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RESEARCH ARTICLE

Evaluation of antimicrobial, antioxidant and antidiabetic properties of green synthesized selenium nanoparticles from *Catharanthus roseus*

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Abstract

The bio-reduction of selenite (Se (IV)) generates nanoparticles with sizes ranging between 30 and 300 nm. Biological properties of Se nanoparticles are dependent on the nanoparticle size; smaller particles have greater activity. The green SeNPs (selenium nanoparticles) are prepared by mixing of plant leaves methanolic extracts of *Centella asiatica*, *Azadirachta indica*, *Ocimum tenuiflorum*, *Murraya koenigii* and *Catharanthus roseus* with sodium selenite. This mixture was stirred which gave a dispersion of SeNPs conjugated with plants phytoconstituents. Among these plants, *C. roseus* showed high yield of SeNPs and purification of the nanoparticles was performed by alkaline lysis. The visualization and characterization of nanoparticles were performed by UV-Visible spectroscopy, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). The antioxidant activity of nanoparticles was determined by ABTS, DPPH and FRAP assays. The anti-bacterial activity was carried out for *Streptococcus mutans*, *Staphylococcus aureus* and *Escherichia coli* and anti-fungal activity was carried out for *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* using well diffusion method. The suspension solution confirms the formation of SeNPs showed (285 nm) by UV analysis. Screening analysis showed enormous phytoconstituents in leaves methanolic extract and simultaneously, the synthesized SeNPs by FT-IR spectrum confirmed the presence of functional groups which were associated with bioactive molecules. XRD study exhibits the nano-crystalline nature of SeNPs. SEM analysis revealed that the biosynthesized selenium nanoparticles were oval with size range of 195 nm. Green synthesized SeNPs were found to possess significant antioxidant activity (IC_{50} 403.571, 477.48 and 60.090 µg/mL) and SeNPs showed strong antimicrobial action, with *A. flavus* showing the lowest zone of inhibition (9 mm) and *S. mutans* showing the highest zone of inhibition (35 mm). The synthesis of thermodynamically stable aqueous SeNPs has been aided by the active phytoconstituents found in *C. roseus* leaves extract, which are effective reducing agents. The present results support the advantages of green method to produce Se NPs having potential activities.

Keywords: antibacterial; antioxidant; *Catharanthus roseus*; selenium nanoparticles

Introduction

In the twenty-first century nanotechnology has emerged as one of the promising approaches for innovations that lead to fulfill the human needs. Nanoparticles have generated intense interest because of their unique electronic, optical, photo responsive, catalytic properties and biomedical application (1). The ability of numerous biological systems, such as plants and algae (2), diatoms (3), bacteria (4), yeast (5), fungi (6) and human cells (7) to convert inorganic metal ions into metal nanoparticles through the reductive capabilities of their proteins and metabolites has been shown during the past decade. Rapid biosynthesis of nanomaterials using plant materials has more advantages over other biological methods, because it is inexpensive, quick in production time, safe and the capacity to scale up production volumes (8). Phytoconstituents are backbone of plants is what gives them their therapeutic qualities with less toxicity (9). Several medicinally valuable plants such as, *Centella asiatica* (L.) Urban (Brahmi), *Azadirachta indica* A. Juss. (Neem), *Ocimum tenuiflorum* L. (Tulsi), *Murraya koenigii* (L.) Sprengel (Curry leaves) and *Catharanthus roseus* (L.) G. Don (Vinca) are some of most commonly used medicinal plants. The primary techniques for producing nanoparticles are physical and chemical procedures, which are frequently expensive and potentially harmful to the environment. Green synthesis has been intensively sought in recent years as a substitute, effective, affordable and eco-friendly way to create nanoparticles with characteristics. This concept offers examination of the several elements influencing the size, shape and yield of metal nanoparticles (10). Understanding plant toxicity and safeguarding people and animals from natural poisons are two benefits of studying medicinal plants.

Centella asiatica, also known as Brahmi in Hindi and Unani, commonly known as Gutu kola, is used in Indian medicine system for enhancing the memory and for the treatment of skin disease. In Ayurvedic medicine the aerial parts and roots are used for medicinal purpose and its chemical constituents have wide therapeutic applications in areas of antimicrobial, anti-

inflammatory, anticancer, neuroprotective, antioxidant and wound healing activities (8). *Azadirachta indica* is also known as neem or kahibevu, the entire neem tree-leaves, blossoms, seeds, fruits, roots and bark have long been used to treat fever, infections, inflammation, skin conditions and dental issues. Additionally, it possesses anti-inflammatory, antiviral, antifungal and antibacterial properties (11). *Ocimum tenuiflorum* or tulsi, is a plant with a complex chemical makeup that includes a variety of nutrients and other biologically active substances. Different parts of tulsi plant like leaves, flowers, stem, root and seeds are known to possess therapeutic potentials and have been used by traditional medical practitioners, as expectorant, analgesic, anticancer, antiasthmatic, antiemetic, diaphoretic, antidiabetic, antifertility, hepatoprotective, hypotensive, hypolipidemic and antistress agents (12). *Murraya koenigii* commonly called as Karibevu, studies revealed the presence of varies phytoconstituents like alkaloids, flavonoids, carbohydrates and sterol (13). The plant is credited with tonic and stomachic properties. Bark and roots are used as stimulant and externally to cure eruptions and bites of poisonous animals (14). *C. roseus* commonly called as nithyapushpa, more than 130 alkaloids have been identified from extract of different plant parts. The most important alkaloids vinblastine and vincristine are derived from leaves, which exhibits anti-cancer and anti-diabetic properties. Another alkaloid rubacine derived from roots is used as hypotensive and anti-arrhythmic agent (15). Where the GCMS analysis of *C. roseus* methanolic extracts revealed core chemical constituents such as trans-squalene, n-hexadecanoic acid, eicosyl acetate, stearin, 1H-Benz(G)indole-3-carboxylic acid (16). *C. roseus* contains significant amounts of volatile and phenolic compounds including caffeoylquinic acids and flavonol glycosides which are known to antioxidant activity. It has multiple applications in foods, cosmetics and pharmaceutical industries. Besides antioxidant activity, these compounds exhibit antiallergic, anti-inflammatory, antimicrobial, anti-thrombotic, cardio protective and vasodilatory effects (17).

Selenium (Se) is one of the necessary trace elements in the human body. Approximately, 40-300 µg of Se is needed daily as dietary supplements by a normal adult. It is found that elemental Se at nano size carries high biological activity with low toxicity. This exciting property of nano Se has provided its application in medical and pharmaceutical sciences. Conventional method for nanoparticles synthesis includes physical and chemical approaches. Physical methods involve high temperature spraying method, evaporation, laser ablation techniques, plastic deformation, electrodeposition are some widely used physical processes for nanoparticles synthesis are highly expensive as they required high cost. In chemical synthesis, solvents, additives, reductants or a stabilizer such as ethaline, borohydride, dode-canthiolates are commonly used, these chemicals are environmentally unfriendly and toxic for living organisms. The overall environmental concern has influenced researchers to replace this methodology with clean, nontoxic and environment friendly, green chemistry approach. Selenium nanoparticles from biological sources like microbes were advantageous compared to chemical methods. Some of the advantages include highly purified form of nanoparticles, faster production rate (18). In recent times, green synthesis using medicinal plant extract has obtained specific importance in the formation of nanoparticles because phytoconstituents are backbone of plants and easily produce nanoparticles with less toxicity. Particularly, essential dietary micronutrient selenium found in the form of SeNPs is relatively a new member of drug nanocarriers in medicine because SeNPs exhibited strong antioxidative and antibacterial activity (19). Characterization of selenium nanoparticles has always depended upon the source from which it has been produced. Characterization is done by using some methods like Fourier transform resonance spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscope (SEM), transmission electron microscope (TEM), Brunauer-Emett-Teller method, etc. FTIR is a sensitive technique used for identifying organic chemicals such as paints, adhesives, resins, polymers, coatings and drugs. XRD is a unique method used for the determination of crystallinity of a compound. XRD is mainly useful for distinguishing between amorphous and crystalline materials and to determine structural properties such as lattice parameters, strain, grain size, phase composition, preferred orientation, order-disorder transformation and thermal expansion. SEM uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about sample including external morphology like texture, chemical composition and crystalline structure and orientation of materials making up the sample. Transmission electron microscopes are capable of imaging at higher resolution than light microscopes, owing to smaller de Brogolie wavelength of electrons. In the reaction change from colourless to deeply brick-red colour indicated the formation of nanoparticles. The characterization of the nanospheres in relation to size is of great importance, both in industrial and biologic activities. Recent reports describe that Se (0) nanoparticles with a size under 100 nm have a greater bioavailability. In addition, other studies mention that a smaller size increases the ability to trap free radicals with greater antioxidant effect and as expected, 5-200 nm nano-Se can directly scavenge free radicals *in vitro* in a size dependent fashion (20).

The current research aimed to utilize extracts from five distinct medicinal plants as bio-reductants and capping agents for the creation of selenium nanoparticles. The synthesized SeNPs were characterized using various techniques, including UV-Visible spectroscopy, scanning electron microscopy, Fourier transform infrared spectroscopy and X-ray diffraction. Additionally, the selenium nanoparticles were examined for their antimicrobial properties against various pathogens, including both Gram-positive and Gram-negative bacteria, as well as fungal species. This study also focused on assessing the antioxidant effects of SeNPs through DPPH, ABTS and FRAP assays.

Materials and Methods

Plant collection and materials

Healthy *C. asiatica*, *A. indica*, *O. tenuiflorum*, *M. koenigii* and *C. roseus* leaves were collected from local regions of Belthangady Taluk, Dakshina Kannada District of Karnataka and were identified and confirmed by relevant literature reviews. Sodium selenite ($NaSeO_3$) and methanol were purchased from HIMEDIA India. All the experiments were performed with sterile distilled water and de-ionized water (Fig. 1).

Preparation of plant leaves extract and phytochemical analysis

Leaves were washed with distilled water, shade dried and powdered. Extractions of phytoconstituents from these samples were done by Soxhlet apparatus by using methanol as solvent. Twenty grams of each plant leaf powder was taken in a

Whatman filter paper and placed in the thimble containing 200 mL of methanol in 500 mL round bottom flask. The extract obtained in solution was distilled to remove solvent by distillation process for 3 hr. The extracts were collected and dried in glass jars at 40 °C. The extracts were stored at 4 °C for further analysis. The preliminary phytochemical screening analysis was carried out in methanol extracts of plant leaves to check the presence of different phytoconstituents (21, 22).

Synthesis of selenium nanoparticles

An aliquot of 40 mM ascorbic acid was prepared in six separate conical flasks. Ten millilitres of 20 mM sodium selenite were taken in six test tubes separately. Contents in the test tubes were added to six conical flasks containing ascorbic acid. From these, one conical flask was used as control and remaining used for samples. One millilitre of all the above five different plant extracts was added into the remaining five conical flasks separately. After addition of the extracts, both samples and control were analysed spectrophotometrically at regular intervals of time (0, 2, 4 and 6 hr). After completion of the reaction, the synthesized SeNPs were harvested by centrifuging the reaction mixture at 1500 rpm, dried overnight. The red SeNPs were suspended in PBS (pH 7.4) by ultrasonication and then centrifuged and used for further analysis (19, 22, 23). By using *C. roseus* sample, selenium nanoparticles were produced by same method which was mentioned above based on the highest yield.

Characterization of synthesized Se nanoparticles

UV-Visible spectroscopy

The SeNPs were characterized in a PerkinElmer UV-Vis spectrophotometer, Lambda-19 to know the kinetic behaviour of SeNPs. The scanning range of the samples was 200-700 nm at a scan speed of 480 nm/min. The data in the spectrophotometer were recorded and analysed by "BUV Winlab" software. The UV-Vis absorption spectra of the SeNPs were recorded and Origin 8 was used to plot the numerical data (19).

Fourier transform infrared spectroscopy

Two milligrams of the nanoparticles was encapsulated in 100 mg of KBr pellet to prepare translucent sample discs. The pelleted sample specimens were subjected to Fourier transform infrared (FT-IR) spectroscopy (Bruker IR Affinity, Japan) in the range of wavelength 500-4000 cm^{-1} with a resolution of 1 cm^{-1}. A PerkinElmer spectrophotometer was employed to record FT-IR spectrum (19).

X-ray diffraction (XRD)

The crystalline size and purity were characterized by an X-ray diffractometer using Philips Panlytical X'pert Pro X-ray diffractometer using Cu Ka (1.54059 A°) radiation with the X-ray generator operating at 45 kV and 40 mA. The crystalline size of the prepared nanoparticles was determined by using Scherrer's equation as follows $D \approx 0.9\lambda/\beta\cos\theta$, where D is the crystal size, λ is the wavelength of X-ray, θ is the Bragg angle in radians and B is the full width at half maximum of the peak in radians (19).

Scanning electron microscopy (SEM)

The surface morphology of synthesized SeNPs was observed by scanning electron microscopy (ZEISS EVO-MA 10, Oberkochen, Germany) (23).

Biological application

The advancement of the green production of nanoparticles using plant extract performs a significant role on free radical scavenging and microbial-mediated disease.

Antioxidant activity

DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was determined by the method of (24) with some modifications. Different concentrations of the sample and control (100, 200, 300, 400 and 500 µg) and standard ascorbic acid was mixed with equal volume of methanol. Three milliliters of DPPH solution was pipetted and incubated in dark condition for 15 min. After that, absorbance was read at 517 nm spectrophotometrically with methanol as a blank. The percentage radical scavenging activity of the SeNPs were calculated using the following formula:

$$\text{DPPH RSA (\%)} = \frac{(A_C - A_S)}{A_C} \times 100$$

Where A_c = absorbance of control at 517 nm and A_s = absorbance of sample. The concentration of sample required to scavenge 50 % of DPPH free radical (IC_{50}) was calculated from the curve of percent inhibitions plotted against the respective concentration.

ABTS assay

Free radical scavenging capacity of the extracts from different plant samples are estimated by ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay [25]. $ABTS^{+}$ radical cation assay is based on the ability of antioxidants to reduce

Fig. 1. Real time picture of *C. roseus*.

$ABTS^{+}$ (blue/green) to $ABTS^{-2}$ (colorless). ABTS radical cation solution was produced by the reacting of 7.0 mM ABTS with $K_2S_2O_8$ (2.45 mM) at a ratio of 2/1 (v/v), the mixture was kept in the dark at room temperature for 12 hr. Different concentrations of the sample and control (100, 200, 300, 400 and 500 µg) are taken and the volume made up to 100 µL with methanol. An aliquot of 3 mL of ABTS solution is added and incubated in dark for 30 min. After that, the absorbance is read at 734 nm spectrophotometrically with methanol as a blank. The percentage radical scavenging activity of the SeNPs are calculated using the following formula:

$$\text{ABTS RSA (\%)} = \frac{(A_C - A_S)}{A_C} \times 100$$

Where A_c = Absorbance of control at 734 nm and A_s = Absorbance of sample. The concentration of sample required to scavenge 50 % of ABTS free radical (IC_{50}) was determined from the curve of percent inhibitions plotted against the respective concentration.

FRAP assay

Free radical scavenging capacity of the extracts was estimated using the ferric reducing antioxidant power assay (26). Different concentrations of the control and sample (100, 200, 300, 400 and 500 µg) were taken and the volume was made up to 1 mL with methanol. 2.5 mL of 0.2M phosphate buffer (pH 6) and 1.1 mL of 1 % potassium ferricyanide was added and incubated at 50 °C for 20 min. Later, 2.5 mL of 10 % trichloro acetic acid (TCA) was added. The samples and control were centrifuged at 3000 rpm for 10 min if turbidity is observed. After that 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride solution ($FeCl_3$) was added. The absorbance was read at 700 nm spectrophotometrically with methanol as a blank.

Antimicrobial activity

Bacterial and fungal strains

In the present investigation bacterial strains used are *Staphylococcus aureus*, *Streptococcus mutans* and *Escherichia coli* and fungal strains are *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. The pure cultures are collected from the clinical laboratories in and around Mangalore University Mangalagangothri Konaje.

Well diffusion assay

Antimicrobial activity was carried out by using agar well diffusion method (27) with little modification. Potato Dextrose Agar media was autoclaved and poured into the sterilized petriplates and after solidification, 100 µL inoculum of fungal strain was spread over the medium using a glass spreader. Five wells were made using 5 mm diameter cork borer in each plate. The wells were filled with 50 µL aliquots containing 100, 200, 300 and 400 µg/mL of nanoparticles in respective wells and 50 µL of DMSO in centre well which serve as negative control. Streptomycin used as positive control. Plates were incubated at room temperature for 72 hr. The zone of inhibition was recorded in millimeter around the wells. LB Agar was used for antibacterial study. Media was poured into the sterilized petriplates. After solidification, 100 µL inoculum of microbial pathogens were swabbed over the LB Agar plates. Five wells were made using the cork borer in each plate 50 µL of aliquots containing 100, 200, 300 and 400 µg/mL of nanoparticles were dispensed into the wells using micropipette. Central well with DMSO serves as negative control and Penicillin G as positive control. Plates were incubated at room temperature for 72 hr. The antimicrobial activity was evaluated by measuring the inhibition-zone diameter observed.

Antidiabetic activity

α-Amylase inhibition assay was performed using 3,5-dinitrosalicylic acid (DNS) method (28) with modification. Different concentrations of the control and sample (100, 200, 300, 400 and 500 µg) were taken and the volume was made up to 1 mL with DMSO. Then 200 µL of α-amylase was added to each tube. Tubes were incubated at 30 °C for 10 min. 200 µL of 1 % starch (substrate) was added and incubated the tubes at room temperature for 3 min. The reaction was terminated by the addition of 200 µL of DNS solution then boiled the tubes for 10 min in water bath at 85-90 °C. After cooling, 5 mL of distilled water was added. Then absorbance was read at 540 nm spectrophotometrically with DMSO as a blank. α-amylase inhibition assay of SeNPs was calculated by using the formula:

$$\text{\% of α-amylase inhibition} = \frac{(A_C - A_S)}{A_C} \times 100$$

Results and Discussion

Extraction of phytoconstituents from plant leaves done by Soxhlet method

Among five plants *A. indica* showed highest yield while *M. koenigii* lower extract yield (Table 1). A range of yield levels were reported by various authors using various solvent systems. The aqueous technique outperforms methanol in terms of extract yield when compared to acetone, chloroform and ethanol (29). Methanol also produced higher content (14-16 %) in the barks of *Holigarna ferruginea* and *Holigarna arnottiana* than aqueous solvent systems (9-11 %).

Table 1. Yield of extract obtained from five different medicinal plants

Name of plants	Quantity of extract obtained (g)
Centella asiatica	5.063
Azadirachta indica	5.428
Ocimum tenuiflorum	2.137
Murraya koenigii	1.891
Catheranthus roseus	2.384

Qualitative phytochemical analysis

The qualitative phytochemical analysis of *C. roseus* leaves methanol extract is shown in (Table 2). It revealed the presence of enormous active constituents like alkaloids, flavonoids, phenolics, saponins, tannins and terpenoids. Tannins have amazing stringent properties. They are known to hasten the healing of wounds and inflamed mucous membranes (30). In addition to industrial applications as foaming and surface-active agents, saponins have been extensively used as detergents, pesticides and molluscicides. Alkaloids are associated with medicines with the property of cytotoxicity. Several workers revealed the analgesic, antispasmodic and antibacterial properties of alkaloids (31). These active phytoconstituents maybe combined with the metal solution to give the nanoparticles. The plant-mediated synthesis reduces the toxic chemicals and leads to an ecofriendly synthesis of NPs (32).

Synthesis of selenium nanoparticles

Five different leaf extracts are added to ascorbic acid solution. The colour changes from colourless to ruby red color (Fig. 2). After 48 hr of incubation, no further change in colour was observed. This red color of reaction mixture was attributed to the

Table 2. Qualitative phytochemical analysis of *C. roseus* methanolic leaf extract

Tests for	Test reagents	Methanolic extract of leaf
Alkaloids	Wagner's reagent	+
Carbohydrates	Molisch's test	–
Cardiac glycosides	Keller Kelliani's test	–
Flavonoides	Alkaline reagent test	–
Phenolics	Ferric chloride test	+
Phlobatannis	Precipitate test	–
Proteins	1% Ninhydrin solution in acetone	+
Saponians	Foam test	+
Sterol	Liebermann-Burchard test	-
Tannins	Braymer's test	+
Terpenoids	Salkowski's test	–
Quinones	Precipitate test	+

+ symbol indicates presence and – indicates absence with respect to extractive solvents.

Fig. 2. Selenium nanoparticles from sample and control.

excitation of surface plasmon vibrations of the Se nanoparticles and provided an advantageous spectroscopic signature of their formation. Synthesized selenium nanoparticles are characterized by using parameter techniques such as UV-Visible spectroscopy, FTIR, XRD and SEM analysis.

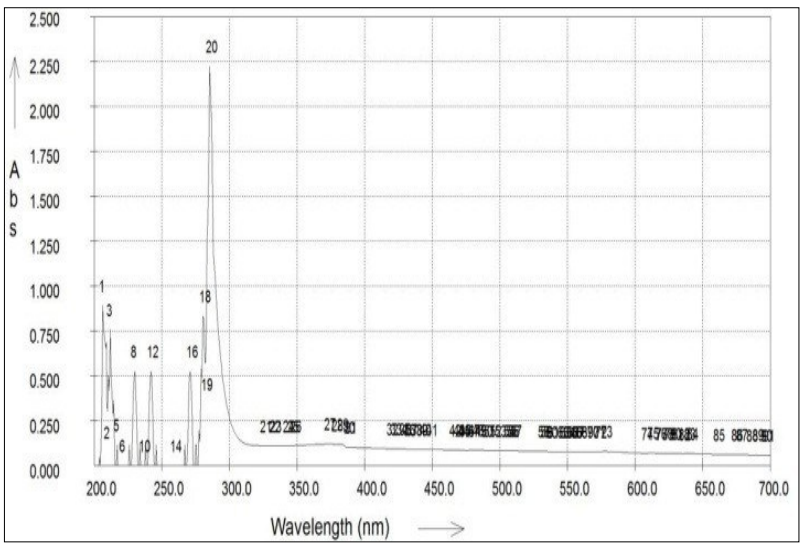
Characterization of selenium nanoparticles

UV-Visible spectrophotometer analysis

The surface plasmon vibrations of SeNPs were also confirmed by UV-visible spectra (Fig. 3). UV-Visible spectra showed the broad peak at 285 nm which confirms the formation of SeNPs. The UV spectra centered between 200 and 300 nm was due to the formation and surface plasmon vibration of SeNPs (18). The broadness of peak also revealed that the synthesized particles were polydisperse (33). The intensity of peak increased with respect to time. The peak intensity was increased with time due to the reduction of SeO_3^{2-} to Se^0. There was no further significant increase in peak after 48 hr, which depicts maximum reduction of SeO_3^{2-} to Se^0. This observation revealed the completely formed SeNPs by phytoconstituents of *C. roseus.*

SEM analysis

Selenium nanoparticle synthesized from the leaves of *C. roseus* shown particle size of 198 nm and are oval (Fig. 4). SEM also showed a well form of a spherical Se nanoparticle at a resolution of 10 nm scale. These particles were well distributed with



Spectrum Peaks And Valleys

	-- PEAKS --			-- VALLEYS --	
Sr. No	Wv(nm)	Absorbance	Sr. No	Wv(nm)	Absorbance
10	236.0	0.000	13	245.0	-0.155
12	242.0	0.526	15	268.0	-0.155
14	259.0	0.000	17	274.0	-0.155
16	271.0	0.526	19	282.0	0.569
18	280.5	0.832	21	325.5	0.110
20	285.5	2.222	24	342.5	0.110
22	332.0	0.110	26	347.5	0.110

Fig. 3. UV-Visible spectrum of SeNP.

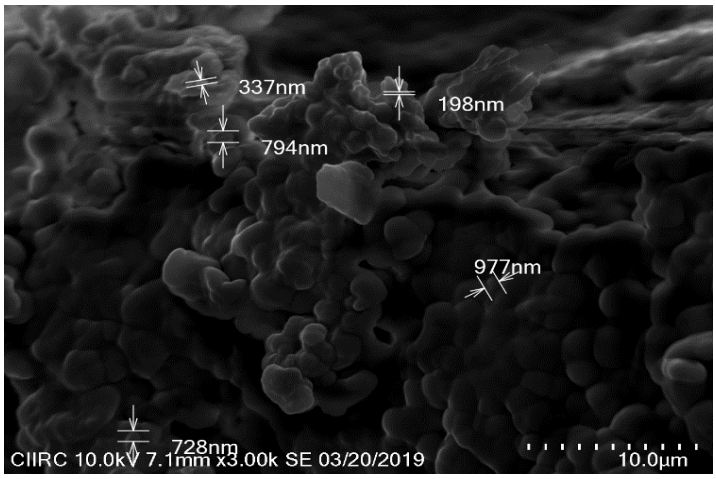


Fig. 4. SeNP of *C. roseus* after SEM analysis.

aggregation. Therefore, it has been suggested that nanoparticle aggregation is dominant over the process of reduction and primary nucleation of reduced atoms. The most accessible metal ions are apparently involved in a smaller number of nucleation events, which leads to agglomeration of the metal (33). Previous report showed agglomerate NPs exhibit higher biological activity (34, 35). The synthesized *C. roseus* leaves methanol extract-mediated SeNPs may have efficient applications in pharmacology.

FTIR analysis

The result of FT-IR analysis of synthesized SeNPs is depicted in Fig. 5. FTIR analysis showed a long stretched peaks at 490-500 cm^{-1} indicates the presence of halogen compound, stretched peaks at 800-900 cm^{-1} indicates the presence of aromatic compounds, stretched peaks at 1100-1250 cm^{-1} indicates the presence of alkyl and aryl halides and a group of stretched peaks at 1300-1350 cm^{-1} indicates the presence of nitro compounds, stretched peaks at 1700-1850 cm^{-1} indicates the presence of amides, carboxylic acids, esters and anhydrides and the absorption peak at 3200-3350 cm^{-1} shows the presence of alcohol group were responsible for selenium nanoparticles synthesis from leaf methanol extract of *C. roseus*. Previous studies revealed that alcoholic groups and aromatic ring were responsible for the formation of selenium nanoparticles from *parsley* extract (36). Hydroxyl group and C-H groups were responsible for the formation of selenium nanoparticles (37). This result indicates the presence of various functional groups as biomolecules which may be responsible for both reduction and stabilization of the SeNPs. Previous reports have also suggested the role of phytoconstituents as a stabilizing agent for the synthesis of metal NPs (38). However, the identity of the active molecules responsible for the synthesis of the SeNPs needs further validation.

XRD analysis

In XRD analysis, the crystallinity of the nanoparticles can be revealed. Selenium nanoparticles synthesized from *C. roseus* showed broad peak at 2θ angles of 20-25° which determines that the sample is nano-crystalline (Fig. 6). XRD validates the creation of SeNPs with crystalline structure. The successful synthesis of SeNPs was confirmed by powder XRD (39). The well-crystalline nature of the produced SeNPs is demonstrated by the strong peaks in the diffraction pattern. The XRD pattern revealed 2θ values spanning from 10° to 80°. Several distinct peaks corresponding to SeNPs were observed at angles such as 100°, 101°, 111°, 201° and 210° and these angles matched the Miller indices.

Antimicrobial activity

At the present time, several pathogenic microorganisms have developed resistance to currently available commercial antibiotics and cause adverse impact on health. Recent attention has been paid to the use of nanoparticles to control diseases due to the antimicrobial properties of these nanoparticles. The anti-microbial activity of SeNPs was carried out on three fungal strains *C. albicans*, *A. niger* and *A. flavus* as well as against three pathogenic bacterial strains, *S. mutans*, *S. aureus* and *E. coli* by using well diffusion method. The biosynthesised SeNPs demonstrated strong antimicrobial action, with *A. flavus* showing the lowest zone of inhibition (9 mm) and *S. mutans* showing the highest zone of inhibition (35 mm) (Table 3). It was investigated the inhibitory effects of different concentrations of Se NPs (100-400 µg/mL). The findings showed that the MIC of Se NPs against *C. albicans*, *A. niger* and *A. flavus* was 300 µg/mL.

Conversely, the MIC of Se NPs against *S. mutans*, *E. coli*

Table 3. The minimum inhibition concentration of selenium nanoparticle

Concentration of nanoparticle (µg)	Bacterial zone of inhibition (mm) for organisms					
	Streptococcus mutans		*Staphylococcus aureus*		*Escherichia coli*	
	Control	Sample	Control	Sample	Control	Sample
100	10	09	12	06	04	09
200	08	13	2	09	02	09
300	04	3	12	17	01	15
400	06	35	12	20	01	18
Concentration of nanoparticle (µg)	**Fungal zone of inhibition (mm) for organism**					
	Aspergillus niger		*Aspergillus flavus*		*Candida albicans*	
	Control	Sample	Control	Sample	Control	Sample
100	10	07	08	08	17	09
200	10	10	08	09	17	10
300	12	12	14	09	03	13
400	13	12	14	11	03	13

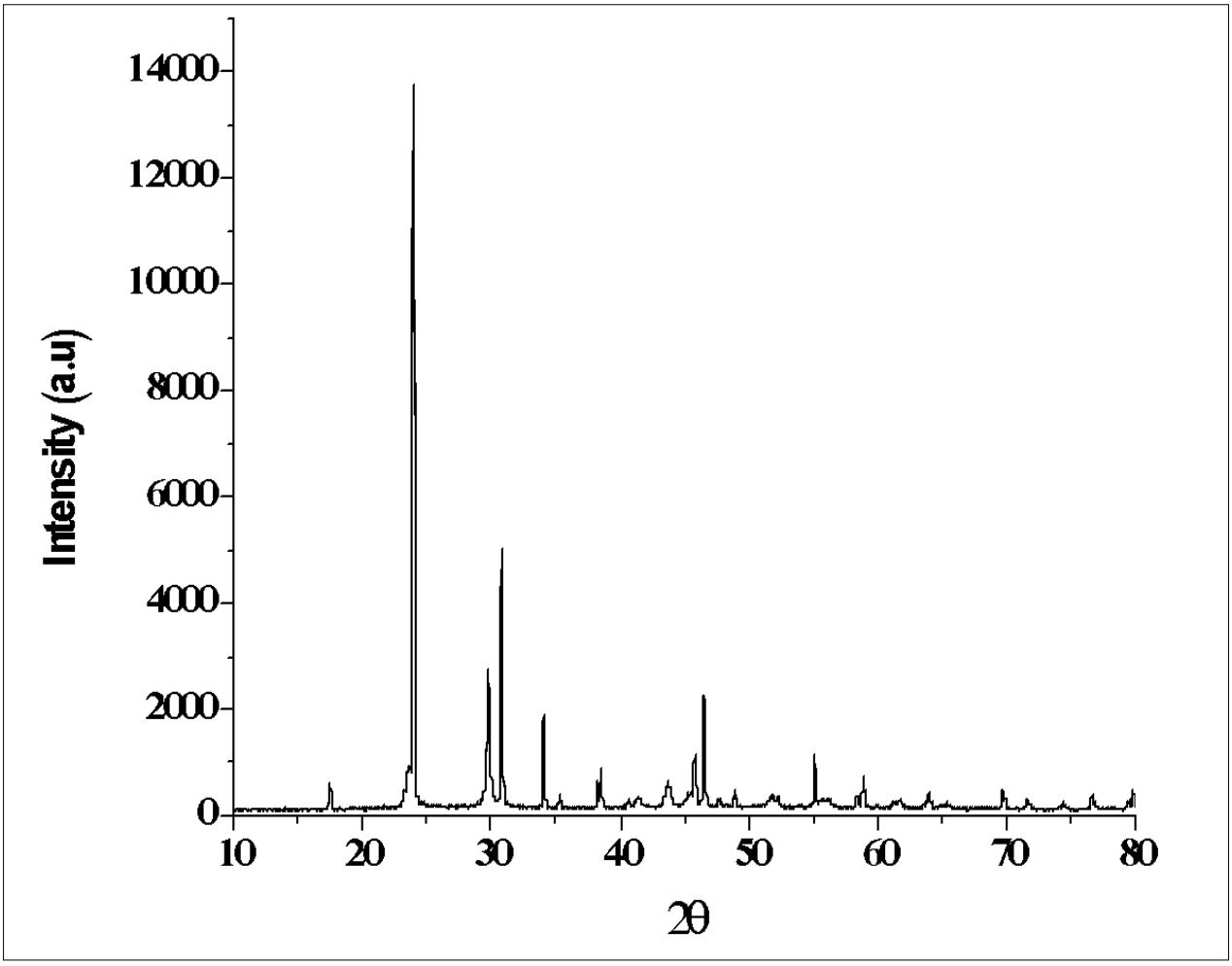


Fig. 5. FTIR analysis of selenium nanoparticles.

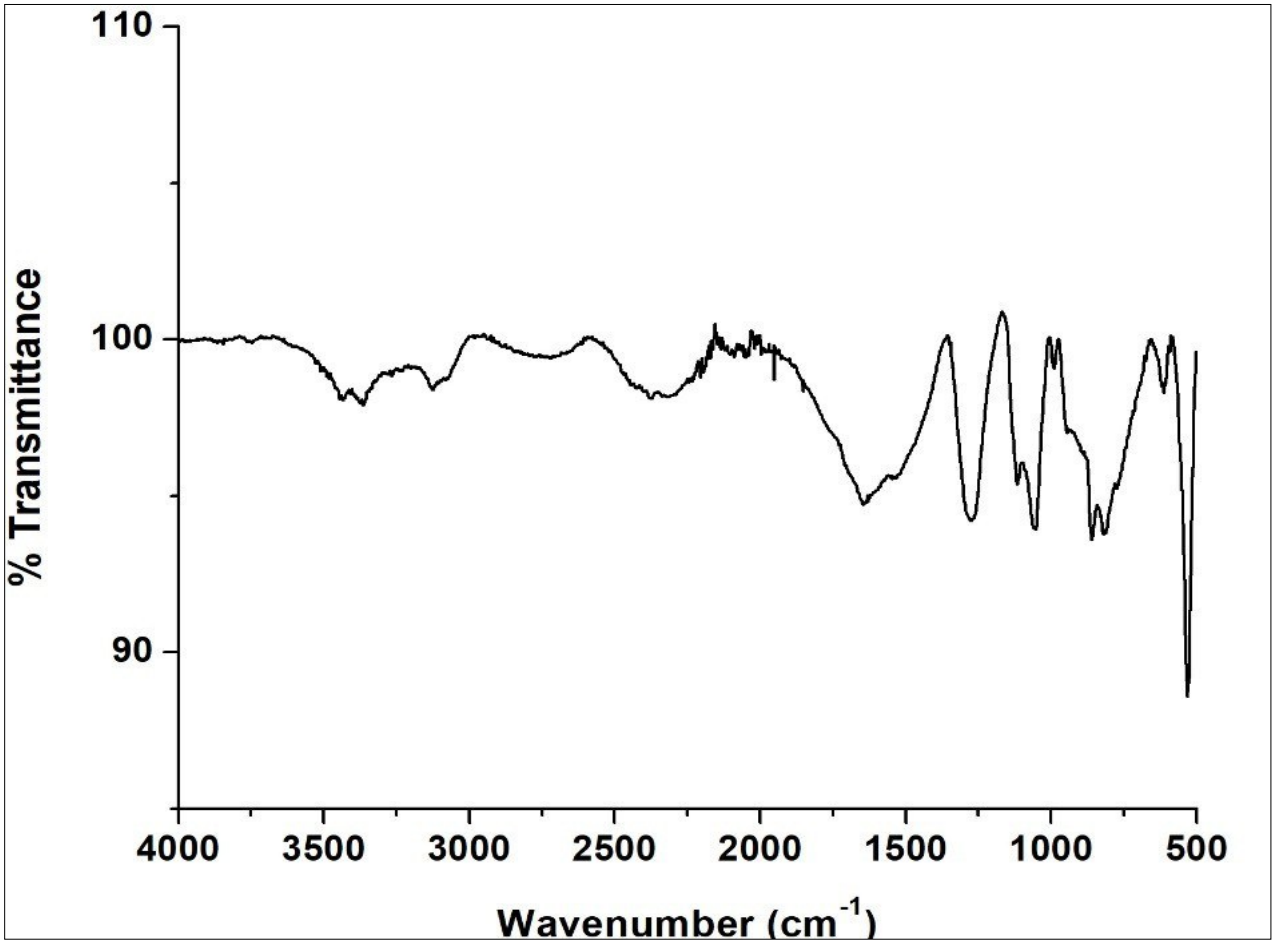


Fig. 6. XRD analysis of selenium nanoparticle.

and *S. aureus*, were 400 µg/mL and 300 µg/mL. The Agar diffusion test has a correlation with the MIC values. The antibacterial efficacy of those NPs made from plant extracts may be due to their existence of phytoconstituents including flavonoids, terpenoids, alkaloids, as well as additional bioactive components. Moreover, these substances cause pathogenic cell death by switching on the permeability of the cell walls along with cell membrane (40, 41). Another reason why SeNPs could be antibacterial is that they might cause microbial cells to die by inactivating enzymes or producing oxygen species that are reactive (42, 43). SeNPs were discovered to have dose-dependent action toward all evaluated bacterial strains when their effectiveness as antibacterial agents was investigated against a variety of gram-positive (*S. aureus* and *S. mutants*) along with gram-negative (*P. aeruginosa* and *E. coli*) (44). SeNPs bio-synthesized via leaf extract of *Mountain persimmon* exhibited antibacterial action toward *S. aureus* and *E. coli* (45). The use of green synthesis of metal nanoparticles is going to be significant in medicinal field.

Antioxidant activity

DPPH free radical scavenging activity

DPPH free radical scavenging assay is shown in Table 4. The antioxidant activity of formulated SeNPs was estimated by comparing the percentage inhibition of DPPH radicals and ascorbic acid. The DPPH radical scavenging activity of SeNPs increased with increase in concentration. Both SeNPs and ascorbic acid showed the activity to scavenge DPPH free radicals in a dose-dependent manner at 100 to 500 mg/mL. The results showed that the IC_{50} of SeNPs was 403.571 µg/mL and ascorbic acid was 6624.91 µg/mL. Further studies evaluated the antioxidant activity of SeNPs made with different techniques and these studies showed that NPs have excellent antioxidant capabilities (46). Previous studies showed that selenium nanoparticles from *Allium sativum* showed high antioxidant activity when compared to control (47). Selenium probably correlates towards the strong antioxidant properties of SeNPs and it is necessary for increasing the effectiveness of selenium-containing enzymes such as glutathione peroxidase helps in protecting tissues and cells from radical destruction (46).

ABTS assay

ABTS free radical scavenging assay is shown in Table 4. The antioxidant activity of formulated SeNPs were estimated by comparing the percentage inhibition of ABTS radicals and quercetin. The inhibition of selenium nanoparticles was determined by calculated IC_{50} value of control and sample. The IC_{50} value of control was more than the sample. The IC_{50} of control was 2132.11 µg/mL and sample were 477.48 µg/mL. The nanoscale size of the particles increases the surface area, enhancing their interaction with free radicals. The presence of bioactive phytoconstituents from the *C. roseus* may act synergistically with selenium to boost antioxidant capacity. Selenium, as an essential trace element is known for its role in redox regulation and free radical scavenging.

FRAP assay

The obtained percentages were plotted in bar graphs as shown in Fig. 7. The inhibition percentage of sample and control was determined using IC_{50} values. The IC_{50} value of sample were 60.090 mg/mL and control were 119.856 mg/mL. The superior antioxidant capacity of the sample could be attributed to the presence of bioactive compounds such as phenolics, flavonoids, or other phytoconstituents capable of donating electrons and reducing ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. These results support the potential of the sample as a therapeutic agent targeting oxidative stress-related disorders.

Antidiabetic activity

The obtained results were shown in Table 5. The inhibition percentage of sample and control was determined using IC_{50} values. The IC_{50} values of sample were 477.48 µg/mL and control were 569 µg/mL. The α-amylase enzyme starts the digestion of carbohydrates present in food; this digestion begins in the mouth and is completed in the small intestine. Since alpha-amylase is a carbohydrate-metabolizing enzyme, inhibiting this enzyme will block carbohydrate breakdown, resulting in decreased postprandial hyperglycaemia. Therefore, inhibition of this enzyme has been shown to be a substantial and well-targeted therapeutic strategy for controlling hyperglycaemia in type 2 diabetes (48). In the current investigation, it was found that the SeNPs suppressed alpha-amylase activity by 75.68 % at 100 g/mL. The findings of this study are consistent with a previous report (48) regarding the evaluation of a new medication against type 2 diabetes based on a polyherbal extract.

Conclusion

Table 4. Radical scavenging activity of the SeNPs synthesized by the methanol extracts of *C. roseus* leaf expressed in µg of ascorbic acid equivalents (AAE) and quercetin/mL

Concentration of nanoparticle (µg)	DPPH	Ascorbic acid	ABTS	Quercetin
100	8.08 ± 0.11	10.94 ± 0.58	9.11 ± 0.13	5.25 ±0.63
200	18.40 ± 0.18	11.82 ± 0.60	17.57 ± 0.33	7.34 ±0.48
300	26.33 ±0.15	12.40 ± 0.65	28.56 ± 0.52	10.07 ±0.12
400	44.67 ± 0.07	13.00 ± 0.41	45.32 ± 0.35	11.30 ±0.33
500	51.74 ± 0.01	13.57± 0.16	52.00 ± 0.73	14.22 ±0.59
IC_{50}	403.571 µg/mL	6624.91 µg/mL	477.48 µg/mL	2132.11 µg/mL

Notes: Values are expressed as mean ± SD (n = 3).

Table 5. α-Amylase inhibition activity of the SeNPs synthesized by the methanol extracts of *C. roseus* leaves expressed in µg of control/mL

Concentration of nanoparticle (µg)	Sample	Control
100	9.473 ± 0.23	2.55 ± 0.38
200	21.72 ± 0.19	16.81 ± 0.32
300	29.75 ± 0.73	34.9 ± 0.17
400	46.10 ± 0.56	35.76 ± 0.39
500	55.69 ± 0.21	39.31 ± 0.69
IC_{50}	477.48 µg/mL	569 µg/mL

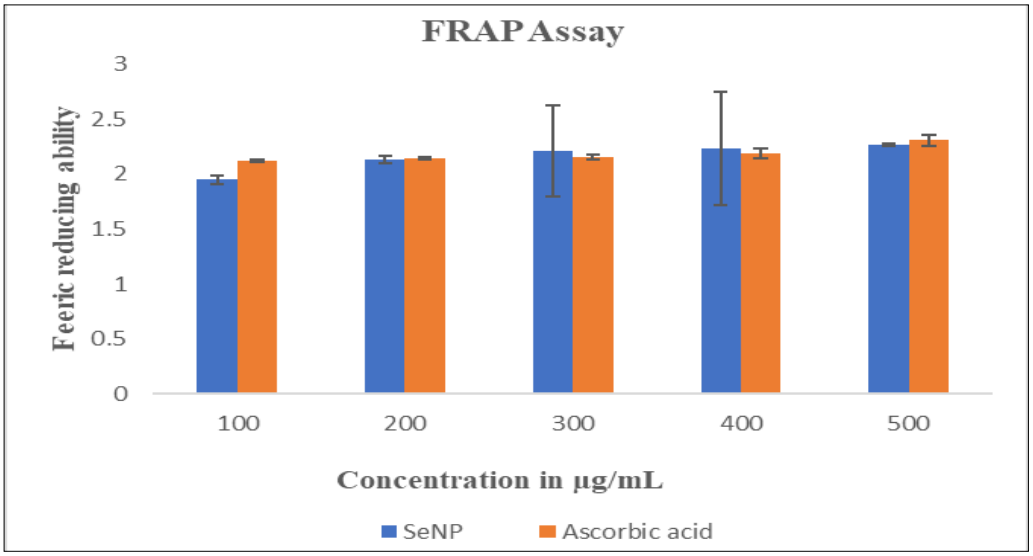


Fig. 7. The ferric reducing ability of the SeNPs synthesized by the methanol extracts of *C. roseus* leaves expressed in µg of ascorbic acid equivalents (AAE)/mL. Values are expressed as mean ± SD (n = 3).

C. roseus leaves extract possesses active phytoconstituents, which are good reducing agents, has helped in the preparation of thermodynamically stable aqueous SeNPs. Aqueous SeNPs has higher radical scavenging activity and antimicrobial activity. Aqueous SeNPs are nontoxic and as has antidiabetic potential which was estimated through alpha amylase activity. As the SeNP are low toxic and have bioavailability they can be used as nutraceuticals. Since the synthesis of SeNPs are done through green synthesis method scalability and reproducibility is the major challenge.

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Authors' contributions

All the authors have made substantive intellectual contributions to the content of this manuscript in the following areas: AR and SNB contributed to concept and design, as well as data acquisition and analysis; AR prepared the original draft; AR, SNB and ASB were involved in review and editing of the manuscript; AR helped in supervision, RBP has done the final approval of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that there is no conflict of interest.

Ethical issues: None

References

1. Ramamurthy CH, Sampath KS, Arunkumar P, Kumar MS, Sujatha V, Premkumar K, et al. Green synthesis and characterization of selenium nanoparticles and its augmented cytotoxicity with doxorubicin on cancer cells. Bioprocess Biosyst Eng. 2013;36 (8):1131-9. https://doi.org/10.1007/s00449-012-0867-1
2. Kasivelu G, Basha SK, Kumar VG, Ganesan S. Silver, gold and bimetallic nanoparticles production using single-cell protein (*Spirulina platensis* Geitler). J Mater Sci. 2008;43(15):5115-22. https://doi.org/10.1007/s10853-008-2745-4
3. Scarano G, Morelli E. Properties of phytochelatin-coated CdS nanocrystallites formed in a marine phytoplanktonic alga (*Phaeodactylum tricornutum* Bohlin) in response to Cd. Plant Sci. 2003;165(4):803-10. https://doi.org/10.1016/S0168-9452(03)00274-7
4. Lengke MF, Fleet ME, Southam G. Biosynthesis of silver nanoparticles by filamentous cyanobacteria from a silver (I) nitrate complex. Langmuir. 2007;23(5):2694-9. https://doi.org/10.1021/la0613124
5. Kowshik M, Deshmukh N, Vogel W, Urban J, Kulkarni SK, Paknikar KM. Microbial synthesis of semiconductor CdS nanoparticles, their characterization and their use in the fabrication of an ideal diode. Biotechnol Bioeng. 2002;78(5):583-8. https://doi.org/10.1002/bit.10233
6. Rautaray D, Ahmad A, Sastry M. Biosynthesis of $CaCO_3$ crystals of complex morphology using a fungus and an actinomycete. J Am Chem Soc. 2003;125(48):14656-7. https://doi.org/10.1021/ja0374877
7. Anshup, Venkataraman JS, Subramaniam C, Kumar RR, Priya S, Kumar TS, et al. Growth of gold nanoparticles in human cells. Langmuir. 2005;21(25):11562-7. https://doi.org/10.1021/la0519249
8. Prakash V, Jaiswal N, Srivastava M. A review on medicinal properties of *Centella asiatica*. Asian J Pharm Clin Res. 2017;10 (10):69-74. https://doi.org/10.22159/ajpcr.2017.v10i10.20760
9. Reddy S, Suraj S, Poorav D. An overview of phytochemicals seen in plant sources. Int J Pharmacogn Life Sci. 2023;4(1):55-9. https://doi.org/10.33545/27072827.2023.v4.i1a.76
10. Makarov VV, Love AJ, Sinitsyna OV, Makarova SS, Yaminsky IV, Taliansky ME, et al. "Green" nanotechnologies: synthesis of metal nanoparticles using plants. Acta Naturae. 2014;6(1):35-44. https://doi.org/10.32607/20758251-2014-6-1-35-44
11. Balaji TK, Shivaji KR, Krishna RK, Balasaheb KN. Medicinal uses of neem (*Azadirachta indica*) in human life: a review. Int J Life Sci. 2018:A10:181-4.
12. Prakash PA, Gupta N. Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: a short review. Indian J Physiol Pharmacol. 2005;49(2):125-31.

13. Gahlawat DK, Jakhar S, Dahiya P. *Murraya koenigii* (L.) Spreng: an ethnobotanical, phytochemical and pharmacological review. J Pharmacogn Phytochem. 2014;3(3):109-19.
14. Saini SC, Reddy GB. A review on curry leaves (*Murraya koenigii*): versatile multi-potential medicinal plant. Am J Phytomed Clin Ther. 2015;3(4):363-8.
15. Kaushik SH, Tomar RS, Gupta MO, Mishra RK. An overview of *Catharanthus roseus* and medicinal properties of their metabolites against important diseases. Eur Acad Res. 2017;5(2):1237-47.
16. Syeda AM, Riazunnisa K. Data on GC-MS analysis, *in vitro* anti-oxidant and anti-microbial activity of the *Catharanthus roseus* and *Moringa oleifera* leaf extracts. Data Brief. 2020;29:105258. https://doi.org/10.1016/j.dib.2020.105258
17. Kabesh K, Senthilkumar P, Ragunathan R, Kumar RR. Phytochemical analysis of *Catharanthus roseus* plant extract and its antimicrobial activity. Int J Pure Appl Biosci. 2015;3(2):162-72.
18. Srivastava N, Mukhopadhyay M. Green synthesis and structural characterization of selenium nanoparticles and assessment of their antimicrobial property. Bioprocess Biosyst Eng. 2015;38(9):1723-30. https://doi.org/10.1007/s00449-015-1413-8
19. Alagesan V, Venugopal S. Green synthesis of selenium nanoparticle using leaves extract of *Withania somnifera* and its biological applications and photocatalytic activities. Bionanoscience. 2019;9 (1):105-16. https://doi.org/10.1007/s12668-018-0566-8
20. Torres SK, Campos VL, León CG, Rodríguez-Llamazares SM, Rojas SM, González M, et al. Biosynthesis of selenium nanoparticles by *Pantoea agglomerans* and their antioxidant activity. J Nanopart Res. 2012;14(11):1236. https://doi.org/10.1007/s11051-012-1236-3
21. Shaikh JR, Patil M. Qualitative tests for preliminary phytochemical screening: an overview. Int J Chem Stud. 2020;8(2):603-8. https://doi.org/10.22271/chemi.2020.v8.i2i.8834
22. Ghutke TD, Kadam RG, Rengarajan C, Nausheed R. Phytochemical analysis and therapeutic profiling of *Helianthus annuus*: a comprehensive qualitative and quantitative study. Plant Sci Arc. 2024;9(3):31-7. https://doi.org/10.51470/PSA.2024.9.3.31
23. Wadhwani SA, Gorain M, Banerjee P, Shedbalkar UU, Singh R, Kundu GC, et al. Green synthesis of selenium nanoparticles using *Acinetobacter* sp. SW30: optimization, characterization and its anticancer activity in breast cancer cells. Int J Nanomedicine. 2017;12:6841-55. https://doi.org/10.2147/IJN.S139212
24. Garcia EJ, Oldoni TL, Alencar SM, Reis A, Loguercio AD, Grande RH. Antioxidant activity by DPPH assay of potential solutions to be applied on bleached teeth. Braz Dent J. 2012;23:22-7. https://doi.org/10.1590/S0103-64402012000100004
25. Madhanraj R, Eyini M, Balaji P. Antioxidant assay of gold and silver nanoparticles from edible basidiomycetes mushroom fungi. Free Radic Antioxid. 2017;7(2):137-42. https://doi.org/10.5530/fra.2017.2.20
26. Noreen H, Semmar N, Farman M, McCullagh JS. Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*. Asian Pac J Trop Med. 2017;10 (8):792-801. https://doi.org/10.1016/j.apjtm.2017.07.024
27. Perez C. Antibiotic assay by agar-well diffusion method. Acta Biol Med Exp. 1990;15:113-5.
28. Balan K, Qing W, Wang Y, Liu X, Palvannan T, Wang Y, et al. Antidiabetic activity of silver nanoparticles from green synthesis using *Lonicera japonica* leaf extract. RSC Adv. 2016;6(46):40162-8. https://doi.org/10.1039/C5RA24391B
29. Yadav M, Chatterji S, Gupta SK, Watal G. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm Sci. 2014;6(5):539-42.
30. Thite SV, Chavan YR, Aparadh VT, Kore BA. Preliminary phytochemical screening of some medicinal plants. Int J Pharm Chem Biol Sci. 2013;3(1):87-90.
31. Malhotra S, Welling MN, Mantri SB, Desai K. *In vitro* and *in vivo* antioxidant, cytotoxic and anti-chronic inflammatory arthritic effect of selenium nanoparticles. J Biomed Mater Res B Appl Biomater. 2016;104(5):993-1003. https://doi.org/10.1002/jbm.b.33448
32. Reddy V, Torati RS, Oh S, Kim C. Biosynthesis of gold nanoparticles assisted by *Sapindus mukorossi* Gaertn. fruit pericarp and their catalytic application for the reduction of p-nitroaniline. Ind Eng Chem Res. 2013;52(2):556-64. https://doi.org/10.1021/ie302037c
33. Sathishkumar M, Sneha K, Yun YS. Immobilization of silver nanoparticles synthesized using *Curcuma longa* tuber powder and extract on cotton cloth for bactericidal activity. Bioresour Technol. 2010;101(20):7958-65. https://doi.org/10.1016j.biortech.2010.05.051
34. Zook JM, MacCuspie RI, Locascio LE, Halter MD, Elliott JT. Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. Nanotoxicology. 2011;5 (4):517-30. https://doi.org/10.3109/17435390.2010.536615
35. Bantz C, Koshkina O, Lang T, Galla HJ, Kirkpatrick CJ, Stauber RH, et al. The surface properties of nanoparticles determine the agglomeration state and the size of the particles under physiological conditions. Beilstein J Nanotechnol. 2014;5:1774-86. https://doi.org/10.3762/bjnano.5.188
36. Fritea L, Laslo V, Cavalu S, Costea T, Vicas SI. Green biosynthesis of selenium nanoparticles using parsley (*Petroselinum crispum*) leaves extract. Studia Univ Vasile Goldis Arad Ser Stiintele Vietii. 2017;27 (3):203-8.
37. Zhang H, Zhou H, Bai J, Li Y, Yang J, Ma Q, et al. Biosynthesis of selenium nanoparticles mediated by fungus *Mariannaea* sp. HJ and their characterization. Colloids Surf A Physicochem Eng Asp. 2019;571:9-16. https://doi.org/10.1016/j.colsurfa.2019.02.070
38. Coccia F, Tonucci L, Bosco D, Bressan M, d'Alessandro N. One-pot synthesis of lignin-stabilised platinum and palladium nanoparticles and their catalytic behaviour in oxidation and reduction reactions. Green Chem. 2012;14(4):1073-8. https://doi.org/10.1039/c2gc16524d
39. Panahi-Kalamuei M, Salavati-Niasari M, Hosseinpour-Mashkani SM. Facile microwave synthesis, characterization and solar cell application of selenium nanoparticles. J Alloys Compd. 2014;617:627-32. https://doi.org/10.1016/j.jallcom.2014.07.174
40. Indhira D, Krishnamoorthy M, Ameen F, Bhat SA, Arumugam K, Ramalingam S, et al. Biomimetic facile synthesis of zinc oxide and copper oxide nanoparticles from *Elaeagnus indica* for enhanced photocatalytic activity. Environ Res. 2022;212:113323. https://doi.org/10.1016/j.envres.2022.113323
41. Murali M, Kalegowda N, Gowtham HG, Ansari MA, Alomary MN, Alghamdi S, et al. Plant-mediated zinc oxide nanoparticles: advances in the new millennium towards understanding their therapeutic role in biomedical applications. Pharmaceutics. 2021;13(10):1662. https://doi.org/10.3390/pharmaceutics13101662
42. Abdel-Moneim AM, El-Saadony MT, Shehata AM, Saad AM, Aldhumri SA, Ouda SM, et al. Antioxidant and antimicrobial activities of *Spirulina platensis* extracts and biogenic selenium nanoparticles against selected pathogenic bacteria and fungi. Saudi J Biol Sci. 2022;29(2):1197-209. https://doi.org/10.1016/j.sjbs.2021.09.046
43. Nayak V, Singh KR, Singh AK, Singh RP. Potentialities of selenium nanoparticles in biomedical science. New J Chem. 2021;45(6):2849-78. https://doi.org/10.1039/D0NJ05884J
44. Boroumand S, Safari M, Shaabani E, Shirzad M, Faridi-Majidi R. Selenium nanoparticles: synthesis, characterization and study of their cytotoxicity, antioxidant and antibacterial activity. Mater Res Express. 2019;6(8):0850d8. https://doi.org/10.1088/2053-1591/ab2558
45. Kokila K, Elavarasan N, Sujatha V. *Diospyros montana* leaf extract-mediated synthesis of selenium nanoparticles and their biological applications. New J Chem. 2017;41(15):7481-90. https://doi.org/10.1039/C7NJ01124E

46. Gunti L, Dass RS, Kalagatur NK. Phytofabrication of selenium nanoparticles from *Emblica officinalis* fruit extract and exploring its biopotential applications: antioxidant, antimicrobial and biocompatibility. Front Microbiol. 2019;10:931. https://doi.org/10.3389/fmicb.2019.00931

47. Vyas J, Rana SH. Antioxidant activity and biogenic synthesis of selenium nanoparticles using the leaf extract of *Aloe vera*. Int J Curr Pharm Res. 2017;9(4):147-52. https://doi.org/10.22159/ijcpr.2017v9i4.20981

48. Riaz Z, Ali MN, Qureshi Z, Mohsin M. *In vitro* investigation and evaluation of novel drug based on polyherbal extract against type 2 diabetes. J Diabetes Res. 2020;2020(1):7357482. https://doi.org/10.1155/2020/7357482

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