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Biosynthesized Selenium Nanoparticles from Micropropagated *Plectranthus Amboinicus* Extract Revealing Antibacterial Activity

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Abstract

Green synthesis of selenium nanoparticles using an aqueous extract of *in vitro*-derived *Plectranthus amboinicus* leaves has been biosynthesized. To produce in-vitro-derived plants of *P. amboinicus* to act as an efficient reductive solution for biosynthesizing SeNPs, a new tissue culture protocol was conducted using *Spirulina plantensis* extract as a natural growth regulator and secondary metabolites elicitor compatible with the adequate concentration of carbon source. Reaching the highest shoot length at 9.0 ± 0.42 and the maximum number of leaves per shoot at 15.0 ± 0.57, the enhancement of total phenolic contents revealed the highest level at 96.4 mg/g on MS supplemented with 1.0 mg of *S. plantensis* extract and 20 gm of sucrose. The enhancement of total phenolic compounds resulted in biosynthesized SeNPs with spherical-like shape and an average diameter of 17.9 ± 1.34 which was verified with Edx, SEM, TEM, and FTIR analyses. The biosynthesized SeNPs revealed antibacterial activity against the oral pathogens *Escherichia coli* and *Staphylococcus aureus* indicating the potential of biosynthesized selenium nanoparticles to be future alternatives to antibiotics losing their activity over time and to be combined with laser applications to intensify their activity in enchanting material delivery to targeted tissues and organs.

Keywords: Biosynthesized SeNPs, *Plectranthus amboinicus, Spirulina plantensis.*

1. Introduction

Selenium (Se) exists in different forms in the environment as ionic selenite (Na_2SeO_3) and selenate, solid-state Se, selenocysteine, and selenomethionine [1, 2]. There is a maximum probability of successfully synthesizing the selenium nanoparticles by using plant extracts that are rich in polyphenols, flavonoids, alkaloids, polysaccharides, saponins, etc. since they are very good reducing and stabilizing agents for nanoparticle preparations. Several research experiments reported the content of phytochemicals contained in prepared plant extracts such as total phenolics and flavonoids [3, 4, 5] tannins [3] and polysaccharides [5]. Moreover, the screening of phytochemicals shows qualitatively the presence of main active compounds [6, 7, 8]. Plant extracts such as Hawthorn [9] Lemon plant [10], Ginger fruit [11], Ashwagandha [3], Avaram [12], Java tea [6], Garlic [13], Lavender leucas [14] and Aloe vera [15] were succeeded as reductive reagents for synthesizing of selenium nanoparticles. Recently preparation of nanoparticles from *in-vitro* derived plants was conducted to record the advantages of using tissue culture techniques for different applications of nanotechnology, *in-vitro* derived plants and callus cultures of *Costus speciosus* extract were used in the rapid biosynthesis of stable silver nanoparticles (AgNPs), and the synthesized silver nanoparticles were found to be highly toxic against different multi-drug resistant such as bacteria *Bacillus subtilis* and *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* compared to selenium nanoparticles (SeNPs) possess antibacterial activity towards the same strains but nontoxic. Cultivation of plant cells, tissues, and organs *in- vitro* has an important role in plant biotechnology and the improvement of many crops, endangered, medicinal, and economically valuable plants. Clonal multiplication *in-vitro* offers scalable options for large-scale plant propagation, conservation, and sustainable utilization [16, 17]. Elicitation is an applicable method for improving the production of secondary metabolites in in vitro cultures. Several investigations for *in-vitro* micropropagation of *Plectranthus species*, specifically *P. amboinicus*, have been reported [18-28]. The current research aimed to study the effects of different treatments of *Spirulina platensis* extract combined with the different concentrations of sucrose on shoot induction and propagation of *P. amboinicus* followed by evaluation of total phenolics for biosynthesizing of selenium nanoparticles using the produced extracts of in-vitro derived *P. amboinicus* applying on antibacterial activity assaying of the produced biosynthesized SeNPs against two common oral pathogens *Escherichia coli* and *Staphylococcus aureus.*

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2. Materials and methods

2.1 Plant Materials and Explants Preparation

Shoots of the *Plectranthus amboinicus* were harvested from plants maintained in the greenhouse at the National Research Centre, Egypt, which was full of healthy growth. The excised shoots were thoroughly cleaned in water for 10 minutes to eliminate any dust and dirt, then rinsed thoroughly under running tap water. Shoot were cut into pieces 2-3 cm in size and were washed with distilled water three times. Under the aseptic conditions in a biosafety laminar-air flow hood, the next sterilization steps were conducted. The washed pieces were immersed into 0.1 % (w/v) mercury chloride ($HgCl_2$) solution for 30 sec., followed by three times washing by distilled water to eliminate any residues of $HgCL_2$. The sterilized shoots (nodal segments) were sterilized with sodium hypochlorite 20% for 5 to 10 mins and then washed three times with sterilized distilled water.

2.2 Culture Media and Growth Condition

The sterile nodal segments were cut into pieces measuring 0.5–0.7 cm in length and cultured on plant MS basal medium with vitamins [29] containing 3 % (w/v) sucrose, and 0.8 % (w/v) agar. The pH was adjusted at 5.7. The explants were transferred to the incubator under growth conditions with a photoperiod of 16 h light and 8 h dark at 22±2°C for 6 weeks.

2.3 Experimental Treatments of *Spirulina platensis* Extract

After six weeks, the shoot-lets were revealed and transferred to full-strength MS medium with vitamins solidified by 8 g/L agar and supplemented at three different concentrations of sucrose (10, 20, 30) g/L and three different concentrations of *Spirulina platensis* extract (0.5, 1.0, 1.5) mg/L (table 1). After 6 weeks of culture, plant height, and number of leaves were recorded to determine the best treatment for morphological parameters.

Table 1. Different treatments of *Spirulina plantensis* extract and Sucrose

Treatment	*Spirulina* Extract Concentrations (mg/L)	Sucrose (g/L)
M0	Basel MS+ Vitamins without *S. plantensis* extract	30
M1	0.5	10
M2	1.0	10
M3	1.5	10
M4	0.5	20
M5	1.0	20
M6	1.5	20
M7	0.5	30
M8	1.0	30
M9	1.5	30

2.4 Sample preparation and determination of total phenolic contents

The best treatment which developed shoots with the highest shoot length and number of leaves per shoot from *in- vitro* treated *Plectranthus amboinicus* were recorded and selected for the determination of total the phenolic contents compared to control plants without any treatments. The *in-vitro* shoots plants were harvested after 2 months of culturing from the best treatment of *Spirulina platensis* extract contained the appropriate concentration of sucrose as a carbon source and in-vitro control plants which cultured on basal medium without any treatment of SE extract and contained the standard concentration of sucrose for *in-vitro* growing plants at 30 g/L. The harvested shoots were carefully cut into small pieces and dried in a ventilated oven at 40ºC for 3 days. After drying, the plants were ground in a domestic coffee grinder and sieved. The powdered samples were extracted for determination of total phenolics using the folin polyphenolic method [30].

2.5 Preparation of *in-vitro* Plant Extract

The plant extract was prepared by transferring 10 g of dried *in-vitro* shoots of *Plectranthus amboinicus* (collected from the best treatment of *Spirulina platensis* extract and carbon source) in a glass beaker contained 400 mL of sterile distilled water. The mixture was then boiled for 5 minutes. After cooling the mixture at room temperature, it was filtered with Whatman No.1 filter paper then removing biomaterials by centrifuging at 1200 rpm for 2 minutes. The extract was stored at room temperature to be used for further experiments.

2.6 Biosynthesis of Selenium nanoparticles (SeNPs)

Green synthesis of SeNPs was conducted by mixing of 20 ml of *in-vitro Plectranthus amboinicus* extract with 100 mM sodium selenite solution. The mixture was allowed to react for 2 hours till the color changed from colorless to light red under stirring conditions at 600 rpm Fig 1. After completion of the reaction, the synthesized SeNPs were collected by centrifugation at 3000 rpm for 15 mins. The Se Nano pellets were washed twice with distilled water, and then the selenium nano pellets were dried overnight.

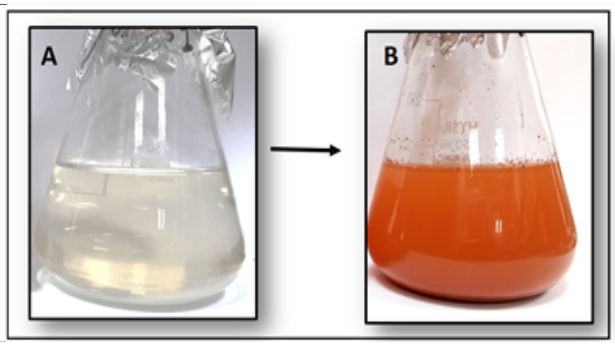

Fig.1 Schematic illustration of synthesized selenium nanoparticles using the extract of *in-vitro Plectranthus amboinicus* produced from the best treatment M5.

2.7 Characterization of the Reductive Selenium nanoparticles

The morphology of SeNPs was characterized and conducted at National Center of Radiation Research and Technology, Atomic Energy Authority, Egypt. The high magnification microscope SEM (Scanning Electron Microscopy) was imaged on the surface of a sample using scanning by electron beams in addition to revealing the elemental composition of the green synthesized (SeNPs) based on the conduction of EDX analysis. The sample was mounted into a sample holder or a stub and then inserted inside the microscope chamber to be analyzed. The sample was subjected to an energetic electron beam (25 keV) which results in emission of X-ray lines of the constituent elements. These X-ray lines were detected with an EDX detector attached to ZEISS - EVO 15 - UK scanning electron microscope. High Resolution Transmission Electron Microscopy was measured using JEOL (JEM-100CX).The sample was measured using FTIR-ATR spectrometer (Vertex 70 spectrometer manufactured by Bruker German to determine the functional groups present in the *in in-vitro Plectranthus amboinicus* extract and SeNPs.

2.8 Antibacterial assay

The assay was performed on two different bacterial strains *Escherichia coli* and *Staphylococcus aureus* isolated from the oral cavity of different patients and sub-cultured into nutrient agar plates before being incubated for 24 h at 37°C, then were activated by sub-culturing one more time to be prepared for the assay. Disk diffusion assay was conducted according to [31, 32] For this experiment, the solution of the biosynthesized SeNPs in two different concentrations was absorbed into the diffusion disks and then transferred to both bacterial culture-plated of the selected strains under aseptic conditions.

2.9 Statistical analysis

Randomized complete blocks of three replications were applied for the experimental design of this study. Statistical analyses were conducted using the program IBM SPSS (SPSS Inc; IBM Corporation, NY, USA) Statistics Version 25 (2017) for Windows. Data were inserted into ANOVA with a P-value of <0.05 being considered statistically significant. Means of the experimental treatments were compared by the least significant difference post-hoc test [33] with a P-value of <0.05 which is considerably statistically significant [34].

3. Results and Discussion

3.1 Explants and Shoot initiations

Different concentrations of sterilization agents and protocols were applied to start the *in-vitro* culture of *Plectranthus amboinicus* which recorded the highest contamination content on the plant surface (these data are not mentioned in this study), and the best sterilization protocol was applied to initiate *in-vitro* plant shoot culture establishment without any contamination. For initiation and shoot establishment of *Plectranthus amboinicus*, MS medium 4.4g/L [29], catalog No., 0222, Duchefa Com., Harrlem the Netherland) including vitamins and free of plant growth regulators were used for all cultures, and 30 g/l sucrose with 8 g/l agar for solidification was added to the medium. The medium pH was adjusted to 5.8 before autoclaving at 121°C and 1.1 kg/cm^2 for 25 mins. The cultures were incubated in a growth chamber at 25 + 2°C with a photoperiod of 16/8 hours (light/dark cycles). Each subculture took 8 months of incubation before transferring to the next experimented treatments Fig 2.

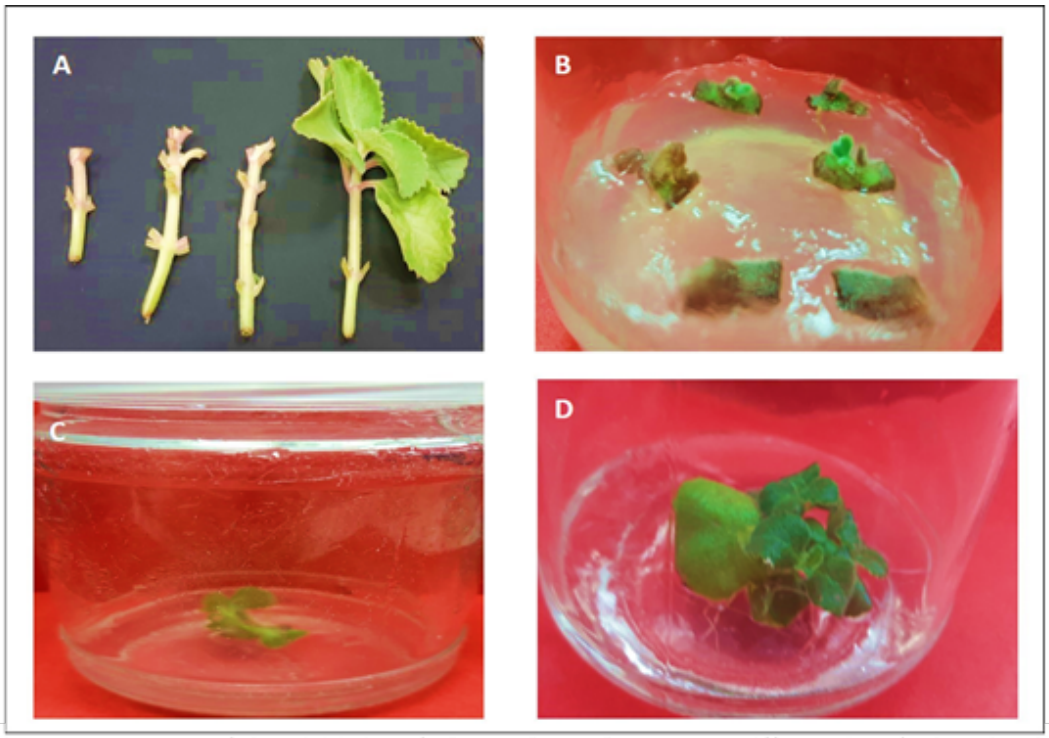

Fig 2. *In-vitro* stages of shoot initiation of *Plectranthus amboinicus*. A. Different size of selected nodal segments before cutting for *in-vitro* culturing, B. Shoot initiation of nodal segments cultured on MS basal medium after 2 weeks. C. The initiated shoot after culturing for 4 weeks and D. Shoot culturing after 6 weeks on basal medium.

3.2 Effects of different Treatments of *Spirulina platensis* extract and concentration of carbon source.

The effects of different treatments of *Spirulina platensis* extract and the concentration of sucrose on in-vitro shoots of *Plectranthus amboinicus* were studied in this research instead of the plant growth regulators which are synthetic and expensive to be used for several studies to establish micropropagtion protocols for commercial and large scale production. The influence of different concentrations of plant growth regulators Naphthalin Acetic Acid (NAA), Benzyl Adenine (BA), and Kinetin (Kin) on *Plectranthus amboinicus* to develop plant growth [35]. The crude extract of *Spirulina platensis* was used as a source of phytohormones, based on the HPLC analysis, the methanol extract of *S. platensis* contained gibberellins at 241.6, kinetin at 150.2, and adenine at 140.6 ppm with positive effects on the *in-vitro* growth of *Capparis cartilaginea* as recorded by Marwa *et al.* 2022 [36]. In this study, the highly significant results using *S. platensis* extract were also confirmed, the effects were implied not only on plant growth but also on the accumulation of total phenolic and the nodal segments of *P. amboinicus* responded differently *in-vitro* depending on the different concentrations of *Spirulina platensis* extract and sucrose as shown in Table 2. The highest shoot length and the maximum number of leaves per shoot were observed on M5 medium supplemented with 1.0 mg of *Spirulina plantensis* extract and containing 20 g/L sucrose at 9.0 ± 0.42 and 15.0 ± 0.57 respectively (Fig 3). The increase of the SE extract concentration of 1.5 mg with the same concentration of sucrose at 20 gm/l led to a remarkable decreasing the shoot length at 6.80 ± 0.65 and the number of leaves per shoot at 12.0 ± 1.00 to verify the specified response of the plant based on the contained phytohormones into SE extract.

Table 2. Effects of different Treatments of *Spirulina platensis* extract and concentration of sucrose on *in-vitro* growing of *Plectranthus amboinicus*

Treatment	Shoot Length (mean ± SE)	Leaves Number /Shoot (mean ± SE)
M0	5.0 ± 0.44	11.3 ± 0.33
M1	4.6 ± 0.25	9.0 ± 0.57
M2	5.5 ± 0.22	9.6 ± 0.33
M3	5.3 ± 0.39	9.0 ± 0.33
M4	8.5 ± 0.59	13.3 ± 0.33
M5	9.0 ± 0.42	15.0 ± 0.57
M6	6.80 ± 0.65	12.0 ±1.00
M7	6.5 ± 0.52	11.6 ± 0.88
M8	6.0 ± 0.27	12.33 ± 0.66
M9	5.9 ± 0.23	12.0 ± 0.57

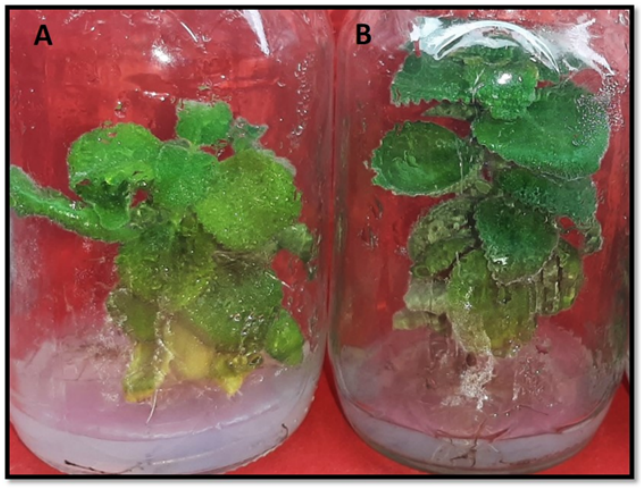

Fig 3. The difference of in-vitro growth of Plectranthus amboinicus cultured on the best treatment M5 (B) MS basal medium supplemented with 1.0 mg of Spirulina platensis extract and 20 g/l sucrose compared to M0 (A) control when shoot elongation occurred on MS basal medium without any treatments.

3.3 Total Phenolic contents

The effects of different treatments of *Spirulina Platensis* extract and the concentration of carbon source on in-vitro shoots of *Plectranthus Amboinicus* were implied not only on the plant growth but also on the accumulation of total phenolics content which is responsible for directly or indirectly increasing the reductive factoring for syntheses of nanoparticles with idealistic characteristics. As shown in Figure 4 the total phenolics content recorded the highest level at 96.4 mg/g on the M5 medium supplemented medium with 1.0 mg of *Spirulina Plantensis* extract and 20 gm of sucrose in comparison to the lowest level at 63.6 mg/g on control plants without any treatments and contained the standard concentration of sucrose at 30 gm/l which commonly used for *in-vitro* plant grow in laboratories. In comparison, the total phenolic content level at 81.23 mg/g was recorded by [37] when they based on Thidiazuron (TDZ) as a plant growth regulator for enhancement of the total phenolic contents. These results will lead the scientific experiments toward using natural extracts from different sources specifically Algae extracts in combination with appropriate concentrations of carbon sources to develop *in-vitro* plants with high levels of phytoconstitutents.

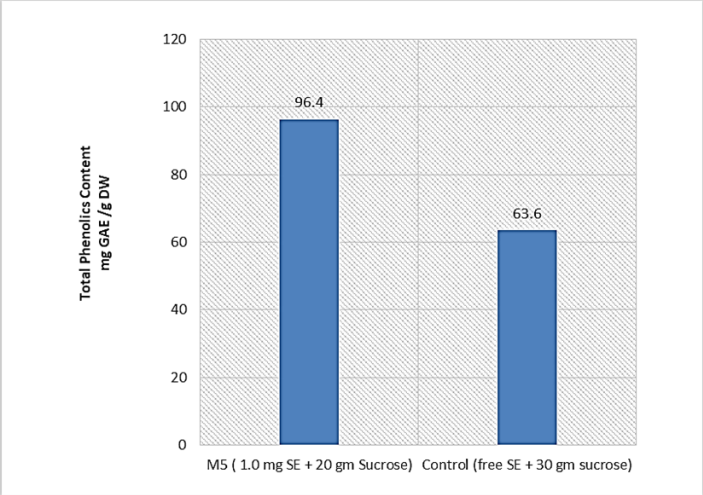

Fig. 4. Total phenolic content in the *in-vitro* shoots of *Plectranthus amboinicus* on M5 medium compared with those of control plants.

3.4 Characterization of the *in-vitro* derived green mediated selenium nanoparticle

SEM and TEM were conducted to analyze the size and morphology of *in-vitro* green synthesis of the SeNPs. The morphology and the elemental composition of SeNPs were observed using SEM and EDX studies as shown in Figs. 5B and 5C. The electron micrograph revealed semi-spherical like shaped particles with a smooth surface. Verifying with TEM analysis Fig. 5A revealed the average diameter of 17.9±1.34 of Se NPs which were synthesized using an aqueous extract of *in-vitro* derived *Plectranthus amboinicus* leaves and stems that were harvested from the best treatment of SE extract. The elemental composition as demonstrated by EDX spectra revealed a strong signal for selenium and a very weak carbon signal might have resulted from the grid of oxides during sample preparation.

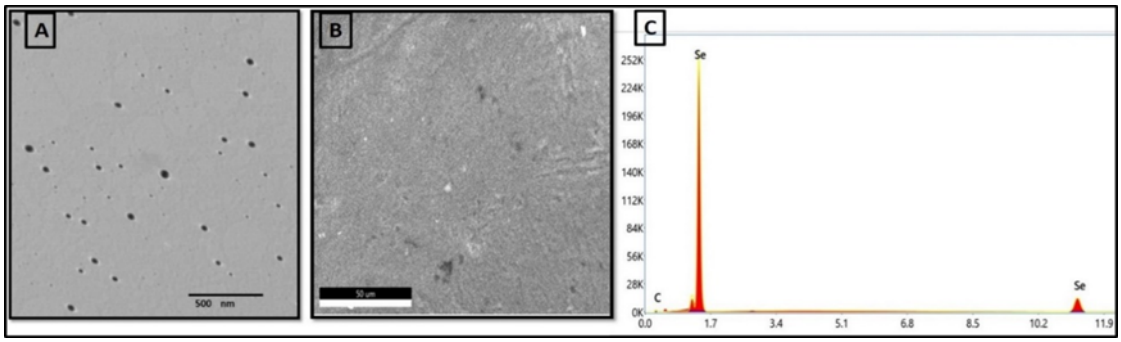

Fig.5 showing the characteristic of the green synthesized nano selenium. 5A TEM analysis revealed the semi-spherical like-shaped particles with a smooth surface and dimeter particle size from 6 to 37 nm, Fig 5B presented the electron micrograph of SEM analysis revealing the surface of the *in-vitro* green synthesis of the produced nano selenium and 5C EDX analysis shows a strong signals for selenium and a very weak carbon signal.

3.5 FT-IR analysis

FT-IR analysis recorded the major absorption bands at 2893, 2113, 1062, and 605 cm^{-1}. The band at 2893 cm^{-1} is due to O-H stretch carboxylic acids. The band at 2113 cm^{-1} is due to C≡C Alkyne (triple bond), The band at 1651 cm^{-1} is due to the C=C stretch in the aromatic ring, N-H stretching in amine, and C=O stretch in polyphenols, 1062 is attributed to the C-O stretching in primary alcohol and the weak band at 605 cm-1is the result of C-H out of plane bending, Figure 6.

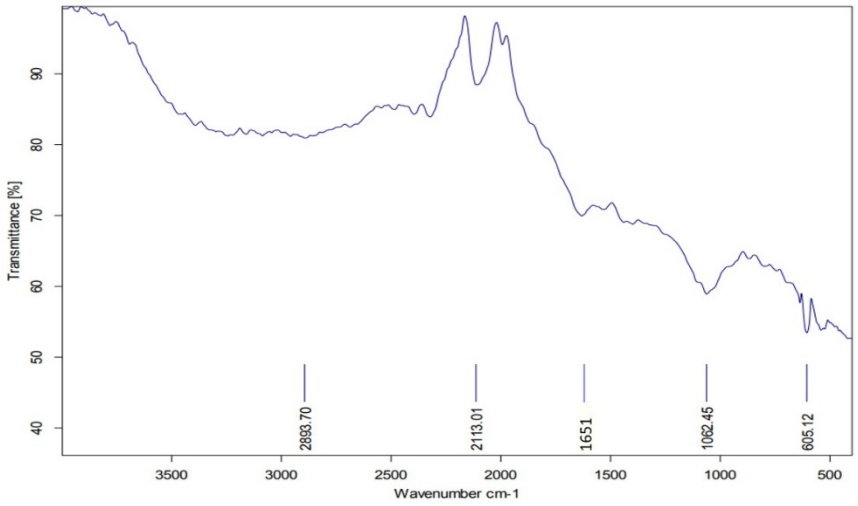

Fig 6. FT-IR spectrum of *in-vitro* derived *Plectranthus Amboinicus* leaves and stems aqueous extract mediated selenium nanoparticle

The phytoconstituents contained *in-vitro* derived *Plectranthus Amboinicus* leaves and stems aqueous extract such as polyphenol components and many bioactive chemicals, including terpenoids, alkaloids, amino acids, glycosides, flavonoids, and tannins in addition to sesquiterpenoids which are responsible for numerous beneficial effects [38, 39]. The water-soluble heterocyclic components act as reductive factors for the reductive system of the selenium ions of nanoparticles based on the capping and reductive capacity of these vital constituents. Therefore, the FT-IR results indicated that the Se-NPs were synthesized and covered with bio-compounds present in the *in-vitro* derived *Plectranthus amboinicus* leaves and stems aqueous extract.

3.6 Antibacterial Activity

The antibacterial activity of the biosynthesized selenium nanoparticles from *in-vitro* derived extract of *Plectranthus amboinicus* in two different concentrations (20 ppm) and (30 ppm) compared to the standard kanamycin discs (30 mg/disc). The result of antimicrobial activity was measured in terms of the zone of inhibition (mm), resulting in 29 and 27 mm zones of inhibition at 30 ppm and 20 ppm of the biosynthesized SeNPs, respectively for *Staphylococcus aureus* strain Fig 7.A and 28 and 25 mm zone of inhibition at (30 and 20 ppm) of the biosynthesized SeNPs respectively for *Escherichia coli* strain Fig 7.B which verified the significant antibacterial activity against both oral pathogen strains.

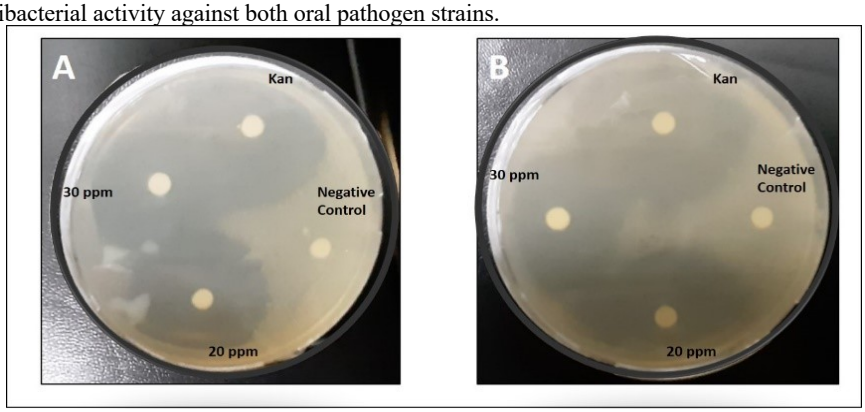

Fig.7 Antibacterial activity of biosynthesized SeNPs using two different concentrations 20 ppm and 30 ppm compered to positive control Standard Kanamycin discs and sterilized dis H_2O as a negative control: A. Antibacterial activity against *Staphylococcus aureus and B.* Antibacterial activity against *Escherichia coli*

These results agreed with the other study by Nenad Filipovic *et al.* 2021 [40], the study recorded the antibacterial activity of SeNPs against two groups of bacterial strains, Gram positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Kocuria rhizophila*) and Gram negative as *Escherichia coli*, *Salmonella Abony*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The mechanism of antibacterial activity was reported in previous studies by Wang *et al.* 2017 [41] that nanoparticles' antimicrobial activity is based on ROS generation, inhibition in the synthesis of proteins and DNA,

expression of metabolic genes, interaction with cell barrier (cell wall disruption and alteration in permeability) .The previous study by Cermonini *et al.* 2016 [42] conducted an antimicrobial assay and recorded that biogenic selenium nanoparticles possess excellent antimicrobial activity against clinical isolates of *P. aeruginosa.* Their comparative study between the antimicrobial activity of biogenic and chemically synthesized SeNPs recorded significantly better results for the antimicrobial activity of the biogenic SeNPs, as a result of the special biogenic surface coating of the biosynthesized SeNPs. Several research papers state that multidrug-resistant bacterial strains were developed because of the continuous usage of commercial antibiotics. This study recommends with agrees with other Nano biosynthesized studies that nanoparticles are considered promising alternative antimicrobial agents specifically when treating chronic and nosocomial infections instead of common-use antibiotics.

4. Conclusion

This study concluded for the first time the successful synthesizing of selenium nanoparticles using the extract of the treated *in-vitro* medicinal plant *Plectranthus amboinicus*. Instead of using synthetic and expensive plant growth regulators, a new eco-friendly trend was applied by using the effective extract *Spirulina plantensis* resulted in a significant influence on *in-vitro* plant growth of *Plectranthus amboinicus* and enhancing the accumulation of total phenolics. The selected medium M5 contained 1.0 mg of the SE extract and 20 gm/L sucrose improved the shoot length and number of leaves of the *in-vitro* plantlets moreover increasing the level of total phenolics compared to the control. These positive results occurred as plant response to the addition of SE extract in specific concentrations due to its phytohormone contents. The designed experimental was conducted mainly to improve the efficiencies of in-vitro derived *Plectranthus amboinicus* extract to be used as an effective reduction agent for rapid biosynthesis of selenium nanoparticles, resulting in the idealistic green synthesized SeNPs with spherical-like shape and average diameter of 17.9±1.34 which were verified with Edx, SEM, TEM, and FTIR analyses. The positive antibacterial activity against the two oral pathogen strains (*Escherichia coli* and *Staphylococcus aureus*) emphasizes the importance of biosynthesized selenium nanoparticles in the future for medical purposes furthermore, Nanoparticles derived from plant origin are eco-friendly and health-friendly. It is recommended for further research and laser applications in enchanting material delivery for targeting tissues and organs.

Conflict of interest

The authors have declared no conflict of interest.

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