

Biosynthesis and Characterization, Antioxidant and Antimicrobial Activities of Selenium Nanoparticles from Ethanol Extract of Bee Propolis

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Abstract

Aim: Biosynthesis of selenium nanoparticles (SeNPs) has gained significant interest due to their distinctive chemical and biological properties that is essential for potential application in various fields.

Methods: In the present study, propolis, the beehive product collected from 5 different Indian states like Haryana, Himachal Pradesh, Uttaranchal, Karnataka and Kerala were used for the biosynthesis of SeNPs and characterized by using UV-vis spectrophotometer, fourier transform spectroscopy (FT-IR), X-ray diffraction (XRD) and scanning electron microscopy (SEM).

Results: SeNPs biosynthesized by propolis were observed as crystalline, oval shaped and smooth surface particles. Study also reports the efficiency of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Fluorescence recovery after photobleaching (FRAP) and superoxide dismutase (SOD) assay to estimate the antioxidant potential. The antimicrobial assay was evaluated only for SeNPs synthesized from propolis extracts obtained from Karnataka state showed high antioxidant activity. The antibacterial activity against pathogenic gram-positive bacterial strains (*Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus mutans*), gram-negative bacterial strains (*Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*) was evaluated by resazurin microtiter plate method to check the minimum inhibition concentration (MIC). The antifungal activity of pathogenic fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* were determined by well diffusion method.

Conclusion: It is the first report describing the biosynthesis of selenium nanoparticles using bee propolis. This study demonstrated the antioxidant and antimicrobial potential of SeNPs biosynthesized by using ethanol extract of propolis.

Keywords: Antibacterial; Antifungal; Bee propolis; Biosynthesis; Nanoparticles; Selenium

Introduction

Biosynthesis of nanoparticles is an interdisciplinary application of metal science and technology through biology. The main reaction of this technique is oxidation or reduction using biomolecules [1]. The metal nanoparticles with a definite shape, size, and crystal nature have a considerable application in medicine including cancer therapy [2], DNA analysis [3], drug delivery [4], biosensor [5] and magnetic resonance imaging [6], apart from agricultural [7], environmental remediation [8], pharmaceutical [9], electronics and commercial industries [10]. Currently, the development of biocompatible nanoparticles is of great concern among the scientists worldwide, because of their unique physical, chemical and biological characteristics [11]. To overcome the limitations like the generation of hazardous toxic chemicals from the conventional methods, biological processes have been considered as an alternative and efficient method for the nanoparticles synthesis, that exhibits significant electromagnetic and catalytic property, large surface to volume ratio, less toxic and cost-effective [12,13].

Selenium is a non-metallic essential trace element exists in three well defined allotropic forms such as red coloured amorphous selenium in powder form, black in coloured crystalline trigonal having helical chains and crystalline monoclinic (α , β , γ) is also red in colour [14]. Selenium as a micronutrient at an appropriate concentration is essential for human health due to its antioxidative effect. Selenium deficiency can lead to heart disease, hypothyroidism and a weakened immune system [15]. It is reported that SeNPs have excellent biocompatibility, bioefficacy and low toxicity as compared to various organic and inorganic forms of selenium compounds [16]. The biosynthesis of SeNPs has gained much interest due to its excellent therapeutical and nutraceutical potential

among the other nanoparticles [17-19]. Biosynthesis of SeNPs, using the diverse range of microorganisms [20] and different plant parts including leaves, flower, fruits, peel and seed extracts have been studied extensively [21,22]. However, there are no reports on the biosynthesis of SeNPs from bee propolis that are widely used in folk medicine since ancient time.

Propolis is a natural resinous beehive product collected by honey bees from leaf buds and exudates of various plants, mixed with bee enzyme, pollen and wax. Its complex bioactive compounds depend on plant sources in local flora and exhibit a broad spectrum of biological and pharmacological properties [23]. It was reported that bee propolis can synthesize nanoparticles, due to its broad range of biomolecules like phenols, flavonoids, alkaloids, steroids, terpenoids etc. [24,25]. In this study, the ethanol extract of bee propolis collected from different states of India has been used to evaluate its antioxidant potential and the extract of bee propolis collected from Karnataka was used as reducing, capping, and stabilizing agent for the biosynthesis of SeNPs and its antimicrobial activity.

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Received: December 17, 2018; **Accepted:** February 11, 2019; **Published:** February 15, 2019

Citation: Shubharani R, Mahesh M, V.N.Yogananda Murthy (2019) Biosynthesis and Characterization, Antioxidant and Antimicrobial Activities of Selenium Nanoparticles from Ethanol Extract of Bee Propolis. J Nanomed Nanotechnol 10: 522. doi: [10.4172/2157-7439.1000522](https://doi.org/10.4172/2157-7439.1000522)

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Materials and Methods

Fresh propolis sample belonged to *Apis mellifera* colonies were collected from 5 states like Haryana, Himachal Pradesh, Uttaranchal, Karnataka and Kerala in India. The samples were cleaned to remove the debris and extracted with 70 % ethanol for further investigations [26].

Antioxidant assay

The antioxidant potential of ethanol extracts of propolis samples collected from 5 different states of India were used.

ABTS free radical-scavenging activity

ABTS radical scavenging was carried out by the reaction of ABTS (7 mM) solution in different aliquots of ethanol extracts (100, 200, 300, 400, 500 µg/ml). After the incubation in dark for 15 min, extent of decoloration was measured at 734 nm. ABTS reagent without sample was used as control. Percentage of ABTS radical inhibition was plotted against gallic acid and IC₅₀ values were determined [27].

DPPH free radical scavenging activity

DPPH scavenging activity was estimated by addition of different concentration of ethanol extracts to 0.1 mM of 1-1-diphenyl-2-picrylhydrazyl in methanol. The solution was incubated in dark at 37°C for 30 min and change in absorbance was measured at 517 nm using spectrophotometer. Percentage of DPPH free radical scavenging activity of each concentration was calculated and IC₅₀ values were determined [28].

FRAP assay

Different concentrations (100-500 µg/ml) of ethanol extracts were separately mixed with FRAP reagent and allowed to react at 37°C in dark for 30 min. An increase in the absorbance with increasing concentration is directly proportional to the reducing power of samples. Results expressed as the concentration of samples having ferric-tripyridyltriazine reducing ability in gallic acid was assessed by measuring the absorbance at 593 nm with some modifications [29].

Superoxide dismutase (SOD) activity

In this assay, the reaction mixture containing 250 mM phosphate buffer (pH 7.8), EDTA (0.1 mM), NBT (75 µM), methionine (13 mM) and riboflavin (2 µM) was mixed with different concentrations of ethanol extracts and incubated at room temperature for 15 min, absorbance was measured at 560 nm. Inhibition percentage of superoxide anion generated was calculated [30].

Biosynthesis of SeNPs from ethanol extract of propolis

The highest antioxidant activity was observed in propolis extract collected from Karnataka state and used for the biosynthesis of SeNPs. In this process, 10 ml of freshly prepared ethanol extract was added drop wise to 30 mM sodium selenite solution, along with 40 mM ascorbic acid. The mixture was allowed to react at room temperature for 24 hrs till the colour changes from light brown to orange. The biosynthesised SeNPs were centrifuged at 10,000 rpm for 30 min at 4°C and then the separated nanoparticles were washed thrice with double distilled water. The nanoparticles were then washed by using ethanol and dried overnight. The powder form of SeNPs was used for further characterization and analysis [31].

Characterisation of selenium nanoparticles

UV-vis spectrum analysis: UV-vis spectrum is the most important

technique for identification and characterization of nanoparticles. The wavelength of biosynthesised SeNPs was recorded from 200-800 nm using Genesys 10S UV-visible spectrophotometer and analysed for the maximum absorption peaks.

Fourier transform infrared spectroscopy analysis: FT-IR spectrum was obtained using spectrophotometer (Shimadzu-00463 model) in the spectral region of 4000-600 cm⁻¹ using a resolution of 4 cm⁻¹ and 64 coadded scans. FT-IR spectrum confirms the surface chemistry and organic functional group responsible for stabilization of nanoparticles [32].

X-ray diffraction analysis: XRD reading was taken on a Bruker D8 Advanced Eco X-ray diffractometer having Bragg-Brentano Focusing geometry. XRD data was analyzed using X'pert high score software for the identification of the crystalline phases. X-ray diffraction is used for the phase identification and structure of nanoparticles [33].

Scanning electron microscopy analysis: A Hitachi (Model SU 3500) ultrahigh resolution field emission scanning electron microscope was used to observe the morphological characteristics and size of biosynthesised SeNPs [34].

Antimicrobial assay

Antimicrobial assay was evaluated only for the SeNPs biosynthesised from propolis extracts obtained from Karnataka. The antibacterial activity against pathogenic gram-positive bacterial strains (*Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus mutans*) and gram-negative bacterial strains (*Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*) were evaluated by resazurin microtiter plate method to check the minimum inhibition concentration. Antifungal activity of pathogenic fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* were determined by well diffusion method [35,36].

Antibacterial assay by resazurin microtiter plate: Resazurin microtiter plate method was used to study the antibacterial activity of the biosynthesised SeNPs. Resazurin solution (10 %) was prepared in sterile water, stored at 20°C as a stock solution and diluted to 1:10 with sterile water when required. In each well of the microtiter plate, 100 µl of sterilized Luria Bertani (LB) broth, 30 µl of 0.1% resazurin solution, 100 µl of serially diluted biosynthesized SeNPs (1000, 500, 250, 125, 62.5, 31.25, 15.25, 7.81 µg/ml) was added. Subsequently, 100 µl of each bacterial culture was inoculated and 200 µl of deionized water was added to prevent the sample from drying. The control wells were prepared with culture media, resazurin dye, and bacterial suspension. Color blank wells were prepared only with broth and dye. The plates were sealed and incubated for 24 hrs at 37°C. After incubation, the color change from blue to pink was observed, whereas blue colored wells with no growth of organisms, pink and orange colors were positive with growth of organisms.

Antifungal assay by well diffusion method: Well diffusion method was applied to study the antifungal activity of biosynthesised SeNPs from propolis extract. Fungal inoculums of *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* were spread thoroughly on the sterilized petriplates with solidified potato dextrose agar media. On each agar plates, five wells with 5.5 mm diameter were made using sterile cork borer. The wells were filled with 50 µl of different concentrations of sample. Standard fungicide cycloheximide used as positive control and ethanol used as a negative control. The culture plates were incubated at 25°C for 72 hrs and the zone of inhibition for all the three organisms was measured in diameter (mm) around the wells.

Statistical analysis

The experiment was conducted in triplicates and the results were statistically analysed. All the standards were calculated by regression value and Pearson's correlation is less than 0.05.

Results

Antioxidant activity

The antioxidant potential of ethanol extracts of propolis collected from the different locations of India was measured by ABTS, DPPH, FRAP and SOD assays.

ABTS assay

ABTS radical cation scavenging activity is an excellent method to determine the antioxidant capacity of the samples, which has the characteristic absorbance maxima at 734 nm. In the results, ethanol extracts showed a significant ABTS radical scavenging activity. Among all the samples, the propolis collected from Karnataka exhibited highest activity with IC₅₀ value of 15.1 µg/ml, which was comparable with the standard gallic acid. The antioxidant activity of ethanol extracts is represented in Figure 1A.

DPPH assay

DPPH (1-1-diphenyl-2-picrylhydrazyl) was used to measure the free

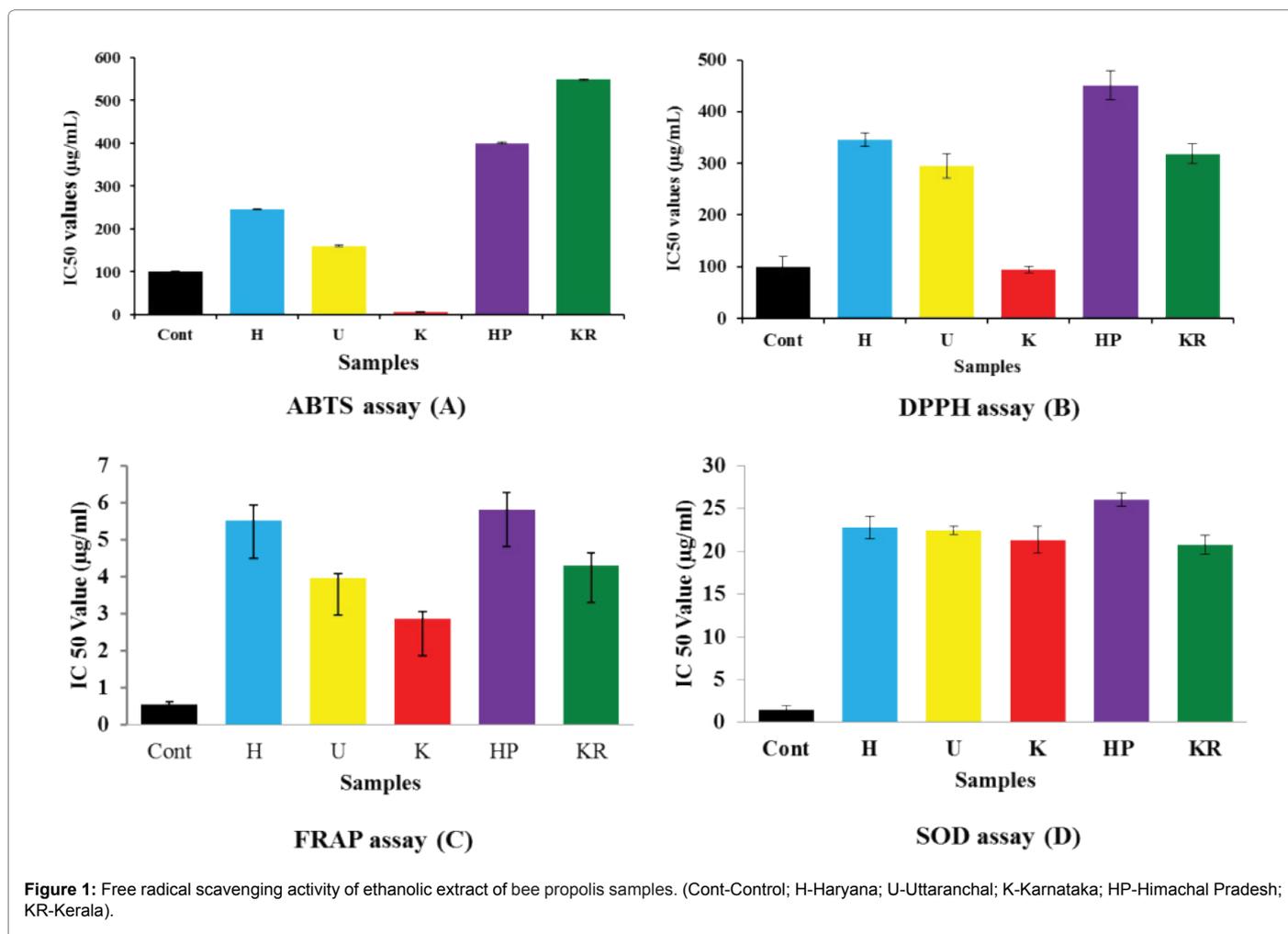
radical scavenging activity which was stable at room temperature and accepts electron or hydrogen free radical to form a stable diamagnetic molecule. This ability of DPPH to undergo reduction by an antioxidant is measured in terms of decrease in its absorbance at 517 nm. Maximum free radical scavenging activity was determined by the sample collected from Karnataka with IC₅₀ value of 78.9 µg/ml followed by Uttaranchal (217.2 µg/ml), Kerala (223.5 µg/ml) and Haryana (263.8 µg/ml). The minimum activity was recorded from Himachal Pradesh sample with 358.2 µg/ml of IC₅₀ value. The results of DPPH radical scavenging activity of ethanol extracts is represented in Figure 1B.

FRAP assay

FRAP is a simple assay that measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-S-triazine complex (TPTZ-Fe (III)) to blue coloured ferrous complex (TPTZ-Fe(II)) in acidic medium. All the ethanol extracts tested exhibited higher antioxidant capacity and the IC₅₀ values ranged from 2.86 µg/ml (Karnataka) to 5.71 µg/ml (Himachal Pradesh). The potential antioxidant activity of ethanol extracts are presented in Figure 1C.

SOD assay

Superoxide is a highly reactive toxic species, induces oxidative damages in the living system. The reduction of riboflavin generates superoxide radical which reduced NBT, resulting in the formation of



blue formazine. The inhibition of formazine was maximum in extracts collected from Karnataka with IC_{50} value of 21.65 $\mu\text{g/ml}$ followed by Kerala (22.22 $\mu\text{g/ml}$), Uttaranchal (22.32 $\mu\text{g/ml}$), Haryana (22.52 $\mu\text{g/ml}$) and Himachal Pradesh (25.91 $\mu\text{g/ml}$). Gallic acid was used as positive control in the entire antioxidant assay. The superoxide radical scavenging assay of propolis samples are presented in Figure 1D.

Biosynthesis of selenium nanoparticles

The reduction of selenium ions into SeNPs induced by propolis extracts was evident by change in color from light brown to reddish orange, which is the most significant property of nanoparticles. The ethanol extract of propolis from was brown in color and after the addition of acidic sodium selenite solution, color changed to orange within 24 hrs indicating the formation of SeNPs.

Characterization of selenium nanoparticles

The characteristics of nanoparticles such as size, structure and shape are essential for the determination of particles property. There are a number of methods to characterise the biosynthesised nanoparticles among which UV-visible spectrophotometry, Fourier transform infrared, X-ray diffraction and Scanning Electron Microscopy are widely used.

UV-vis spectrum analysis

UV-vis spectrum of SeNPs biosynthesised from ethanol extract of propolis is shown in Figure 2. The absorbance spectrum was recorded in the range of 200-800 nm. A strong absorption peak was observed between 250-280 nm with maxima at 265 nm, confirming the presence of selenium in the samples.

FT-IR analysis

FT-IR spectra of SeNPs are presented in Figure 3 and results showed the peak value around 2950 and 2850 cm^{-1} may be due to the presence of organic polymer lignin. The absorption peak around 1750 and 1470 cm^{-1} can be the peak of carboxyl group. The peak at 1030 and 730 cm^{-1} represent phenolic group. Spectra confirmed the presence of different functional group like alcohol and polyphenols, may be responsible for reduction and stabilization of SeNPs.

XRD analysis

XRD pattern of SeNPs studied is portrayed in Figure 4, where the diffraction intensity was observed from 2θ angles varying from 10° to 100° . The distinct peak at 23.2° , 29.5° , 45.5° , 55.5° and 59.8° determined the crystalline nature of SeNPs.

SEM analysis

Scanning electron microscopy (SEM) is widely used for inspecting the particles morphology, i.e. to determine the physical dimensions, shapes and size distribution of nanoparticles. Result obtained revealed that the morphological characteristics of biosynthesised SeNPs by propolis extract was found to be oval in shape with a smooth surface and the size ranged from 52.9-118 nm (Figure 5).

Antimicrobial activity

Antibacterial potential of SeNPs biosynthesised from Karnataka propolis sample was evaluated according to colour change against the bacterial strains such as *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus mutans*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* by resazurin microtiter plate method and summarised in Table 1. Results revealed that, an antibacterial potential of SeNPs sample showed a significant antibacterial activity against 4

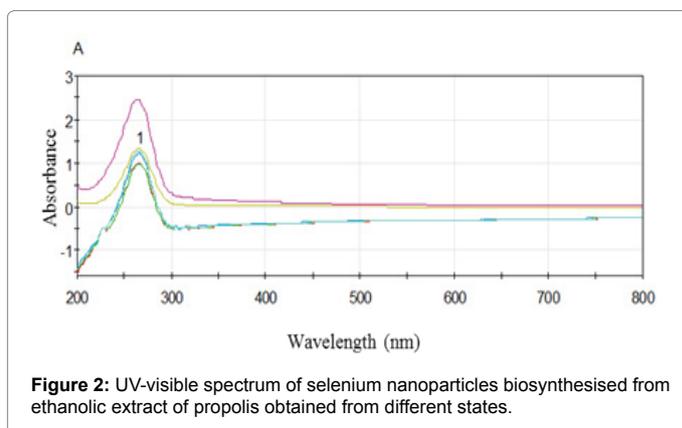


Figure 2: UV-visible spectrum of selenium nanoparticles biosynthesised from ethanolic extract of propolis obtained from different states.

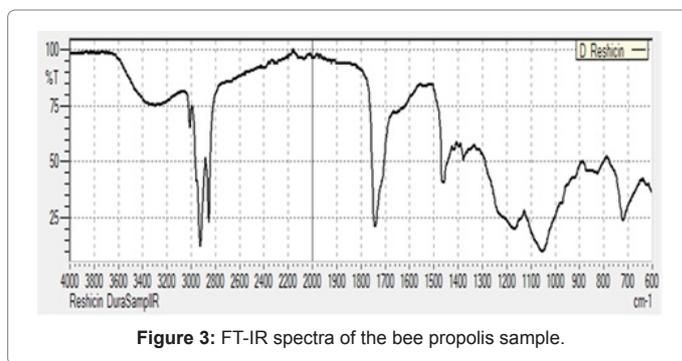


Figure 3: FT-IR spectra of the bee propolis sample.

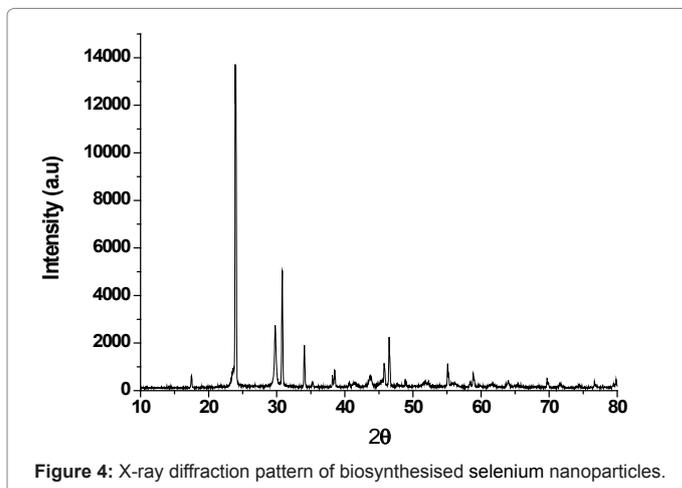


Figure 4: X-ray diffraction pattern of biosynthesised selenium nanoparticles.

bacterial strains. Highest antibacterial activity was observed against *Bacillus cereus* and *Staphylococcus aureus* (gram-positive bacteria) at a concentration of 250 $\mu\text{g/ml}$. The moderate activity was observed against *Streptococcus mutans* (gram-positive bacteria) followed by *Salmonella typhi* (gram-negative bacteria) at the concentration of 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ respectively. SeNPs did not inhibit the growth of *Escherichia coli* and *Pseudomonas aeruginosa* up to the highest concentration of 1000 $\mu\text{g/ml}$. The antifungal effect of SeNPs was evaluated against *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* by well diffusion method. Maximum zone of inhibition was found in *Aspergillus niger* (20 mm) followed by *Aspergillus flavus* (18 mm) at 500 $\mu\text{g/ml}$ concentration, where as *Candida albicans* did not show any significant zone of inhibition (Table 2).

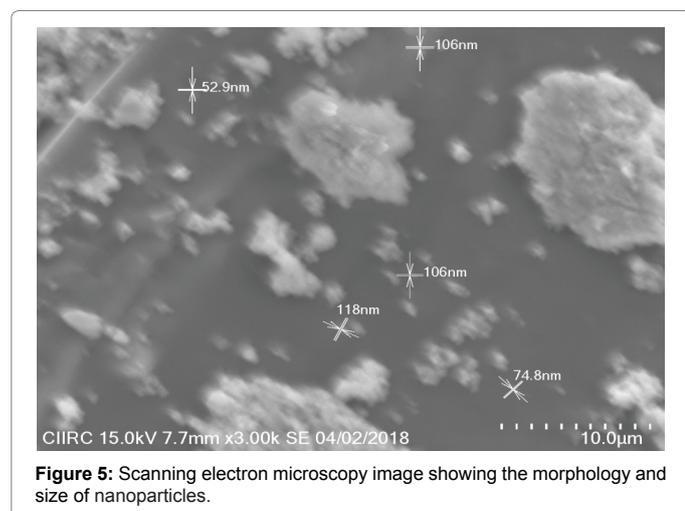


Figure 5: Scanning electron microscopy image showing the morphology and size of nanoparticles.

Sl. No.	Test organisms	Minimum Inhibition Concentration (MIC) of SeNPs (µg/ml)
1.	<i>Bacillus cereus</i>	250
2.	<i>Escherichia coli</i>	000
3.	<i>Salmonella typhi</i>	1000
4.	<i>Pseudomonas aeruginosa</i>	000
5.	<i>Staphylococcus aureus</i>	250
6.	<i>Streptococcus mutans</i>	500

Table 1: *In vitro* antibacterial activity of ethanol extract of propolis by resazurin microtiter plate method.

Sl. No.	SeNPs Concentrations	Fungal Zone of Inhibition (mm)		
		<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Candida albicans</i>
1.	100 µg	10	9	9
2.	200 µg	11	9	9
3.	300 µg	13	9	9
4.	400 µg	15	10	9
5.	500 µg	20	18	9
6.	Ethanol	00	00	00
7.	Cycloheximide	22	22	18

Table 2: *In vitro* antifungal activity of ethanol extracts of propolis by well diffusion method.

Discussion

Although the previous report on biosynthesis of SeNPs using plant extract [37] and microorganisms [38] are available, there is a lack of scientific literature on biosynthesis of SeNPs mediated by propolis extract. Hence, this report gives an alternative approach for biosynthesis of SeNPs using an ethanol extract of propolis. The reduction of selenium ions into SeNPs induced by propolis extracts was confirmed by the conversion of color from light brown to reddish orange after the addition of acidic sodium selenite solution which is the most significant property of nanoparticles. Result is in accordance with the previous report [39] on synthesis of SeNPs mediated by aqueous extract of *Aloe vera* leaf. Present results also correlates with UV-visible spectra of SeNPs biosynthesised from the aqueous extract of orange peel and *Petroselinum crispum* leaf showed the peak at 265.5-270 nm respectively [40,41].

The functional group such as alcohol and polyphenols involved in the reduction of selenium ion to SeNPs were detected with FT-IR analysis. Recent reports on synthesis of SeNPs by the aqueous extract

of *Leucas lavandulifolia* leaf, *Bougainvillea spectabilis* flower and dried *Vitis vinifera* fruits highlighted the probable role of phytochemicals such as terpenoids, sugar, amines, alcohols, phenols and carboxylic acids in reduction of selenium ions and stabilisation of SeNPs [42-44]. X-ray diffraction is an effective technique used to characterise the nanoparticles. Present results confirmed the biosynthesised SeNPs were in the form of nanocrystals, which corresponds to the reported data of biosynthesised SeNPs mediated by *Diospyros montana* leaf extract [45]. Size of the biosynthesised SeNPs was analysed by scanning electron microscopy. Size of nanoparticles has a significant effect on the UV-vis absorption spectra, i.e. small size particles absorb light of a lower wavelength and vice versa [46]. The particles were found to be oval shaped with diameter range of 52.9-118 nm. The present observation is in accordance with the report [47] where *Bacillus mycoides*, isolated from the rhizosphere of legume *Astragalus bisulcatus* was used to synthesise SeNPs by reducing sodium selenite with size range of 50-400 nm. Similarly, the shape of SeNPs synthesised from fenugreek seed extract in aqueous medium was oval in shape with smooth surface, but the size ranging from 50-150 nm. They have been found to be active against human breast cancer cells [48]. There is a clear correlation between the nanoparticles size and their biological activity [49]. Thus, these reports clearly justify the efficient synthesis and stabilisation of SeNPs from the ethanol extract of propolis.

The radical scavenging activity of ethanol extract of propolis was found to be concentration dependent, the activity increased with increasing concentrations. Ethanol extracts showed a comparable ABTS, DPPH, FRAP and SOD radical scavenging activities with gallic acid as a standard [50]. However, the propolis samples collected from Karnataka exhibited an effective radical inhibition in all the assays compared to other states. SeNPs are less toxic form of selenium, has been proved to have antioxidant activity and is being used as chemopreventive agent in cancer treatment [39]. Nanoparticles are endorsed as sustainable antimicrobial agents having remarkable perspective to resolve the microbial multidrug resistance problem [51,52] and microbial biofilm formation [12,53]. Resazurin microtiter plate method has provided a potentially useful technique for determining maximum inhibitory concentration (MIC) of a large number of test samples. MIC in microbiology is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after required incubation. It is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agents and also to monitor the activity of new antimicrobial agent [54,55]. Resazurin is blue compound, has been potentially used for a novel antibacterial therapy where the active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink) which can be further reduced to hydroresorufin [56]. The biogenic nanoparticles have been found to be active against a wide range of gram-positive and gram-negative bacteria [57-61]. According to the results of the present study, it is observed that the increasing quantity of SeNPs have comparatively higher antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* probably due to thinner peptidoglycan layer and presence of porins [62]. From the present results, it is indicated that the biosynthesised SeNPs mediated by propolis extract has the efficient antioxidant and antimicrobial activity against pathogenic organisms. The antifungal activity of SeNPs extract was enhanced by increasing the concentration, confirming the inhibition activity of the extract was concentration dependent. Furthermore, the antifungal activity of standard antifungal cycloheximide was more potent than the biosynthesised SeNPs extract against tested organisms in the present study [63]. However, much work is needed to control the morphology

and particle size, important issues in the evaluation of nanoparticle synthesis.

Conclusion

The SeNPs can be used for various applications especially in medicine, due to its medicinal properties such as low toxicity, better reactivity, low dosage, etc. The formation of SeNPs and their functionalization with ethanol extract of bee propolis were evident from UV-vis. Spectra, XRD, FT-IR and SEM images. Metabolites from propolis extracts i.e alcohol and phenolic compounds were predominantly responsible for the reduction of selenium ion to SeNPs. The biosynthesized SeNPs shows oval shape with a smooth surface and the size range of 52.9-118 nm. SeNPs exhibited significant activity against antioxidant properties, gram-positive and gram-negative bacterial and fungal strains. The biosynthesized SeNPs could be a potent antioxidant and antimicrobial agent to treat diseases caused by gram-positive and fungal strains may be used to kill the pathogens for preventing diseases. Antioxidant assays of all the propolis samples studied indicated that, the propolis extract collected from Karnataka showed a significant radical scavenging activity. However, further research is needed to improve the synthesis efficiency, control of particle size and its application in medicine and healthcare.

Conflict of Interest

All the authors declare that they have no conflict of interest.

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