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Enhancing the biochemical constituents in avocado callus using encapsulated chitosan nanoparticles

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Abstract

Chitosan nanoparticles (ChNPs) as a biodegradable and biocompatible substance were employed as a bio-elicitor to stimulate the biosynthesis of bioactive metabolites in avocado callus. Effect of *in-situ* encapsulation of different active compounds (Salicylic acid, Phenylalanine, Methionine and Ca-Pyruvate) onto ChNPs during preparation was performed using ionic gelation method. The prepared encapsulated nanoparticles were characterized using transmission electron microscope (TEM) and dynamic light scattering (DLS) techniques. The formulated ChNPs had a beneficial effect on the majority of the indicators tested on Murashige and Skoog (MS) medium. The results indicated that maximum mean value of callus fresh weight before the addition of nano-Chitosan was 215 mg/jar, and ChNPs encapsulated methionine 40 mg/L had the greatest mean record of callus fresh weight and glutathione content with low malondialdehyde (MDA) concentration of 0.993 nmol/g. However, ChNPs encapsulated salicylic acid at 100mg/L has the best antioxidant capacity due to a 40.88% rise in the content of the essential oil 3,4-Dihydro-2H-1,5-(3''-T-butyl) benzodioxepine and the largest accumulation of polyphenolic components. The current approach involved analysing molecular amplified fragment-length polymorphism (AFLP) using genomic DNA with two restriction enzymes (EcoRI and MseI) EcoR I-ACA and MseI-CTC primer pairs. The present work provides an efficient method for enhancing the bio-active compounds bioaccumulation in avocado callus using encapsulated ChNPs.

***Keywords**:* Chitosan nanoparticles; Tissue culture; Antioxidants; Avocado callus; *Persea americana*; Polyphenols; AFLP

1. Introduction

Chitosan is a linear homopolymer comprised of N-acetylglucosamine units that are (1,4) connected [1]. It is a partially deacetylated polymer obtained from chitin, a glucose-based unbranched polysaccharide found in crustaceans and insect exoskeletons, as well as some bacterial and fungal cell walls. Chitosan is a positive-charged biodegradable natural polymer with such a variety of advantages, including nontoxicity, mucoadhesion, hemocompatibility, biodegradability, and anticancer, antioxidant, and antibacterial properties make it a promising candidate for several bio-applications as plant tissue culture elicitation, agriculture, and medicine [2, 3]. The capacity of chitosan to dissolve, entrap, encapsulate, and/or have bioactive substances adhere to its nanoparticle matrix is improved when it is formulated in nanoparticles because of its highly surface area. Their nanoscale size also eases the burden through cell membranes effectively [4].

1. Plants have long been employed as a trustworthy source of food, flavoring, agrochemicals, and medicinal compounds. Plant biotechnology uses a plant in vivo system to give a long-term solution for the bioproduction of secondary metabolites in plants [5]. Herbal medicine has become a global phenomenon with medical and economic ramifications in recent decades. Herbs' widespread use around the world has prompted serious questions about their quality, safety, and usefulness. As a result, exact scientific evaluation has become a need for herbal health claims to be accepted. Avocado (*Persea americana* Mill.) is a member of the Lauraceae botanical family. The species is classified into four races based on its genetic makeup, each possessing its own origin. Because of the significant levels of different oils, proteins, carbohydrates, minerals, and

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vitamins in its fruits, FAOSTAT found that they had a high nutritional value [6]. It can treat hypertension, stomach pains, bronchitis, diarrhoea, and diabetes, among other things. Peptone, b-galactoside, glycosylated abscisic acid, alkaloids, cellulose, polygalactourease, polyuronoids, cytochrome P-450, and volatile oils are all commonly detected in this plant [7].

In response to external stress, plants produce secondary metabolites as a natural defense mechanism. These metabolites are active materials and can be used in the food, cosmetics, agrochemicals, and pharmaceutical sector. Elicitors have recently been employed in a novel method for increasing metabolite production in plants [8, 9]. There are numerous methods for increasing secondary metabolite biosynthesis in plant tissue and cell culture [10]. Elicitors are chemicals that cause metabolic changes in the plant to enhance the synthesis of secondary metabolites [11]. Elicitors accomplish this by triggering signalling pathways [12]. Different elicitors, either biotic or abiotic, that serve as stress agents and stimulate the synthesis of secondary bioactive substrates in plant callus [13]. Plant growth and secondary metabolites were improved in tissue grown plants provided with elicitors [14, 15]. Elicitation could help overcome some of the challenges that arise with producing the most economically significant bioactive secondary metabolites on a large scale from wild and cultivated plants, undifferentiated or differentiated cultures [16].

2. As organic substrates, for instance amino acids play an important part in cell development. They operate as buffers because they contain both acid and basic groups, allowing the plant cell to maintain a steady pH while also removing ammonia [17]. They can also aid plant growth by enhancing photosynthesis, mRNA transcription, sugar and protein biosynthesis [18], as well as acting as co-enzymes or precursors to a variety of plant hormones [19]. They bind to metal cations primarily through carboxylate (-COO) and amine ($-NH_2$) groups, changing metals.

3. Despite the fact that Phenylalanine (Phe) is a crucial component of plant development and growth, Phe is a precursor of phenylpropanoids, flavonoids, anthocyanins, lignin, tannins, and salicylate, which are all necessary for plant growth, reproduction, and defense against abiotic and biotic stresses [20, 21]. Methionine (Met) amino acid is a sulfur-containing amino acid that is nutritionally required for humans and animals, but its low quantity in plants reduces its utility as a source of dietary protein. Methionine is also an important metabolite in plant cells because its first metabolite, S-adenosylmethionine (SAM), regulates the levels of numerous important metabolites like ethylene, polyamines, and biotin [22]. As a phenolic compound, Salicylic acid (SA) is found in plants, it has a role in a variety of physiological and biochemical processes. Exogenously applied SA is beneficial to plant growth and development [23, 24]. Pyruvic acid is a junction molecule that gives energy to cells via the citric acid cycle (known as the Krebs cycle) when oxygen is present (aerobic respiration) and ferments to generate lactate (lactic acid) when oxygen is not available (anaerobic respiration) [25, 26].

4. The present research's goal is to activate the role of successfully prepared ChNPs individually and encapsulated some plant cells' substrates like (methionine, phenylalanine, calcium pyruvate, and salicylic acid) in growth, proliferation and bioactive compounds in avocado callus production enriched with antioxidants. The impact of NPs on avocado callus genetic response and changes in its profile by using molecular analysis was studied.

2. Materials and methods

2.1. Preparation of chitosan encapsulated organic substrates:

Chitosan NPs and their encapsulated analog were prepared using ionic gelation method [27] with some modifications in fig (1). The benefits of ion gelatin method, it is easy to process and conduct with simple steps, using aqueous environment, no heat consuming and can replace the risk of cross-linking reagents, it is also controllable and tunable as well as non-toxic. Chitosan solution with concentration of 0.4 % in (1%) acetic acid aqueous solution 50 ml was prepared. The organic compound (Salicylic acid, or Phenylalanine, Methionine and Ca-Pyruvate) with predetermined concentration was added to the chitosan solution under stirring for complete dissolving. Sodium tripolyphosphate (TPP) solution (20 ml) was added dropwise to the above solution under magnetic stirring and after complete addition it was left under stirring for another 1 hour. The ratio of chitosan to TPP was kept to be 3:2.

For encapsulation of substrates in details, 20 ml of (1% chitosan dissolved in 2% acetic acid) was diluted to 50 ml. 0.04g of salicylic acid was added to chitosan solution under stirring till complete dissolving. Stock TPP solution was prepared with 0.65g in 100 ml distilled water. 20 ml of TPP was added dropwise to chitosan solution to obtain nano-chitosan encapsulated salicylic acid. The other organics (Phenylalanine, Methionine and Ca-Pyruvate) were prepared using the same procedure (and the weight of organic was doublet of the required).

The obtained milky or white turbid suspension was indicated by the formation of ChNPs with their encapsulated combination. The NPs were characterized using TEM, DLS, and Zeta potential

assessments to determine their size, stability, shape, and charges, as in previous literature [28, 29].

Table 1) Abbreviation of the nano-capsule used in this study.

Treatments	Abbreviation
Control (no Chitosan or other substrates)	----------
Chitosan 100 mg/L NPs	ChNPs
Chitosan NPs 100 mg/L @Met 40 mg/L	ChNPs@Met
Chitosan NPs 100mg/L @PA 40 mg/L	ChNPs@PA
Chitosan NPs 100 mg/L@Ca-pyruvate 60 mg/L	ChNPs@Ca-Py
Chitosan NPs 100 mg/L@Salicylic acid 100 mg/L	ChNPs@SA

2.2. Plant material

2.2.1. Optimization of plant material

Leaf explants were obtained from mature trees grown in Al Qanater El- Khayrya Research Station, Horticulture Research Institute, Agriculture Research Center Egypt. At the laboratories of tissue culture, Desert Research Centre, leaves were washed under running tap water for 30 mins. Surface sterilization of leaves was carried out in transfer hood by soaking them in 30% (v/v) Clorox bleach solution (2.5% sodium hypochlorite) for 25 min, providing gentle agitation, followed by three sequential rinses in sterilized distilled water, followed by 3 min in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution then washed 6 times with sterile distilled water to remove the traces of mercuric chloride. All leaves were placed directly on the surface of full strength Murashige and Skoog (MS) [30] (Duchefa, Haarlem, the Netherlands) supplemented with 100mg/L myo inositol of and 30g/L sucrose. The pH of the medium was adjusted to 5.7 – 5.8 before being solidified with 2.8 g/L phytagel (Duchefa,Haarlem the Netherlands). Media were dispensed in large jars capped with autoclavable polypropylene lids, then autoclaved at a pressure of 1.06kg/cm^2 and 121°C for 20 min. The cultures were incubated at 25+- 2°C with a 16- h photoperiod under cool white florescent tubes.

2.2.3. Callus initiation and proliferation

Leaves were cut into small pieces (2.0-3.0cm), then cultured aseptically on MS medium supplemented with various plant growth regulators; 2,4-dicloro-phenoxyacetic acid (2,4-D) (1.0, 2.0, 3.0 and 4.0mg/L) or naphthalene acetic acid (NAA) (1.0, 2.0, 3.0 and 4.0mg/L) or indole acetic acid (IAA) (1.0,2,0, 3.0 and 4.0mg/L) and a cytokinin; 6-benzylaminopurine (BAP) (0.5mg/L) were used for callus initiation, in addition to MS medium without growth regulators. Callus tissues were transferred to freshly prepared medium for proliferation. Each treatment contained at least 9 replicates and was repeated twice. Percentage of callus initiation, fresh weight of callus (mg) and callus colour and texture were recorded after eight weeks of culture.

2.2.2. Elicitation

Fresh weight of callus (2g) was transferred to the most favourable callus initiation in addition to different elicitors, ChNPs, ChNPs@Met, ChNPs@PA, ChNPs@Ca-Py and ChNPs@SA. Callus was collected and the fresh and dry weights were determined.

2.3. Biochemical determinants

2.3.1. Lipid peroxidation content

With certain changes, the amount of lipid peroxidation in fresh callus samples was assessed in terms of calculating the end product, MDA (malondialdehyde) [31]. Avocado callus samples weighing 0.5 g were homogenised in 2.5 mL trichloro acetic acid 0.1% (w/v) (TCA). The homogenate was centrifuged for 15 mins at 14,000 rpm. 0.5 mL of the liquid supernatant was combined with 2 ml 5% thiobarbituric acid and in 20 % TCA solution. The slurry was heated in a water bath at 95°C for 30 mins before cooling in an ice bath for 5 mins. The supernatant was measured at 532 nm after centrifugation. Each sample's nonspecific turbidity was also measured at 600 nm and subtracted from the absorbance measured at 532 nm. The concentration of MDA-TBA complex was calculated from MDA standard curve and converted to nmol g^{-1} fresh weight.

2.3.2. Antioxidant capacity

To investigate avocado extract's ability to scavenge the synthetic radical DPPH (2,2-di-phenyl-1-picrylhydrazyl), the following approach was used: 0.5 mL avocado fresh callus methanolic extract was mixed with 2 mL of a 0.004 % solution of DPPH in methanol 80%. The reaction mixture was mixed and kept in dark at room temperature for 30 mins. According to **Oktay *et al*.** [32], spectropotometry was employed to estimate the wavelength of mixture absorption which was 517 nm. The following formula was used to calculate the ability to scavenge DPPH radicals:

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

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

2.3.3. Total 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. GSH was determined using the **Moron *et al.*** method [33], which involves reacting GSH with DTNB (5,5'-dithiobis nitro benzoic acid) to create a yellow product that absorbs at 412 nm.

3. Identification of polyphenolic compounds by HPLC

3.1. Extraction of polyphenolic compounds

In a brief, 1 g of avocado callus tissue was combined with 60 mL of 56 % ethanol (v/v) and held in a thermostatic water bath at 63°C for 20 mins while shaking. The mixture was centrifuged at 2500 rpm for 10 mins after cooling, and the supernatant was filtered and kept at 4°C until HPLC analysis [36].

3.2. HPLC conditions

HPLC analysis was performed using an Agilent 1260 series. The column used for separation was Eclipse C18 column (4.6 mm x 250 mm i.d., 5 μm). The mobile phase composed of two solvents, water and (0.05% trifluoroacetic acid in acetonitrile) with a flow rate 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A); 15-16 min (82% A) and 16-20 (82%A). The wavelength of the detector was monitored at 280 nm. The injection volume was 5 μl for each of the sample solutions. The column temperature was maintained at 40 °C.

4. Identification of fatty acids by Gas chromatography–mass spectrometry (GC-MS)

4.1. Extraction of fatty acids:

Samples were homogenised in a 2:1 chloroform: methanol (v/v) mixture, and cell debris was removed by filtering in the Folch approach. The homogenizer and recovered cell debris were washed with a fresh solvent combination, which was then pooled with the preceding filtrate before being added to a 0.73 % NaCl water solution, producing a final solvent system of 2:1:0.8 chloroform: methanol: water (v/v/v). Evaporation was used to remove the solvents, and the dry extracts were then methylated [37].

4.2. GC-MS conditions

The chemical components of callus samples were identified using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). Column oven temperature was initially held at 50ºC and then raised by 5°C /min to 230°C hold for 2 min. increased to the final temperature 290°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using auto-sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200 °C. The compounds were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral databases.

5. Molecular studies

5.1. DNA extraction

Total DNA was extracted using techniques developed for medicinal and aromatic plants [34]. RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37°C for 30 mins to eliminate RNA contamination. The concentration of DNA in different samples was calculated by measuring optical density at 260 nm and applying the following equation:

Conc. (ug/ml) = OD260 × 50× dilution factor.

5.2. Amplified fragment length polymorphisms (AFLPs)

AFLP is a method that employs PCR amplification to detect genomic restriction fragments and may be used on DNA of any origin or complexity. Using a restricted set of genetic primers, the fingerprints are created without any prior knowledge of the sequence. AFLP allows for the selective amplification of restriction fragments from a complete digest of genomic DNA, which is particularly beneficial for detecting variation between genotypes that are closely related.

AFLP procedure was applied according to [35]. Samples were prepared by cutting the genomic DNA with two restriction enzymes (EcoRI and MseI) and ligating with double stranded EcoR 1and Mse1 adaptors. The adaptors were ligated with the overhanging sticky ends produced by the restriction enzymes. On other hand Genomic template stability (GTS) value calculated for each treatment, using the formula:

$$\mathbf{GTS\% = (1-d/n) \times 100}$$

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

6. Statistical analysis:

The data was analyzed to a one-way ANOVA, and Duncan's multiple range test was used to detect the differences between means at the 0.05 probability level. Version 16 of the SPSS software (Richmond, USA) was deployed [38].

3. Results and discussion

3.1. Characterization of encapsulated chitosan nanoparticles.

3.1.1. Transmission electron microscope

Figure 1a showed the TEM micrograph of ChNPs at two different magnifications. It is clearly seen from

the images that the particles showed semi-spherical shape interconnected with each other with appearance of some aggregation. The size of ChNPs was within the range of 15-30 nm with narrow particle size distribution. Whereas the ChNPs encapsulated SA (Fig 1b) showed particles with definite spherical shape with relatively larger size than blank ChNPs within the range of 30-60 nm. The image also showed small aggregations resemble nuclei that attract some particles around it; that is may be due to the presence of SA molecules. The obtained results of TEM measurement are in agreement with previous reported articles [39, 40] that give spherical shape NPs but with larger particles size due to the different in preparation condition as concentration of materials.

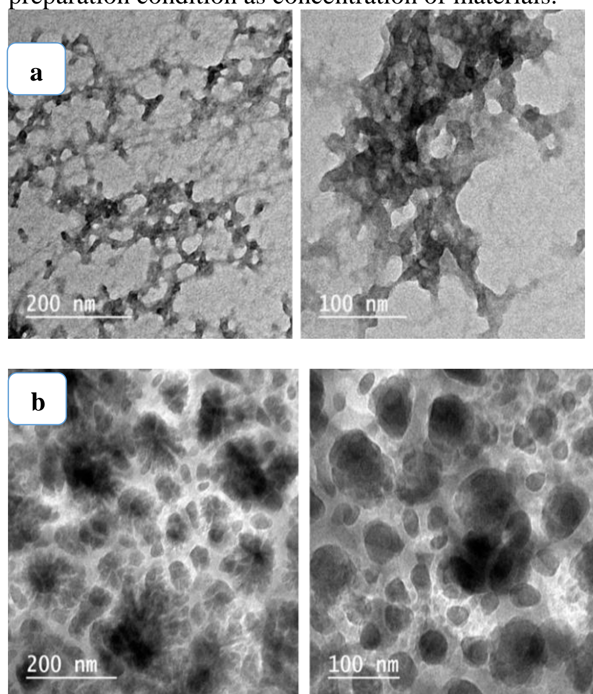

Figure (1) TEM micrographs of A) ChNPs and B) ChNPs@SA at two different magnification

3.1.2. Zeta potential and dynamic light scattering

The particle size obtained from DLS measurements for ChNPs, ChNPs@SA and ChNPs@Ca-Py were 98, 80 and 82 nm, respectively as shown from Figure 2. The relative larger particle size of the prepared nanoparticles in DLS rather TEM may be due to the DLS provides a hydrodynamic radius of nanoparticles and ChNPs are in swelled state in aqueous solution, whereas, TEM provide the dried diameter of nanoparticles [41]. In addition the TEM give an image for selected area for measurement while DLS give an overall image of the nanoparticles and their aggregations. The zeta potential of the prepared ChNPs, ChNPs@SA and ChNPs@Ca-Py are 25.6, 28.6 and 25.3 mV as presented in Figure 3. The obtained results indicated that there is no great difference between the zeta potential of the measured samples and the zeta potential no affected by presence of organic compound as Salicylic acid or Ca- pyruvate.

3.2. Induction of callus cultures

Table 2 illustrates the effects of different combinations of PGRs on callus initiation. Data clearly shows that the leaf explants of *P. americana* induced 100% of white callus on all tested MS media supplemented with PGRs. The control medium without PGRs gave a negative response. Growth regulators that are often used in tissue culture to initiate callus and increase the production of secondary metabolites (organogenesis) are auxins and cytokinins [42].

The highest mean fresh weight of callus was 215mg/jar on MS medium supplemented with 3.0 mg/L 2,4-D+1.0 mg/L NAA+1.0 mg/L AA+0.5 mg/L BA (Fig.1), followed by MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA (196.67mg/jar). On the contrary, the lowest mean fresh weight of callus (152mg/jar) on MS medium contains 1.0 mg/L NAA and 0.5 mg/L BA. It can be concluded that 2,4-D played an important role in callus initiation by comparing the effects of different tested concentrations of PGRs on callus growth. It could be noticed that callus fresh weights gradually increased with increasing the concentration of 2,4-D and NAA from 1.0 to 3.0 mg/L. Previous studies have shown 6-BA (as a cytokinin) at a certain concentration can promote callus subculture, differentiation and regeneration in most plants [43].

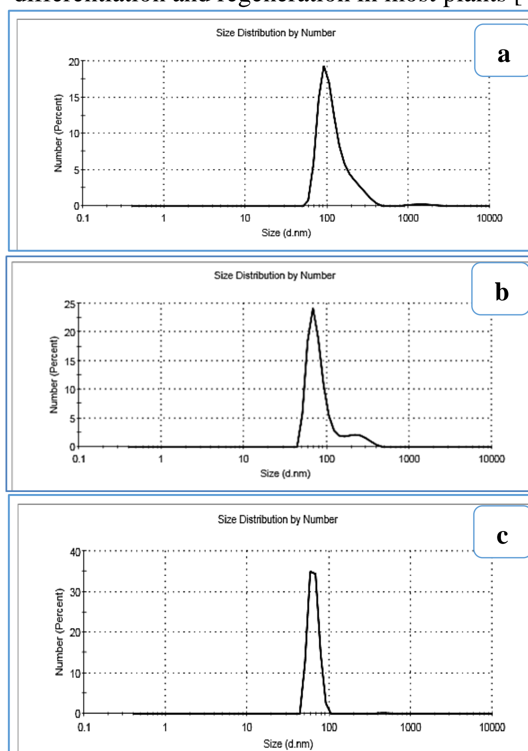

Figure (2): Particles size of a) ChNPs, b) ChNPs@SA and c) ChNPs@ Ca-Py

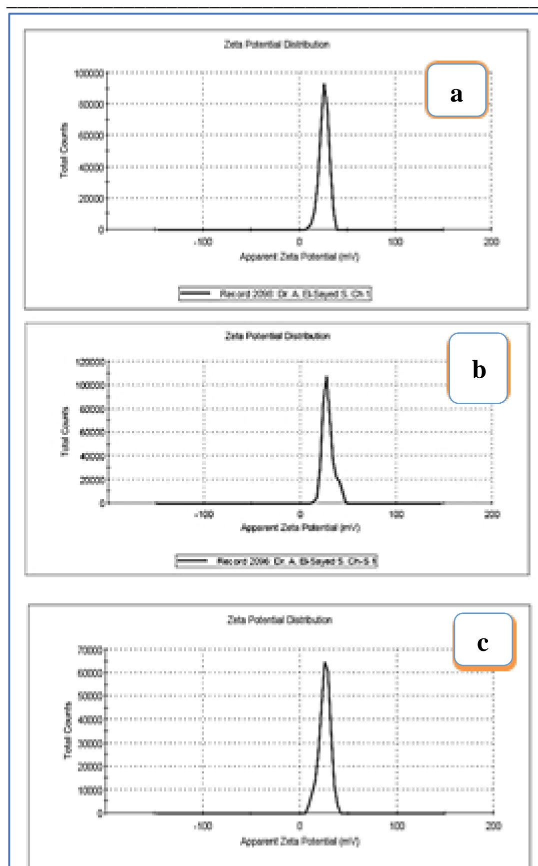

Figure (3): Zeta potential of a) ChNPs = +25.6, b) ChNPs@SA = +28.6 and c) ChNPs @ Ca-Py = +25.3

Jamshieed reported a high callusing percentage of *T. officinale* leaf explants with BAP and IAA combinations in concentrations ranging from 1.0 to 2.0 mg/L after 4 weeks of induction [44]. It was also noticed that the high concentration of 2,4-D and NAA decreased the mean fresh weight of the callus. It was found that higher concentrations of 2,4-D inhibit callus proliferation and lower concentrations allow morphogenesis to take place. Therefore, in order to reduce the inhibitory effect of high concentrations of 2,4-D, it is wise not to use high concentrations of 2,4-D in the callus induction process of *R. hybrida* [45]. The lack of ability to generate callus by all three explants may be due to the increase in PGR levels in the seaweed when the exogenous PGRs were introduced as in the study conducted by [46]. Similar results were obtained by several authors, who reported that the induction rate in relation to the combination of PGR (auxin and cytokinin) greatly depends on the type of combination, which varies alongside genotype.

Optimal callus induction could be obtained through manipulating 2,4-D concentrations and the duration of its presence in the induction medium [47], as different genotypes respond differently to varying 2,4-D levels [48]**.** This is attributed to the different roles of auxins and cytokinins in plant cell division and growth. Reports on the role of PGR indicate that auxins regulate DNA replication while cytokinins control cell processes, including mitosis and cytokinesis [49, 50]. The plant growth regulators and their concentrations have obvious effects on callus initiation and development in cotton [51]. It is well known that auxins play an important role in callus formation and the different types of auxins have various effects, as reported by [52]. The superiority of 2,4-D is supported by [53]**.** This study is in broad agreement with [54], who reported that the highest callus fresh weight of *S. rebaudina* was recorded for leaf explant using 2.0 mg/L 2,4-D and 2.0 mg/L NAA). The maximum callus growth was found with auxins such as 2,4-D and NAA and also with BA among the cytokinins [55]**.** Also, Agarwal and Kamal [56] reported that the presence of 2,4-D has been shown to be essential for callus formation in *Momordica charantia*.

Table (2): Callus initiation from leaf segments of avocado on MS medium in addition to different concentrations of 2,4-D, NAA, IAA and BA.

PGRs concentration (mg/L)				Callus induction rate (%)	Callus fresh weight (mg)	Texture of callus	Color of callus
2,4-D	NAA	IAA	BA				
0.0	0.0	0.0	0.5	0.0	0.00 ± 0.00 f		
1.0	0.0	0.0	0.5	100	175.33± 6.49 cd	Nodular	Creamy to yellowish
2.0	0.0	0.0	0.5	100	196.67 ± 1.202 b	Nodular	White
3.0	0.0	0.0	0.5	100	196.67 ± 6.01 b	Friable	white
4.0	0.0	0.0	0.5	100	185.33 ± 5.21 bc	Compact	Yellowish white
0.0	1.0	0.0	0.5	100	152.00 ± 6.25 e	Nodular	whitish to yellowish
0.0	2.0	0.0	0.5	100	166.33 ± 6.64 de	Nodular	Yellowish white
0.0	3.0	0.0	0.5	100	180.67 ± 2.33 b-d	Friable	Yellowish white
0.0	4.0	0.0	0.5	100	180.67 ± 2.33b-d	Compact	Yellowish white
0.0	0.0	2.0	0.5	100	186.33+. 2.33 bc	Friable	white
0.0	0.0	4.0	0.5	100	196.67+. 5.24 b	Friable	Whitish to yellowish
3.0	1.0	1.0	0.5	100	215.00+. 8.66 a	Compact	white
4.0	1.0	1.0	0.5	100	190.00+.3,00 bc	Compact	White

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.

Figure (4): Initiated callus of *P. americana* on MS medium supplemented with 3.0 mg/L 2,4-D, 1.0 mg/L NAA + 1.0mg/L IAA+ 0.5 mg/L BA

3.3. Growth and biochemical markers of avocado callus:

In Table (3), elicitors were added separately to culture (MS) medium supplemented with the best PGRs combination for callus proliferation of 3.0 mg/L 2,4-D +1.0 mg/L NAA +1.0 mg/L IAA+ 0.5 mg/L BA and augmented with different concentrations of the elicitors; ChNPs, ChNPs@Met, ChNPs@PA, ChNPs@Ca-Py and ChNPs@SA. In the growth parameters, the highest mean value of fresh weight of callus (12.29g/explant) was obtained on MS medium supplemented with ChNPs@Met followed by ChNPs@PA which gave 10.90g/explant. However, maximum value of dry weight was reported in treatment with ChNPs@Ca-Py (0.4g/explant) followed by ChNPs@SA (0.38g/explant), and the lowest record of dry weight was obtained by control. The role of Met in growth was well established by [23] on wheat and [22] as Met is a substrate and a methyl donor for several biochemical pathways inside plant cell.

Concerning biochemical markers, the lowest value of lipid peroxidation product (MDA) was achieved by ChNPs (100mg/L) followed by ChNPs@Met. In the same Table, the maximum mean record of antioxidant capacity % of the synthetic radical DPPH was achieved by ChNPs@SA due to the presence of essential oil component 3,4-Dihydro-2H-1,5-(3"-T-Butyl) Benzodioxepine with 40.88%, then followed by ChNPs@Ca-py 40.09% for the same bioactive metabolite. The content of non-enzymatic antioxidant glutathione reduced was also exhibited in Table (3) and the maximum value was detected by ChNPs@Met which has highest fresh weight of callus and minimum MDA content. The results have the same behaviour with [28], using modified ChNPs with PA and coumarin on *Silybium marianum* callus. Generally, using NPs enhanced the antioxidant capacity, GSH content and decreased the pool of MDA in some plants like our previous literature on *Stevia rebudiana* callus [57].

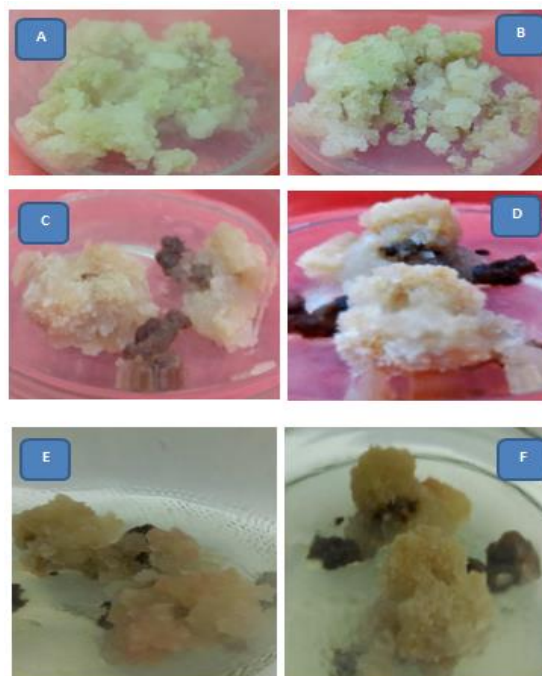

Figure (5): Callus of *P. americana* on MS medium supplemented with 3.0 mg/L 2,4-D +1.0 mg/L NAA+ 1.0 mg/L IAA + 0.5 mg/L BA augmented with: A) control, B) ChNPs 100 mg/L, C) ChNPs@Met, D) ChNPs@PA, E) ChNPs@Ca-Py and F) ChNPs@SA

3.4. Separation of polyphenols from avocado callus by HPLC:

In Table (4) the high performance liquid chromatography separated about 16 polyphenols as following: (gallic acid, chlorogenic acid, catechin, methyl gallate, coffeic acid, syringic acid, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, daidzein, quercetin, cinnamic acid and kaempferol) from avocado callus treated with (ChNPs, ChNPs@Met, ChNPs@PA, ChNPs@Ca-Py, ChNPs@SA). An astonishing increase in gallic acid, ellagic acid was achieved by ChNPs@SA, chlorogenic acid, catechin, methyl gallate maximum values were detected by the same treatments and those bioactive compounds were not detected in control and some applied treatments. The polyphenols syringic acid and rutin were detected only in ChNPs@SA treated callus. The coumaric acid appeared only in control and decreased in its level in ChNPs@PA. The vanillin was detected only by ChNPs@PA and disappeared from control and other treatments. Ferulic acid was detected in ChNPs and ChNPs@Met. The flavonoid naringinin was detected at ChNPs, ChNPs@Met, ChNPs@PA and ChNPs@

SA and the latest treatment recorded the superior value of such phenolic compound.

Naringenin biosynthesized from phenylalanine as a precursor, it is a flavonoid that corresponds to the flavanones category. It may be found in a variety of Citrus fruits, bergamot, tomatoes, and other fruits, as well as in its glycosides form (mainly naringin). This phytochemical has been linked to a number of biological activities, including antioxidant, anticancer, antiviral, antibacterial, anti-inflammatory, antiadipogenic, and cardioprotective properties [60]. Naringenin has also been proposed as a potential new therapy for the treatment of hepatitis C virus (HCV) infection. Indeed, in primary human hepatocytes and mice, this flavanone has been shown to lower HCV release by 80% in infected cells at a concentration below the lethal threshold [61].

Daidzein highest record was recorded by ChNPs@Ca-py and not detected in the control. Daidzein is a nutrient-dense isoflavone that is mostly derived from soy plants. Because of its structural resemblance to the human hormone estrogen, it is also known as phytoestrogen. Daidzein has been attributed to the prevention and treatment of a number of illnesses, including cancer, cardiovascular disease, diabetes, osteoporosis, skin disease, and neurodegenerative disease. Various metabolites, including as equol and trihydroxy isoflavone, are responsible for this pharmacological effect [62].

Cinnamic acid was detected in ChNPs and ChNPs@Met and ChNPs@Ca-Py. The Kaempferol flavonoid was detected in all applied treatments except control and the highest value was achieved ChNPs@SA. These results of ChNPs application were similar to [28] on *Silybium marianu*m callus. ChNPs@SA was the best treatment in accumulation of most polyphenolic compounds and highest antioxidant capacity when compared to control and other treatments in Table (3). This may need further investigations on anticancer activity.

Table (3): Effect of chitosan nanoparticles and its encapsulated organic substrates on avocado callus growth and biochemical markers.

Treatments	Callus growth		Biochemical markers		
	Fresh weight (g)	Dry weight (g)	Malondialdehyde nmol/gm fresh weight	Antioxidnt capacity %	Glutathione reduced µmol/gm fresh weight
Control	7.35 ± 0.54 d	0.09 ± 0.008 d	7.69±0.099 a	76.39 ± 0.178 f	1.797 ± 0.101 d
ChNPs	8.89 ± 0.45 c	0.18 ± 0.009 c	**0.663 ± 0.158 d**	79.60 ± 0.221 d	5.20 ± 0.100 c
ChNPs@Met	**12.29 ± 0.26 a**	0.21 ± 0.015 bc	**0.993 ± 0.049 d**	82.75 ± 0.182 c	**16.82 ± 0.445 a**
ChNPs@PA	10.90 ± 0.43 ab	0.18 ± 0.009 c	1.070 ± 0.06 d	79.06 ± 0.105 e	**6.37 ± 0.406 b**
ChNPs@Ca-Py	8.47 ± 0.57 cd	**0.40 ± 0.044 a**	1.987 ± 0.075 c	84.42 ± 0.032 b	4.97 ± 0.431 c
ChNPs@SA	10.42 ± 0.44 b	0.28 ± 0.038 b	6.01± 0.221 b	**88.36 ± 0.238 a**	**1.66 ± 0.285 d**

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.

Table (4): Effect of chitosan NPs and encapsulated organic substrate in polyphenols separated by HPLC of avocado callus.

Polyphenols	RT (min)	Chitosan nanoparticles and its conjugates application					
		Control	ChNPs	ChNPs@ Met	ChNPs@PA	ChNPs@ Ca-Py	ChNPs@SA
		Concentration µg/g					
Gallic acid	3.44	35.16	40.82	32.24	25.70	37.52	46.29
Chlorogenic acid	4.26	N.D	5.92	6.79	17.48	26.45	27.95
Catechin	4.64	N.D	N.D	5.00	14.99	12.74	70.00
Methyl gallate	5.59	N.D	2.54	0.74	2.55	N.D	19.22
Coffeic acid	6.05	N.D	2.10	1.63	2.81	6.02	3.48
Syringic acid	6.56	N.D	N.D	N.D	N.D	N.D	4.09
Rutin	7.97	N.D	N.D	N.D	N.D	N.D	6.36
Ellagic acid	8.84	3.92	10.29	10.29	11.19	4.73	17.57
Coumaric acid	9.09	1.77	N.D	N.D	0.68	N.D	N.D
Vanillin	9.691	N.D	N.D	N.D	1.04	N.D	N.D
Ferulic acid	10.30	N.D	0.99	0.99	N.D	N.D	N.D
Naringenin	10.72	N.D	4.58	4.58	4.63	N.D	32.95
Daidzein	12.98	N.D	1.20	1.20	1.29	1.93	1.33
Quercetin	13.66	N.D	N.D	N.D	2.55	1.66	N.D
Cinnamic acid	14.98	N.D	0.29	0.29	N.D	0.43	N.D
kaempferol	16.20	N.D	3.29	3.29	2.02	1.78	6.03

- **RT referred to retention time**
- **N.D (not detected) is referred to less than the detection limit of the device.**

3.5. Identification of fatty bioactive metabolites:

The GC-MS analysis has shown the presence of different bioactive compounds in the chloroform callus extract of avocado. The analysis revealed the presence of 25 bio-constituents represented in Table (5) as following: Trichloromethane, dichloromethyl

ethyl sulfone, N,N-Dimethyl-2H-pyran-2-IM Inium chloride, Docosane, Eucalyptol (monoterpenoids), Methane, oxybis[dichloro], Hexanoic acid, 2-Methyl-3-oxo-, Ethyl ester, 3-oxo-20-methyl-11-à-hydroxyconanine-1,4-diene, 2,2,3,3,4,4 hexadeutero octadecanal, 3,4-dihydrO-2H-1,5-(3"-T-BUTYL)Benzodioxepine,2-((2S,4aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,7 octahydronaphthalen-2-yl) propan-2-ol, Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)- or Norgestrel, trimethylsilyl ether, Hexadecanoic acid, 2,3-Dihydroxypropyl ester, pentadecanoic acid, Oleic Acid, 7-Methyl-Z-tetradecen-1-ol acetate, Hexadecanoic acid, methyl ester, 2-Hydroxy-3-[(9E)-9-octadecenoyloxy]propyl, 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 4H-1-Benzopyran-4-one, 2-(3,4-Dimethoxyphenyl)-3,5-Dihydroxy-7-Methoxy-2-hydroxy-3-[(9e)-9-octadecenoyloxy]propyl(9E)-9 Octadecenoate,9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E) Octadecanal, 2-bromo-, Oleic acid, 3-(Octadecyloxy) propyl ester, Oleic acid, Eicosyl ester.

From the results, it is evident that avocado 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 evaluates the chemicals eluted at various times to determine their nature and structure, resulted in the emergence of peaks at various m/z ratios. These mass spectra are chemical fingerprints that may be detected using the data library.

Concerning the effect of ChNPs, ChNPs@Met, ChNPs@PA, ChNPs@Ca-Py and ChNPs@SA, on the area percent of these compounds data revealed that all treatments had an enhancing impact in increasing the area percent of most phyto-constituents compared with the control Table (6). From the results, data showed that the bioactive compounds with maximum peak area in callus extract are the trichloromethane that resulted from the steps of callus preparation for further analysis in GC mass, 3,4-Dihydro-2H-1,5-(3"-T-Butyl) Benzodioxepine with molecular formula, $C_{13}H_{18}O_2$ and molecular weight 206 is from essential oils in plant and has antioxidant activity. Hexadecanoic acid, methyl ester with molecular formula, $C_{17}H_{34}O_2$, molecular weight 270, has antioxidant properties too [63]. From the results, it is evident that, avocado contains various bioactive compounds.

Concerning NPs treatments, in Table (6) it is clear that, application of ChNPs increased most bioactive metabolites when compared to the control except compounds at the retention times (5.54, 21.52, 25.69, and 29.93). In case of ChNPs@Met, it is evident that this treatment enhanced the accumulation as are percent of the bioactive constituents except for the retention times (14.21, 19.86, 21.52, 24.91, 29.46, 35.58, and 37.96). At the application of ChNPs@PA, most active compounds were enhanced except the retention times (21.52, 25.69, 29.93, and 37.96). On the contrary, the application of ChNPs@Ca-Py and ChNPs@SA has the same action where they increased all bioactive compounds except the retention time (29.93). In this concern, GC-mass carried out the chemical profiling of avocado callus as its main secondary metabolite are fatty compounds and monoterpenes which act as antioxidants, anticancer and have profitable characteristics.

Table (5): Effect of chitosan NPs on identified bioactive fatty compounds of avocado callus.

PN	RT	Compound Name	Molecular Formula	Molecular Weight
1	5.08	Trichloromethane	$CHCl_3$	118
2	5.37	Dichloromethyl ethyl sulfone	$C_3H_6C_{l2}O_2S$	176
3	5.54	N,N-Dimethyl-2h-pyran-2-im inium chloride	$C_7H_{10}ClNO$	159
4	7.45	Docosane	$C_{22}H_{46}$	310
5	8.08	Eucalyptol	$C_{10}H_{18}O$	154
6	8.17	Methane, oxybis[dichloro]	$C_2H_2C_{l4}O$	182
7	8.26	Hexanoic acid, 2-methyl-3-oxo-, ethyl ester	$C_9H_{16}O3$	172
8	14.21	3-Oxo-20-Methyl-11-à-Hydroxyconanine-1,4-Diene	$C_{22}H_{31}NO2$	341
9	19.86	2,2,3,3,4,4 Hexadeutero octadecanal	$C_{18}H_{30}D_6O$	274
10	21.52	3,4-Dihydro-2h-1,5-(3"-t-butyl)benzodioxepine	$C_{13}H_{18}O_2$	206
11	23.37	2-((2S,4aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl) propan-2-ol	$C_{15}H_{26}O$	222
12	23.65	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)- Or Norgestrel, trimethylsilyl ether	$C_{27}H_{42}O_4$ C24H36O2Si	430 384
13	24.91	Hexadecanoic acid, 2,3-dihydroxypropyl ester	$C_{19}H_{38}O_4$	330
14	25.69	Pentadecanoic acid	$C_{15}H_{30}O_2$	242
15	27.1	Oleic Acid	$C_{18}H_{34}O_2$	282
16	29.46	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268
17	29.93	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
18	32.16	2-Hydroxy-3-[(9e)-9-Octadecenoyloxy]propyl	$C_{39}H_{72}O_5$	610
19	33.75	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{21}H_{40}O_4$	356
20	35.58	4H-1-benzopyran-4-one, 2-(3,4-Dimethoxyphenyl)-3, 5-Dihydroxy-7-Methoxy-	$C_{18}H_{16}O_7$	344
21	37.96	2-Hydroxy-3-[(9e)-9-octadecenoyloxy]propyl (9e)-9-octadecenoate	$C_{39}H_{72}O_5$	620
22	39.06	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)	$C_{57}H_{104}O_6$	884
23	39.2	Octadecanal, 2-bromo-	$C_{18}H_{35}BrO$	346
24	40.9	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	592
25	40.99	Oleic acid, eicosyl ester	$C_{38}H_{74}O_2$	562

Table (6): Effect of chitosan NPs on bioactive fatty metabolites area percent of avocado callus.

PN	RT	Area percent %					
		Control	**ChNPs**	**ChNPs@Met**	**ChNPs@PA**	**ChNPs@Ca-Py**	**ChNPs@SA**
1	5.08	23.29	44.95	9.89	41.21	42.34	37.1
2	5.37	0.53	1.35	0	1.27	1.78	1.42
3	5.54	1.73	0.84	0	0	0	0
4	7.45	1.27	0	24.19	0	0	1.42
5	8.08	1.21	2.93	0	2.71	0	1.83
6	8.17	0.62	0	0	0	0	1.59
7	8.26	1.21	0	1.36	0	0	1.57
8	14.21	1.84	2.84	0.95	3.34	3.34	3.04
9	19.86	1.89	2.36	0.89	4.86	2.83	2.55
10	21.52	40.02	30.98	1.07	28.36	40.09	40.88
11	23.37	0	0	33.37	4.51	0	0
12	23.65	0	0	0.66	0	0	0
13	24.91	2.1	2.65	0.99	2.12	3.41	2.91
14	25.69	6.13	2.83	5.53	4.82	0	0
15	27.1	0	1.62	0.94	0	0	0
16	29.46	1.4	2.97	1.04	0	0	1.98
17	29.93	7.92	3.69	9.36	5.74	6.21	2.61
18	32.16	0.54	0	4.25	0	0	0
19	33.75	0.9	0	2.02	0	0	0
20	35.58	3.06	0	0.54	0	0	0
21	37.96	1.67	0	0.54	1.06	0	0
22	39.06	0.43	0	0	0	0	0
23	39.2	0.63	0	0.56	0	0	1.1
24	40.9	0.72	0	0.91	0	0	0
25	40.99	0.89	0	0.95	0	0	0
Total		100	100	100	100	100	100

3.6. AFLP markers

AFLP analysis using pairs of primers EcoR I-ACA and MseI – CTC provided a total of 99 bands ranging from 1470 to 120 bp in (Fig.7). About 87 out of 99 bands were polymorphic, 12 of them were monomorphic bands and 53 were polymorphic without unique bands with total 88 % polymorphism. On other hand specific markers based on AFLP were detected between treatments and control as shown in Table (7) which generated by two unique markers in control followed by five markers in treatment one (ChNPs), four markers in treatment two (ChNPs@Met), while the highest markers were eleven in treatment three (ChNPs@ PA), three markers in treatment four (ChNPs@ Ca-Py) and nine markers in treatment five (ChNPs@ SA).

In addition the genomic template stability (GTS) value was calculated for each treatment, which appeared the highest stability number (30%) in both of treatments number one (ChNPs) and two (ChNPs@ Met) while the lowest stability number showed (15%) in treatment three (ChNPs@ PA) as shown in Table (8)

The AFLP profile enabled us to discriminate between all the treatments for studying genetic variability and stability and showed that there are bands that appear in all treatments (common bands). However, other bands were present in some treatments and absent in others (polymorphic), while a unique marker (specific band) appeared within treatments. The appearance of some polymorphic bands and specific unique bands indicated a direct relationship between ChNPs treatments and the genetic gene expression action reflected by treatments.

In general, the survey study of a number of polymorphic and specific unique bands, in addition to the genetic stability value (GTS), which refers to the high ability of an AFLP profile to reveal most of the information in a single locus and can be used for molecular genetic analysis, gives rise us to understand and conclude the genetic translation action of avocado callus treated with ChNPs. We were able to successfully identify significant molecular markers and adequate distinctions among the treated avocado samples under the five NPs treatments and control using bands of DNA markers at the molecular genetics level, which were complemented with other biochemical analysis data and found to have great relevance, such as antioxidants and glutathione content.

These results agreed with those Karp *et al.* [58] who reported that AFLP technique is rapidly becoming the method of choice for estimating genetic action in both cultivated and treatments. Neqi *et al.* also used AFLP markers to study inter- and intraspecific genetic variations using some species which is an important wild medicinal plants [59]. They suggested that AFLP marker is as an efficient tool for estimating genetic effective markers under treatments application which reflect the genetics expression of transcription and translation rate during the treatments through coding and alter some active ingredient genes pathway like in our study.

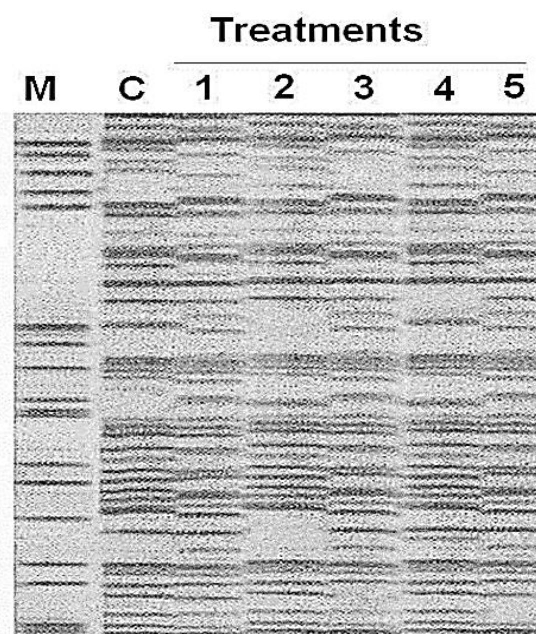

Figure (6): AFLP amplification profile generated from genomic DNA of six samples of avocado under five treatments and control (Where; M: Marker, (C): Control, (1): ChNPs, (2): ChNPs@Met, (3): ChNPs@PA, (4): ChNPs@Ca-Py, and (5): ChNPs@SA)

Table (7): A List of specific markers of avocado callus for AFLP marker profile with number of total bands in treatments and their markers molecular weights (MW) in bp.

Samples	Treatments	Total bands	Marker number	Molecular weights (bp)
(1)	**Control**	**40**	**2**	**1209, 544**
(2)	**ChNPs**	**40**	**5**	**1181, 753, 379, 320, 231**
(3)	**ChNPs@Met**	**40**	**4**	**1343, 753, 580, 280**
(4)	**ChNPs@PA**	**46**	**11**	**1215, 1116, 1085, 742, 636, 522, 496, 376, 367, 286, 138**
(5)	**ChNPs@Ca-Py**	**41**	**3**	**1306, 775, 188**
(6)	**ChNPs@SA**	**44**	**9**	**1157, 1055, 964, 736, 488, 408, 361, 207, 182**

Table (8) Genomic template stability parentage value against AFLP profile

AFLP profile	Genomic template stability %				
	ChNPs	ChNPs@Met	ChNPs@PA	ChNPs@Ca-Py	ChNPs@SA
	30	30	15	27	20

Conclusion

These findings serve as a stepping stone for additional biological study into avocado (*Persea americana*) as a medicinal plant rich in bioactive compounds by utilizing ChNPs or organic substrates encapsulated in ChNPs. ChNPs@Met is indicated for high fresh weight and glutathione concentration, however ChNPs@SA is provide maximal antioxidant capacity, polyphenolic compounds, and fatty bioactive component accumulation. In the future, we may be able to synthesise ChNPs encapsulated a combination of the two active compounds for obtaining bioactive valuable secondary metabolites enriched with antioxidants.

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Conflict of Interests

Authors do not have any conflict of interest.

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