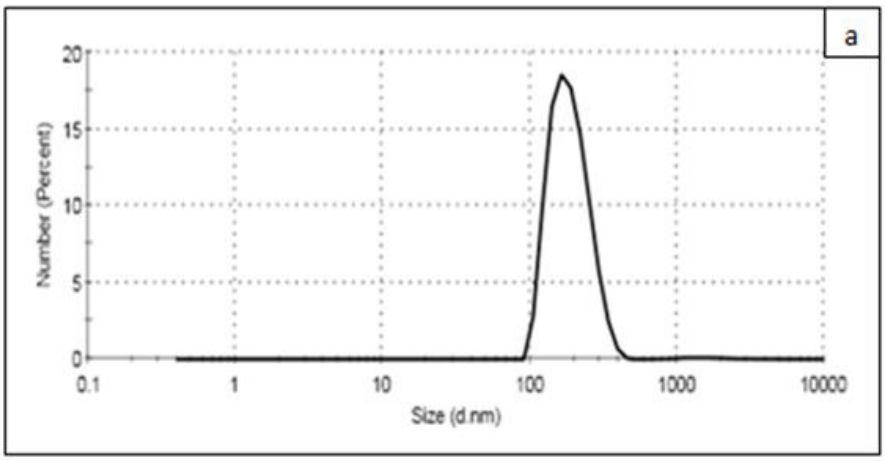


Fig.2. TEM of magnetite NPs prepared with neem aqueous extract

1.2. Dynamic Light Scattering (DLS)

The particles size and zeta potential of the synthesized magnetite nanoparticle were assessed using Zetasizer (Ver 7.04, Malvern instrument Ltd) as shown in (figure 3). As shown in (figure 3a). The particles size of the magnetite nanoparticle was around 190 nm. The particles size measured using DLS instrument were much higher than that measured using TEM. In DLS; the measurement was performed on suspended state of the NPs in the aqueous media with their aggregations and accumulation within the plant extract. While in TEM; the measurement was performed on dried particles and selected area that clearly confirm the particles formation and demonstrated if the particles are aggregated or separated from each other. The figure 3b showed the zeta potential or surface charge of the synthesized NPs. One can conclude that the prepared nanoparticle have negatively charged with a value of -23mV. The negatively charges was due to the oxygen atoms of oxide form of magnetite nanoparticle (Fe_2O_3) and may be also the reactive polar function groups of the plant extract.



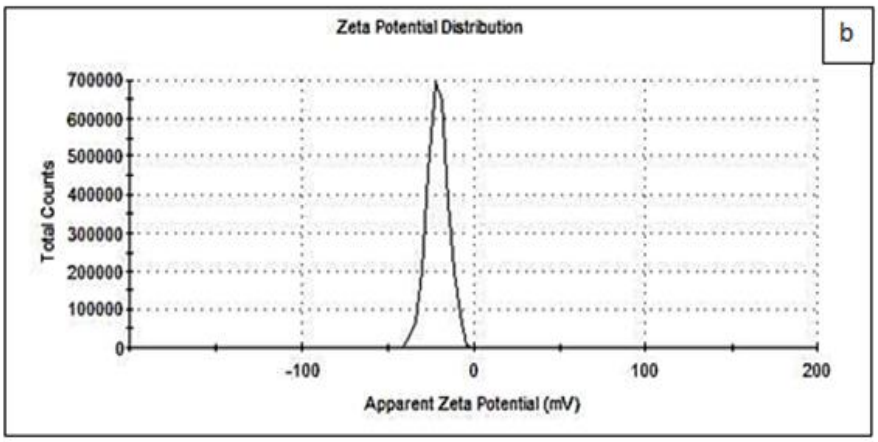


Fig.3. Particle size (a) and zeta potential (b) of magnetite NPs

2. Characterization of the prepared chemically Fe_3O_4 (MC NPs)

It showed well dispersed nearly spherical shape with particle size about 50 ±5 nm as shown in TEM image in figure (4) and for extra- confirmatory identification by dynamic light scattering (DLS) which was performed to evaluate the nano-dispersity and particle size determination of average particle size which were 50 ±5 nm with poly dispersed index pdi= 0.431 indicated narrow size range in figure (5a). Stability and surface charge were estimated by Zeta potential exhibited = - 21.0 mV moderate stable NPs according to figure (5b).

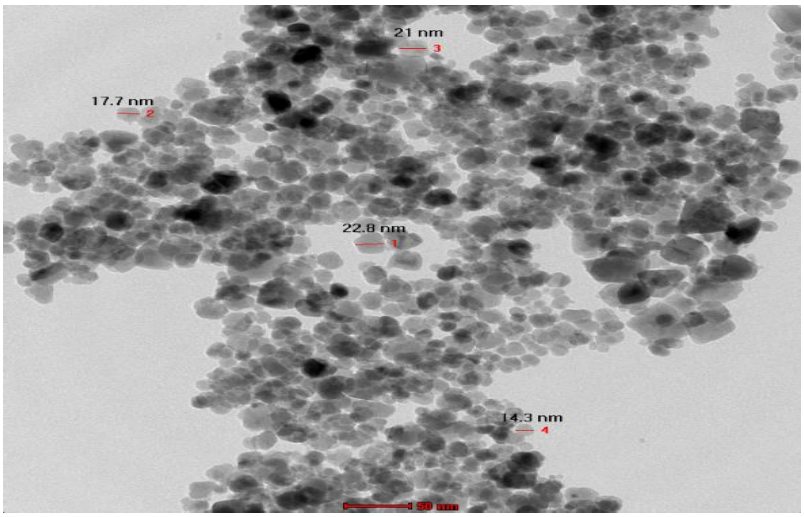
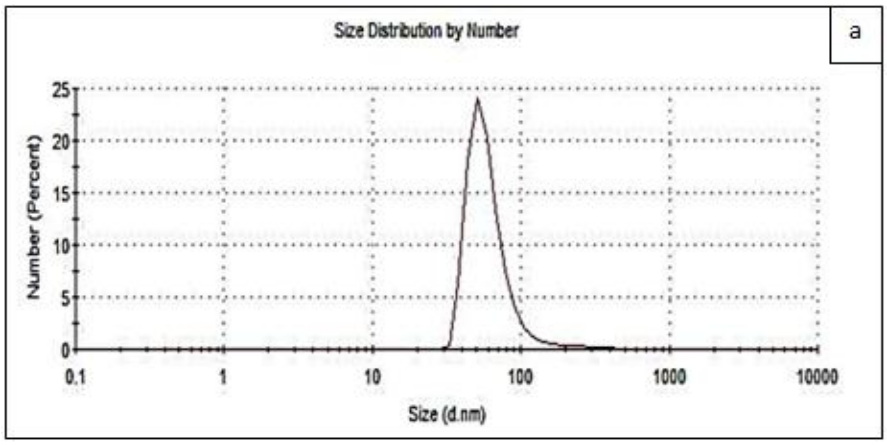


Fig.4. TEM of magnetite NPs prepared with chemical method



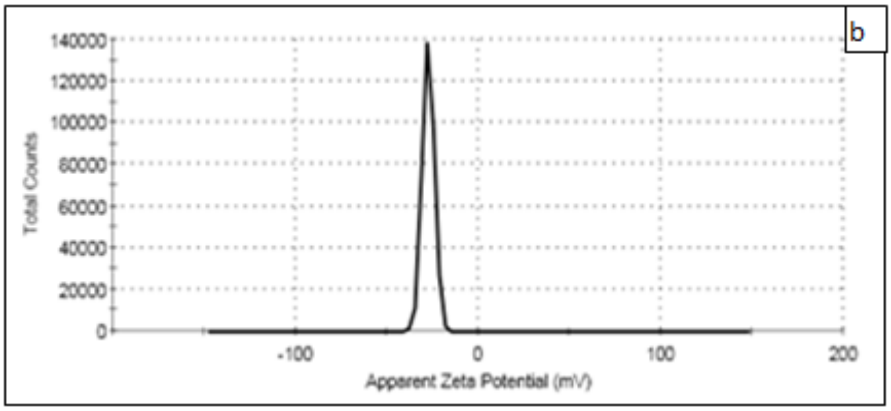


Fig.5. Particle size (a) and zeta potential (b) of magnetite NPs

3. Callus growth and development

Hundred percent of leaf segments of *S. rebaudina* L. produced callus on media containing all growth regulators examined combinations, while the control PGRs free medium gave a negative response as presented in (Table2 and figure 6). Visible callus formation was obtained within two weeks and observations were taken after eight weeks of the culture. Greenish yellow callus was induced from wound sites in the leaf segments. The highest mean fresh weight of callus (225.56 mg/jar) was obtained on MS medium containing 2.0 mg/L 2,4-D and 0.5mg/L BA, followed by 2.0mg/L NAA and 0.5 BA which gave 197.00mg/jar. Comparing the effect of different tested concentrations of PGRs on callus growth, it could be noticed that callus fresh weights were gradually increased with increasing the concentration of 2,4-D and NAA from 0.5 to 2.0mg/L. It is well known that auxins play an important role in the callus formation and the different types of auxins had various effects as reported by **Gang et al. (2003).** The superiority of 2,4-D is supported by **Yang et al. (2008).** This study is in broad agreement with **Tahereh (2018)** who reported that the highest callus fresh weight of *S. rebaudina* was recorded for leaf explant using 2.0mg/L 2,4-D and 2.0 mg/L NAA. Another study by **Abdelmaksood et al. (2017)** showed that a combination of auxin and cytokinin was a better treatment for the high frequency of callus in *S.rebaudina*. The maximum callus growth was found with auxins such as 2,4-D and NAA and also with BA among the cytokinin **(Gopi and Vatsala,2006)**. Also, **Agarwal and Kamal (2004)** reported that the presence of 2,4-D has been shown to be essential for callus formation in *Momordica charantia*. 0.25 mg/LBAP with 2 mg/L 2,4-D concentrations proved to be optimal for the production of maximum callus and also were more effect on callus weight, callus volume and callus color **(Bahman Fazeli-Nasab,2018)**. The need for exogenous growth regulators for callus induction in leaf explants of *B. gasipaes* was previously reported by **Santos et al. (2012).**

Table (2). Callus initiation from leaf segments of *Stevia rebaudiana* using MS medium containing 2,4- D, NAA and BA.

PGRs concentration (mg/L)			Callus induction rate (%)	callus fresh weight (mg)	Texture of callus	Color of callus
2,4-D	NAA	BA				
0.0	0.0	0.0	0.0	0.0	--	--
0.5	0.0	0.5	100	131.3 ± 1.856 f	Friable	Creamy to yellowish
1.0	0.0	0.5	100	168.00 ± 1.528 d	Nodular	Light green
1.5	0.0	0.5	100	189.00 ± 4.163 b	Friable	Greenish white
2.0	0.0	0.5	100	229.67 ± 2.028 a	Compact	Yellowish green
0.0	0.5	0.5	100	106.00 ± 3.215g	granulated	Creamy to yellowish
0.0	1.0	0.5	100	155.00 ± 2.333e	granulated	Yellowish green
0.0	1.5	0.5	100	176.33 ± 3.480 c	Friable	Yellowish green
0.0	2.0	0.5	100	197.00 ± 2.517 b	Compact	Yellowish green

Data are means ± Standard error
Means followed by the same letter in a column are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level.
***Data recorded after 8 weeks.**

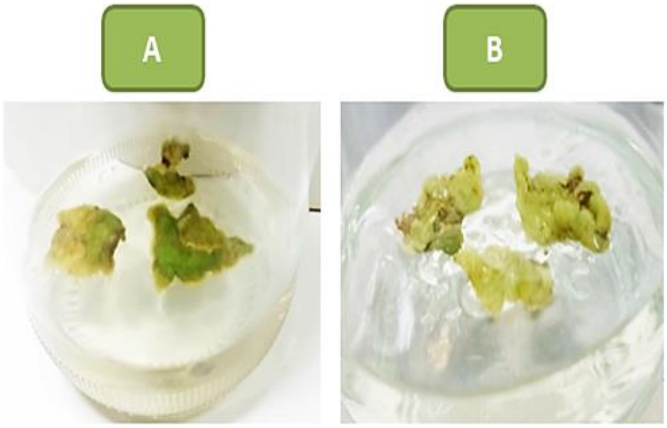


Fig.6. Callus of *Stevia rebaudiana* on MS medium supplemented with 2.0mg/l 2,4-D + 0.5mg/ lBA.(A) After two weeks. (B) after eight weeks

4. Growth and some biochemical markers in *Stevia rebaudina* callus.

Using neem extract individually with two concentrations, chemically and bio-engineered magnetite were added separately, to the culture medium supplemented with the best PGRs combination for callus proliferation of 2mg/l 2,4-D and 0.5mg/l BA was represented in table (3). All the treatments enhanced the growth of callus (fresh weight and dry weight). The highest mean value of fresh callus weight was recorded in case of chemically synthesized magnetite NPs (4 mg/l) but the maximum record of dry weight was determined by chemically engineered magnetite NPs (2 mg/l) followed by magnetite NPs (4 mg/l) at the same table and (figure 7). In this regard, the application of magnetite NPs at a certain level in culture media may have activated the expression of aquaporin genes which enhanced the uptake of water content and thus improved the percentage of moisture content in stevia plant grown *in vitro* **(Khan et al., 2020)**. This finding was compatible with (**Hendawey et al. 2015)** worked on *Stevia rebaudina* and (**Fouda et al. 2021)** on *Salvadora persica*. NPs have reportedly been employed to stimulate plant growth, yield, and secondary metabolites production. Callus culture was a suitable method for the generation of bioactive metabolites in plant cell culture systems (**Plaksenkova et al. 2019).** *Stevia rebaudina* is a valuable plant its leaves contain prevalent amount of stevioside, which is made up of three molecules of glucose and one molecule of steviol, a diterpenic carboxylic alcohol, **(Geuns, 2004)**. The diterpene glycosides stevioside and rebaudioside, found in the leaves of Stevia, are believed to be 100-300 times sweeter than sucrose **(Tanaka, 1982; Ishima and Katayama, 1986).** Concerning glutathione, it is a tripeptide (cysteine, glycine, and glutamic acid). The medicinal importance of such tripeptide was summarized as follows: Singlet oxygen, hydroxyl radicals, and superoxide radicals are all chemically neutralized by it. Several antioxidant enzymes require glutathione as a cofactor. It participates in vitamin C and E regeneration, the neutralization of free radicals generated by chemical toxin metabolism in Phase I of the liver by it. One of around seven liver Phase II processes that conjugate the active intermediates produced by phase I to make them water soluble for kidney excretion. Mercury is transported out of cells and the brain by GSH. Cellular growth and apoptosis are regulated. It is necessary for mitochondrial function and mitochondrial DNA preservation **(Pizzorno, 2014).** In table (3), the maximum mean value of GSH was reported by chemically engineered magnetite NPs (2 mg/L) which previously recorded the maximum dry weight of callus. The same treatment gave lowest MDA toxic product when compared to control and other treatment. However, the highest mean value of antioxidant capacity of scavenging synthetic radical was related to neem extract application on callus in concentration 2% followed by chemically fabricated magnetite NPs (2 mg/L). As a rich source of antioxidants, neem extract increased the antioxidant activity of stevia callus where it contains nimbin, nimbidin, ninbidol, gedunin, sodium nimbinate , quercetin, salannin, and Azadirachtin **(Linton et al. 1997, Nisbet et al. 2001, Biswas et al. 2002, Gupta et al. 2017, Benelli et al. 2018, Chutulo and Chalannavar, 2018, Saleem et al. 2018).** The results were in harmony with **Hendawey et al. (2015)** on stevia treated with magnetite

Table3. Effect of neem extract, chemically synthesized and bioengineered magnetite on growth and some biochemical markers in *Stevia rebaudina* callus.

Treatments	Fresh weight (g)	Dry weight (g)	Glutathione µmol/gm fresh weight	Malondialdehyde nmol/gm	Antioxidant capacity (DPPH %)
Control	6.49 ± 0.736 c	0.233 ± 0.024 c	3.46 ± 0.046 d	16.79 ± 0.047 b	75.50 ± 0.273 e
Neem 2%	6.77 ± 0.300 c	0.370 ± 0.023 b	8.80 ± 0.096 b	15.54 ± 1.21 bc	81.88 ± 0.088 a
Neem 4 %	8.09 ± 0.176 bc	0.307 ± 0.023 bc	7.57 ± 0.370 bc	15.95 ± 0.179 b	77.01 ± 0.224 d
MC NPs 2 mg/L	9.96 ± 0.710 ab	0.560 ± 0.036 a	10.27 ± 0.320 a	12.15 ± 0.043 d	80.83 ± 0.81 b
MC NPs 4 mg/L	10.29 ± 1.195 a	0.513 ± 0.055 a	6.51 ± 0.236 c	20.80 ± 0.239 a	76.53 ± 0.183 d
MG NPs 2 mg/L	8.57 ± 0.551 abc	0.400 ± 0.025 b	7.56 ± 0.133 bc	14.15 ± 0.120 c	75.50 ± 0.137 e
MG NPs 4 mg/L	7.73 ± 0.356 c	0.300 ± 0.038 bc	8.32 ± 0.881 b	19.59 ± 0.127 a	78.83 ± 0.000 c

Data are means ± Standard error
Means followed by the same letter in a column are not significantly different according to Duncan's multiple range tests and the mean difference is significant at the 0.05 level.

Where: MC NPs=Magnetite NPs prepared chemically, MG NPs= Magnetite NPs green synthesized by neem extract

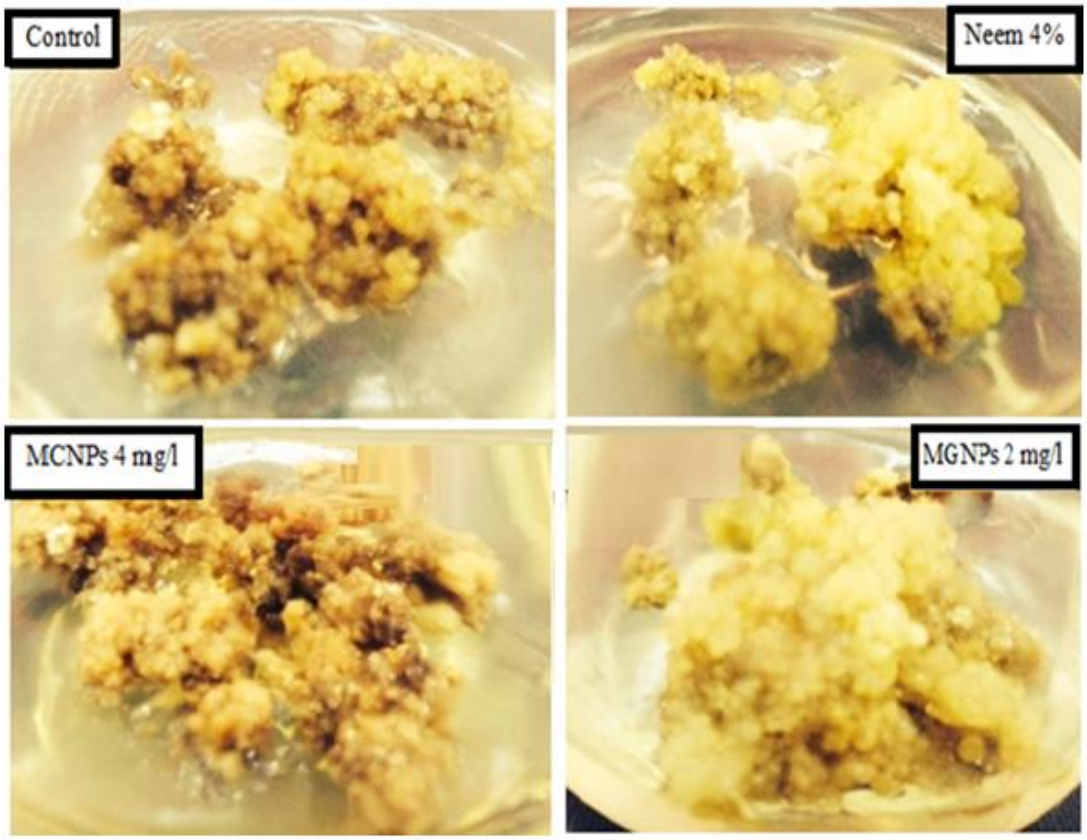


Fig.7. Callus of *Stevia rebaudina* on MS medium supplemented with 2.0mg/l 2,4-D + 0.5mg/l BA with (a) control medium (b) 4% Neem extract (c) MCNPs 4 mg/l (d) MGNPs 2mg/l

5. Active compounds in *Stevia rebudiana* callus by GC-MS

The GC-MS analysis has shown the presence of different active compounds in the chloroform callus extract of *Stevia rebudiana*. The analysis revealed the presence of 54 prominent peaks, but the major compounds identified in the chloroform extract were 26 compounds only (Table 4). Major compounds identified were, (2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II); Phenol,2-[[5-(2-methyl-3-benzofuranyl)-1H-pyrrol-2-yl][5-(2-methyl-3-benzofuranyl)-2H-pyrrol-2-ylidene]methyl]-acetate(ester)(CAS); (5,10,15,20-tetraphenyl[2-(2)H1] prophyrinato)zinc(II); 5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxy calix-4-arene; 1,3-Dimethyl-6-morpholinocyclohepta[c]pyrrol; (2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II); N,N'-Dicyclohexyl-1,7-di pyrrolidinylperylene-3,4:9,10-tetracarboxylic acid bisimide; 2,9-Bis(5-tert-butyl-2-methoxy-3-pyridylphenyl)-1,10-phenanthroline; 6-Hept-6-enyl-2-methoxy-4,2',6'-tris(4-methoxy benzyloxy)-4'-non-8-enylbiphenyl; Dichloro(5,10,15,20-tetraphenylporphyrinato)vanadium; (2-Nitro-5,10,15,20-tetraphenyl[2-(2)H1]prophyrinato)nickel(II); 5-(Dibromomethyl)-1,3-bis (tribromomethyl)benzene; Cyclohexene,1,2,3,3,6,6-Hexadeutero-5-Vinyl-; 2,6-Bis(2,3,5-triphenyl-4-oxocyclopentadienyl)pyridine; 2-endo-Aminomethyl-7-oxabicyclo[2.2.1]hept-5-en-2-exo-ol; Benzene,1-methoxy-4-(2-propenyl)- (CAS); Methoxychromene precocene tetramer; 3,5-Di-t-Butyl-4-hydroxy phenyl bis(1,2-dihydro-2-oxo-N-phenylcyclohepta[b]pyrrol-3-yl) methane; Pentamethyl pentaphenyl cyclopenta siloxane; 2-Dococen-1-al; (2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II); 2,5-Dibromo-1,4-di-n-hexa decylbenzene; (2-hydroxy-5,10,15,20-tetraphenylporphinato)zinc(II); Tetradecanal (CAS); DL-3,4-Dimethyl-3,4-hexanediol; 2(3H)-Furanone,5-heptyldihydro- (CAS); Tetraphenyl porphyrinato dichloro titanium(IV), Methylsulfinato[2,3,7,8,12,13,17,18 octa ethyl porphyrinato]indium and [2-(o-Methoxyphenyl)-5,10,15,20-tetraphenyl porphyrinato]copper(II). From the results, it is evident that *stevia rebudiana* contains various bioactive compounds. The compounds were identified through NSIT and Whiely library of mass spectrometry attached with GC. The GC-MS spectrum confirmed the presence of various components with different retention times. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra are fingerprint of compound which can be identified from the data library. Concerning the effect of neem extract, magnetite NPs prepared chemically (MCNPs), and magnetite NPs green synthesized by neem extract (MG NPs) on the area percent of these compounds data revealed that all treatments had a positive effect in increasing the area percent of phytoconstituents compared with the control (Table 5). From the results, data showed that the bioactive compounds with maximum peak area in callus extract are, Phenol,2-[[5-(2-methyl-3-benzofuranyl)-1H-pyrrol-2-yl][5-(2-methyl-3-benzofuranyl)-2H-pyrrol-2-ylidene]methyl]-, acetate (ester)(CAS);5-(Dibromomethyl)-1,3-bis(tribromomethyl)benzene; Cyclohexene, 1,2,3 ,3,6,6-Hexadeutero-5-Vinyl-; 2,6-Bis(2,3,5-triphenyl-4-oxocyclopentadienyl)pyridine; 3,5-Di-t-Butyl-4-hydroxy phenyl bis(1,2-dihydro-2-oxo-N-phenylcyclohepta[b]pyrrol-3-yl)methane; and Pentamethyl pentaphenyl cyclopenta siloxane. In light of this, neem extract stimulates the increase of active compounds in the stevia callus compared to control at all retention times except (11.02, 11.19, 11.34, 14.07, 15.74, and 18.44), and this was evident in the low concentration of 2% versus 4% neem extract. On the other hand, MC and MG NPs resulted in a higher increase in the area percent of active compounds compared to the treatments of neem extract and control except for the retention times (5.14, 5.36, 5.45, 11.02, 11.19, 11.34, 14.07, 15.74, 18.44, 21.35, 31.8, 34.25). As a comparison between the effect of MC NPs and MG NPs, it was observed that the MG NPs was higher in the content of active compounds in stevia callus with two concentrations than MC NPs at all retention times except (5.45, 5.73, 9.15, 14.87, 15.25, 31.8, 34.25, 39.79, 41.71, 42.32). However, the maximum area percent was recorded at all treatments when stevia callus treated with MG NPs at 4% which is Phenol,2-[[5-(2-methyl-3-benzofuranyl)-1H-pyrrol-2-yl][5-(2-methyl-3-benzofuranyl)-2H-pyrrol-2-ylidene]methyl]-, acetate (ester)(CAS). In this connection, GC-MS carried out the chemical profiling of *Stevia rebaudiana* produces, as its main secondary metabolite diterpene glycosides which are natural sweeteners. As a sweetener, it has advantages that as a terpene it doesn't cause an allergic reaction, unlike most peptide sweeteners **(Abdullateef and Osman, 2012).** Identification of the compounds was achieved by using NSIT and wiley libraries and comparisons of retention time. The steviol glycosides are responsible for the sweet taste of the leaves of the stevia plant has many medicinal properties it is employed for the discussion of various shape such as Cancer **(Balandrin and Klocke, 1988)**, diabetes **(Lailerd et al. 2004)**, Obesity, cavities hypertension (Gupta, 2013). It possesses hypoglycemic, hypotensive, vasodilating, taste improving, anti-fungal, anti-bacterial properties and increase urination function of the body and is devoid of genotoxic effect **(Alan, 2002; Anbazhagan et al. 2010; Luwanska et al. 2015)** Hence it is a plethora of characteristics having commercial and therapeutic value **(Harismah et al. 2018; Myers et al. 2018).**

Table (4) Major peaks identified by GC-MS in chloroform dry callus extract of *Stevia rebaudiana* treated by magnetite NPs prepared chemically and green synthesized.

PN	RT	Compound Name	MF	MW
1	5.14	(2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II)	$C_{58}H_{40}CuN_5O_2$	901
2	5.21	Phenol,2-[[5-(2-methyl-3-benzofuranyl)-1H-pyrrol-2-yl][5-(2-methyl-3-benzofuranyl)-2H-pyrrol-2-ylidene]methyl]-, acetate (ester)(CAS)	$C_{35}H_{26}N_2O_4$	538
3	5.31	(5,10,15,20-tetraphenyl[2-(2)H1]prophyrinato)zinc(II)	$C_{44}H_{27}DN_4Zn$	676
4	5.36	5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene	$C_{44}H_{56}O_4$	648
5	5.41	1,3-Dimethyl-6-morpholinocyclohepta[c]pyrrol	$C_{15}H_{18}N_2O$	242
6	5.45	(2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II)	$C_{58}H_{40}CuN_5O_2$	901
7	5.73	N,N'-Dicyclohexyl-1,7-dipyrrolidinylperylene-3,4:9,10-tetracarboxylic acid bisimide	$C_{44}H_{44}N_4O_4$	692
8	6.32	2,9-Bis(5-tert-butyl-2-methoxy-3-pyridylphenyl)-1,10-phenanthroline	$C_{44}H_{42}N_4O_2$	658
9	8.45	6-Hept-6-enyl-2-methoxy-4,2',6'-tris(4-methoxybenzyloxy)-4'-non-8-enylbiphenyl	$C_{53}H_{64}O_7$	812
10	8.72	Dichloro(5,10,15,20-tetraphenylporphyrinato)vanadium	$C_{44}H_{28}Cl_2N_4V$	733
11	9.15	(2-Nitro-5,10,15,20-tetraphenyl[2-(2)H1]prophyrinato)nickel(II)	$C_{44}H_{27}N_5NiO_2$	715
12	11.02	5-(Dibromomethyl)-1,3-bis(tribromomethyl)benzene	$C_9H_4Br_8$	744
13	11.19	Cyclohexene,1,2,3,3,6,6-Hexadeutero-5-Vinyl-	$C_8H_6D_6$	108
14	11.34	2,6-Bis(2,3,5-triphenyl-4-oxocyclopentadienyl)pyridine	$C_{51}H_{33}NO_2$	691
15	14.07	2-endo-Aminomethyl-7-oxabicyclo[2.2.1]hept-5-en-2-exo-ol	$C_7H_{11}NO_2$	141
16	14.87	Benzene,1-methoxy-4-(2-propenyl)- (CAS)	$C_{10}H_{12}O$	148
17	15.25	Methoxychromene precocene tetramer	$C_{48}H_{56}O_8$	760
18	15.74	3,5-Di-t-Butyl-4-hydroxy phenyl bis(1,2-dihydro-2-oxo-N-phenylcyclohepta[b]pyrrol-3-yl)methane	$C_{45}H_{42}N_2O_3$	658
19	18.44	Pentamethyl pentaphenyl cyclopenta siloxane	$C_{35}H_{40}O_5Si_5$	680
20	21.35	2-DOCECEN-1-AL	$C_{12}H_{22}O$	182
21	22.37	(2,2-Dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato)copper(II)	$C_{58}H_{40}CuN_5O_2$	901
22	28.72	2,5-Dibromo-1,4-di-n-hexadecylbenzene	$C_{38}H_{68}Br_2$	682
23	29.85	(2-hydroxy-5,10,15,20-tetraphenylporphinato)zinc(II)	$C_{44}H_{28}N_4OZn$	692
24	30.73	Tetradecanal (CAS)	$C_{14}H_{28}O$	212
25	31.8	DL-3,4-Dimethyl-3,4-hexanediol	$C_8H_{18}O_2$	146
26	34.25	2(3H)-Furanone,5-heptyldihydro- (CAS)	$C_{11}H_{20}O_2$	184
27	39.79	tetraphenyl porphyrinato dichloro titanium(IV)	$C_{44}H_{28}Cl_2N_4Ti$	730
28	41.71	Methylsulfinato[2,3,7,8,12,13,17,18-octaethylporphyrinato]indium	$C_{37}H_{47}InN_4O_2S$	726
29	42.32	[2-(o-Methoxyphenyl)-5,10,15,20-tetraphenylporphyrinato]copper(II)	$C_{51}H_{34}CuN_4O$	781

PN= Peak number, RT= Retention time (minute), MW= Molecular weight, MF= Molecular formula

Table (5) Active compounds identified by GC-MS in chloroform dry callus extract of *Stevia rebaudiana* treated by magnetite NPs prepared chemically and green synthesized.

PN	RT	Area percent (%) for major compounds in callus of *Stevia rebaudiana*						
		Control	Neem Extract (%)		MC NPs (mg/l)		MG NPs (mg/l)	
			2	4	2	4	2	4
1	5.14	0.58	1.66	4.37	1.86	1.54	2.11	3.28
2	5.21	0.92	2.31	7.12	8.43	8.97	9.52	10.84
3	5.31	0.74	2.59	4.26	2.25	4.17	2.58	4.67
4	5.36	0.65	3.91	3.39	2.13	2.44	2.41	2.69
5	5.41	0.46	4.25	4.88	3.12	4.36	5.34	1.97
6	5.45	0.67	2.38	3.69	3.3	1.27	1.22	1.93
7	5.73	1.14	2.48	1.82	3.15	2.59	3.1	1.77
8	6.32	0.51	1.76	1.91	1.98	1.68	1.38	2.44
9	8.45	0.47	2.21	1.62	1.54	1.78	2.32	2.31
10	8.72	1.57	1.74	1.93	2.09	2.23	2.23	2.45
11	9.15	0.49	2.43	2.25	1.76	3.09	2.13	2.00
12	11.02	7.02	1.36	1.63	1.48	1.42	1.23	1.87
13	11.19	6.68	2.35	2.55	1.93	1.5	2.38	1.65
14	11.34	8.82	2.05	1.47	1.71	1.61	1.52	1.85
15	14.07	4.03	1.63	1.76	1.47	2.24	2.61	1.81
16	14.87	1.56	2.65	2.04	2.25	2.66	1.87	1.7
17	15.25	0.69	1.72	1.47	2.01	2.61	2.57	1.6
18	15.74	9.77	7.33	5.47	7.92	8.46	8.89	9.11
19	18.44	10.59	2.56	1.49	1.59	1.65	1.69	2.11
20	21.35	1.74	1.78	1.59	1.63	1.65	1.77	1.68
21	22.37	1.12	2.39	2.23	2.6	2.43	2.4	2.57
22	28.72	0.76	2.64	2.12	1.37	1.42	2.11	2.66
23	29.85	0.45	1.65	1.89	1.32	1.26	1.23	2.23
24	30.73	0.51	1.59	1.64	1.76	1.4	1.78	1.69
25	31.8	0.46	2.61	1.92	2.58	1.72	1.78	1.84
26	34.25	0.56	4.35	1.65	1.89	2.08	1.79	1.56
27	39.79	1.15	1.92	1.89	2.61	1.88	1.86	2.16
28	41.71	0.89	2.24	2.41	4.25	2.64	2.42	2.26
29	42.32	0.68	1.66	1.5	3.56	3.76	2.23	2.13

MC NPs=Magnetite NPs prepared chemically, MG NPs= Magnetite NPs green synthesized by neem extract

6. Separation of sweet active compounds by HPLC

The table (6) and figure (8) illustrated the natural sweet bioactive compounds, Rebaudioside A and stevioside which are abundant steviol glycoside contained in *Stevia rebaudiana* leaves. These components are widely used as a natural sweetener as a healthier products (**López-Carbón et al. 2019)**. All treatments gave positive effects on natural sweet bioactive constituents' biosynthesis. The highest peak was detected by bioengineered Fe NPs 4 mg/L especially in Rebaudioside A. These results were in harmony with **(Hendawey et al. 2015)** on stevia callus. The increase of active ingridients may be due to the presence of neem plant extracts on the used biogenic magnetite NPs which gave reactive groups in the reaction media especially in biosynthesis of the bioactive compound. its also bound with higher secondary metabolites obtained by GC mass for the same treatment that also has highest GTS% when compared to other treatments.

Table (6): Sweet active compounds identified by HPLC in methanolic fresh callus extract of *Stevia rebaudiana* treated by magnetite NPs prepared chemically and green synthesized.

Treatments	Area percent (%)		
	Stevioside	Rebaudioside A	Unknown
Control	100	N.D	N.D
Neem extract 2%	100	N.D	N.D
Neem extract 4%	89.03	3.48	7.48
MC NPs 2 mg/L	61.27	38.73	N.D
MC NPs 4 mg/L	57.55	42.45	N.D
MG NPs 2 mg/L	100	N.D	N.D
MG NPs 4 mg/L	65.29	34.71	N.D

Where:
MC NPs =Magnetite NPs prepared chemically, MG NPs= Magnetite NPs green synthesized by neem extract, N.D. not detected

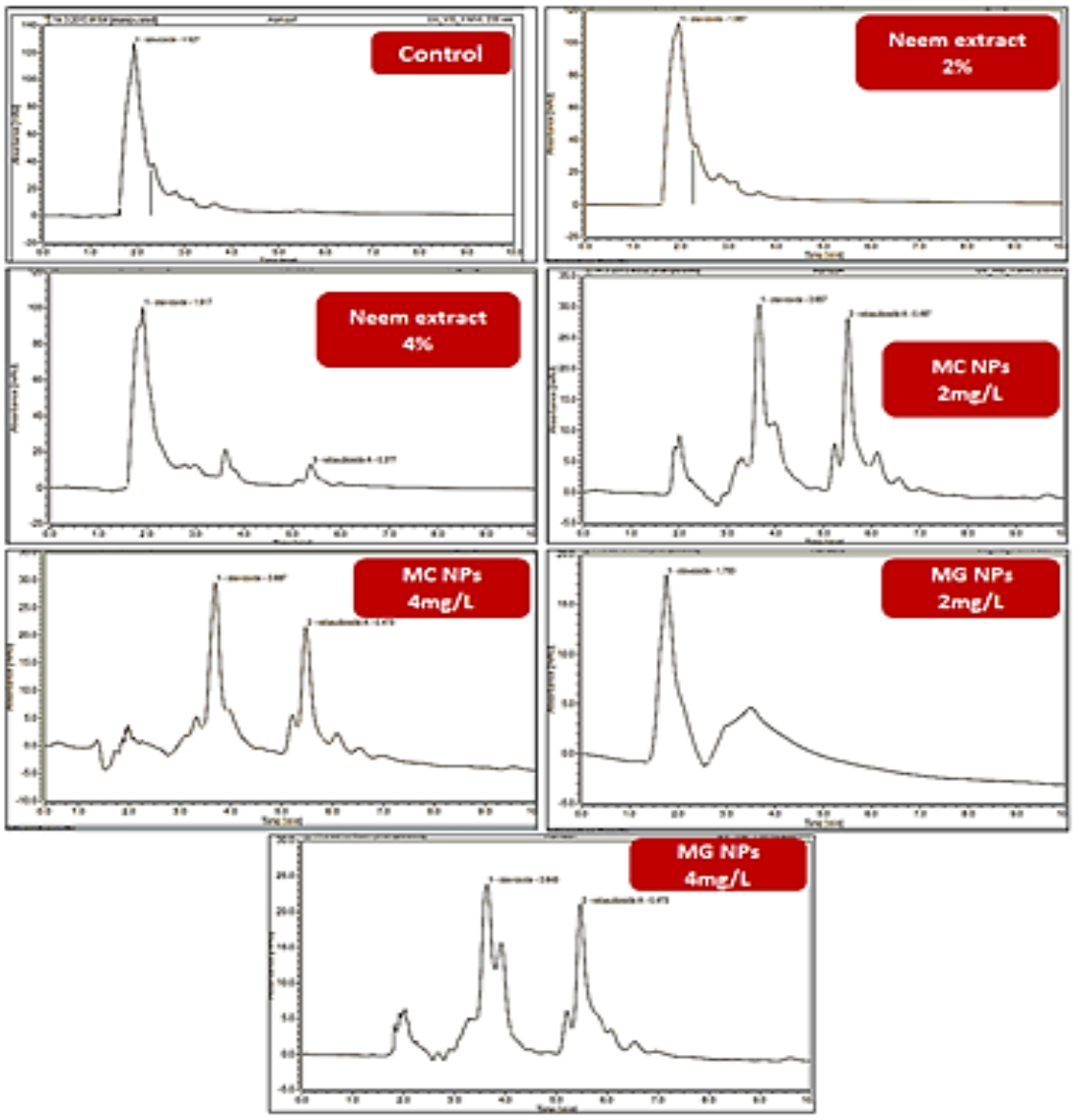


Figure (8): Effect of neem extract, chemically and green nanoengineered magnetite NPs on sweet active compounds of *Stevia rebaudiana* callus.

7. Molecular Analysis

SSR associated with magnetite chemically and green nano- synthesized NPs application

Five SSR primers generated clear patterns with high polymorphism. (Table 7and Figure 9).The five discriminatory primers pairs were succeeded to evaluate the genetic diversity and genetic stability value association of seven samples of Stevia treatments (seven samples, six treatments and control) (Table 7.). For all tested samples, the highest number of bands was developed by the primer SSR4 (nine bands), followed by SSR2 (six bands), followed by SSR5 (five bands) and each of primers SSR1 and SSR3 (four bands). Moreover, the primer SSR4 showed unambiguous bands with 90% polymorphism, while the primer SSR2 produced bands with 83% polymorphism. However the lowest number of polymorphism bands was found by the primer SSR1 which gave 25% polymorphism.

The polymorphism information content (PIC) was a measure of allelic variability and evenness at a particular locus. In this study the PIC values ranged from 0.467 (SSR5) to 0.712 (SSR1) (Table 7). On the other hand the genomic template stability (GTS) value calculated for each treatment, which appeared the highest stability number (50%) in treatment number six (MG NPs 4 mg/L) while the lowest stability number showed (22%) in treatment four (MC NPs 4 mg/L) without biogenic as shown in table (8). The five expressing SSR primers enabled us to discriminate all the treatments for studding the genetic variability and stability and lines, SSR patterns illustrated that there are bands appeared in all treatment (common bands). However other bands were present in some treatment and absent in the others (polymorphic). The appearance of some polymorphic bands indicated a direct relationship of NPs treatments which reflect the genetic of gene expression action to treatments. In the molecular level analysis, the results showed high levels of genetic stability value (GTS) (50%) and 60 % polymorphism among MG NPs 4 mg/L included in this study, which refers to the high ability of SSR markers to reveal most of the information in a single locus and can be used for molecular genetic analysis at nanoparticular treatment on Stevia. In the connection, The interaction of NPs with DNA and/or nuclear proteins, which occurs after their diffusion into the cell and affects the cell cycle, or oxidative stress caused by reactive oxygen species, which affects the ability of DNA repair processes, can explain the induced genetic variation or polymorphism of NPs. The size of NPs has been shown to have an inverse connection with genotoxicity, but exposure length and concentration have a direct association **(Karami et al. 2016)**. According to **Fouda et al. (2021)** worked on *Salvadora persica* and parallel with our study on stevia, the modifications in the ISSR profile showed that the NPs' doses applied generated genetic variation that was dose and NP type dependent (chemical or biogenic source). The emergence of novel DNA bands in the ISSR profile and the absence of normal ones can be defined as a mutation, which is most likely caused by DNA damage or rearrangements caused by NP-induced genetic variation. The stability of genomic templates reflects the changes in the ISSR profile **(Plaksenkova et al. 2019)**. For the three NPs investigated, the GTS % reduced as the NP concentration increased. Fe_3O_4 NPs had the least genetic toxicity in *Salvadora persica* callus, according to earlier studies. For the NPs examined, the GTS % reduced as the NP concentration increased **(Fouda et al. 2021).** Fe_3O_4 NPs had the least genetic toxicity in *Salvadora persica* callus, according to earlier studies. The same NPs had similar effects in flax callus cultures in previous investigations **(Kokina et al. 2017).** These bands are most likely created as a result of NPs' ability to cause genomic variation by interfering with mitosis and modifying DNA by producing chromosomal abnormalities **(Karami et al. 2016).**

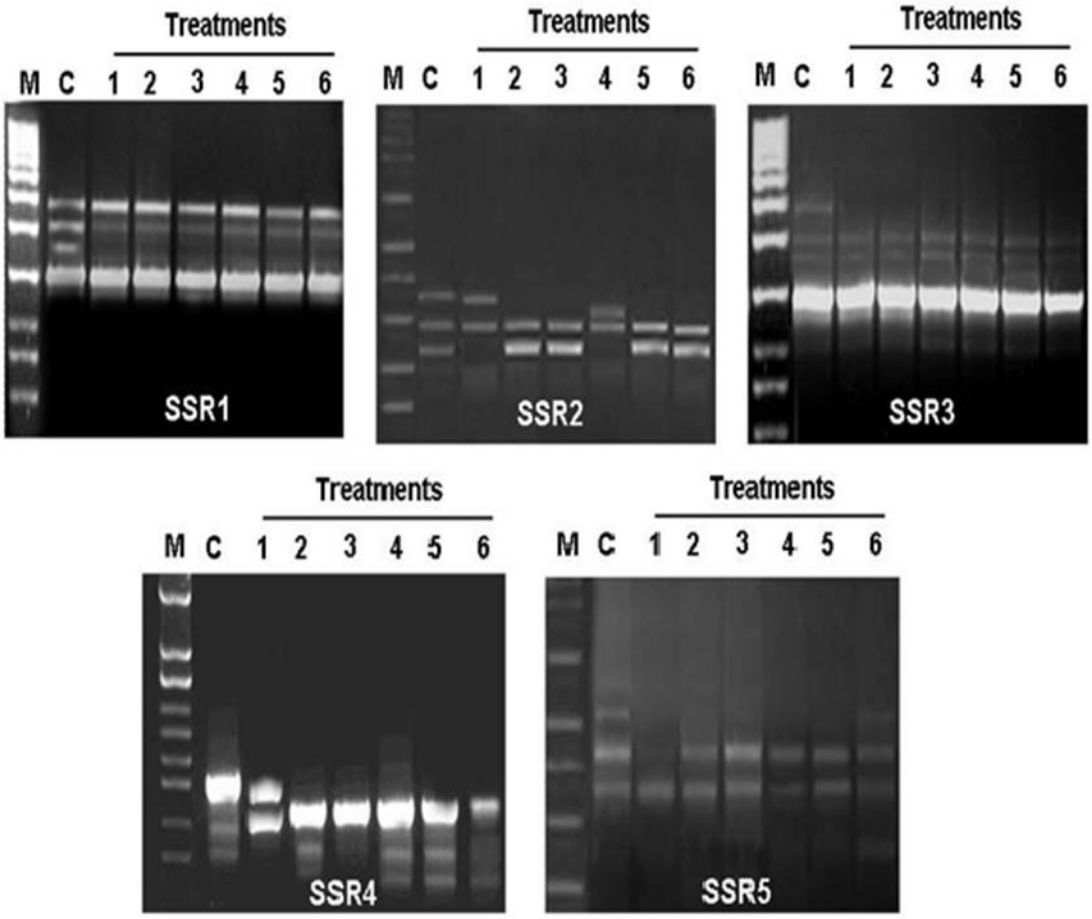
Table (7). SSR primers, their amplified fragments, polymorphic the polymorphism percentage and PIC value.

Primers	Amplified fragments				PIC
	Total number of bands	size range (bp)	Polymorphic	Polymorphism %	
1	4	328-576	1	25	0.712
2	6	286-417	5	83	0.642
3	4	612-703	3	75	0.692
4	9	201-378	8	90	0.596
5	5	352-663	3	60	0.467

Table (8) GTS parentage value against all five SSR primers

	%GTS value						
All five primers	Control	Neem 2%	Neem 4%	MC NPs 2 mg/L	MC NPs 4 mg/L	MG NPs 2 mg/L	MG NPs 4 mg/L
	100	28	33	38	22	27	50

Where:
MC NPs =Magnetite NPs prepared chemically, MG NPs= Magnetite NPs green synthesized by neem extract, N.D. not detected



Where: M=Marker (bp), C=Control, 1=Neem extract 2%, 2= Neem4%, 3= MC NPs 2%, 4=MC NPs 4%, 5=MG NPs 2%, 6=MG NPs 4%

Fig.9. PCR amplification profile generated from genomic DNA of seven samples of *Stevia rebaudiana* under six nanoparticles treatments and control against five SSR primers

IV. Conclusion

In our recent study, the application of treatments enriched stevia callus with antioxidants and could be useful in market use because of the amazing increase in accumulation of bioactive natural sweeteners helpful for people who suffered from some chronic diseases. Particularly the application of green nano-synthesized magnetic NPs 4 mg/L which also has higher genomic templates stability and this opens the door for more research and further applications in the agricultural sector.

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