Indian Journal of Experimental Biology
Vol. 62, December 2024, pp. 1001-1009
DOI: 10.56042/ijeb.v62i12.8081

# Exploring medicinal potential of *Bixa orellana* L. root extract: Synthesis and analysis of silver nanoparticles

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*Received 23 January 2024; revised 18 May 2024*

*Bixa orellana* L., commonly known as "sindoor," has been used for various medicinal applications, which showed its potential use as an active ingredient in pharmaceutical products. The aim of this work was to exhibit the ethnopharmacological and biological activity and the studies on phytochemistry of *Bixa orellana* L root extract. The root extract was subsequently used for the green synthesis of silver nanoparticles (AgNPs), which was confirmed by UV, SEM and XRD analysis. The antimicrobial efficacy of these nanoparticles was assessed against both Gram-positive and Gram-negative bacteria as well as fungi and was compared with the indigenous root extract. The *Bixa* root extract was found to have potential effect against clinical pathogens, free radicals and HEp-2 cell lines. FTIR analysis confirmed the presence of various amine, alcohol, and carboxylic acid groups. UV spectra of AgNPs showed surface plasmon resonance at 425 nm and spherical nanoparticles ranging from 35-53 nm by SEM. X-ray diffraction spectrum exhibited 2θ values corresponding to the silver nanocrystal. The antimicrobial activity of these nanoparticles was compared with that of root extract. This study shows the well-characterized pharmacological actions that may be considered relevant for the future development of an innovative therapeutic agent.

**Keywords**: Antimicrobial, Antioxidant, *Bixa* root extract, HEp-2 cell line

*Bixa orellana* is an evergreen shrub or a small tree native to tropical America and spread in many parts of Asia and Africa. It belongs to the family Bixaceae. In India, it is commonly known as "Sindoor". *Bixa* has economic significance since it is a sole source of the natural reddish orange pigment produced from the aril portion of their seeds. In global market, *Bixa* is the most preferred natural food grade colourant next to saffron[1]. This dye was also used by Amerindians as war paint, dyeing of wool, cotton and silk[2]. Bixin present in *Bixa* is an effective biological singlet molecular-oxygen quencher and shown to protect the cells and tissues against deleterious effects of free radicals[3]. Bixin is also demonstrated to possess activity against clastogenic effects like antitumour[4], is chemo preventive[5], possess antimicrobial[6,7] and antioxidant properties[8]. The root decoction is reported to control asthma, venereal diseases, dysentery, influenza, jaundice and to treat gonorrhea[9,10]. The medicinal value of the root may be attributed to the presence of bioactive compounds like alkaloids, glycosides, tannins, flavonoids *etc*., which produce physiological actions in the human and animal[11].

On the other hand, researchers are now focusing on unique attributes of nanoscale materials by combining traditional medicines with existing technology thereby improving its properties. It was reported that among metals, silver is an effective antimicrobial agent against more than 650 pathogens and having a broad spectrum of antimicrobial activity, allowing its use in a wide range of applications[12]. Therefore, nano-silver is now considered one of the most viable alternatives to antibiotics because it seems to have high potential to solve the problem of multidrug resistance, which is often observed with several bacterial strains[13]. Several approaches are available for the synthesis of silver nanoparticles (AgNPs), *viz*., chemical and physical[14], photochemical reactions in reverse micelles[15] and electrochemical techniques[16]. The major disadvantages associated with these methods include, the use of hazardous chemicals, low material conversions, high energy, temperature, and pressure requirements, and wasteful purifications, which are quite expensive and potentially dangerous to the environment[17]. The advantages associated with plant mediated synthesis of AgNPs are, cost effectiveness,

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environment friendly, easy scalability, no use of high pressure, energy, temperature, toxic chemicals and compatibility for pharmaceutical and biomedical applications[18].

In the present study, the root extract of *Bixa orellana* was employed for various biological applications such as antimicrobial, antioxidant, and anticancer activities. In addition to this, for the first time, we report the green synthesis of AgNPs using *Bixa* root extract. The synthesized AgNPs were further found to be highly effective against clinical pathogens. Hence, *Bixa* root extract could be used as a viable and potential source for several biomedical applications.

## Materials and Methods

### Materials

Nutrient agar/ broth and Potato dextrose agar was obtained from Hi-media, India. TLC plate, Silica gel (60-120 mesh) was purchased Merck, India. Dimethyl sulphoxide (DMSO)-molecular grade, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, Minimal Essential Media (MEM), 100× antibiotic-antimycotic solution, 0.25% Trypsin-EDTA, MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], potassium bromide (KBr) were procured from Sigma Aldrich, USA. All other reagents and chemicals used of analytical grade.

### Plant collection, preparation of its root extract and screening for phytochemicals

Fresh roots of *Bixa orellana* were collected from CSIR-CLRI campus, Chennai, India. Roots were washed with water, dried in shade at 30±2°C and powdered mechanically using pulveriser. 20 g of root powder was extracted using different solvents like water, methanol, ethyl acetate, petroleum ether, and chloroform by Soxhlet extraction method. The extract was concentrated by rotary evaporator and percent yield of crude extracts was calculated. The methanolic root extract was screened for the presence of various phytoconstituents such as alkaloids, phenolics, flavonoids, tannins, saponins, terpenes, steroids, and glycosides[19].

### Bioactivity of root extract

#### *Antimicrobial activity*

Different microbial strains (bacteria and fungi) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh. The *Bixa* root extract was tested for its antibacterial activity against pathogens *viz*., *Bacillus subtilis, Proteus vulgaris, Salmonella typhii, Staphylococcus epidermis, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* by standard disc method[20]. Briefly, 100 µL of freshly grown overnight cultures (~18 h old) were spread on nutrient agar plates. 10 µg/mL of root extract dissolved in DMSO was loaded on sterile discs (5 mm diameter) and allowed to dry before being placed on agar plates. Streptomycin disc (10 µg/disc) was used as reference drug. The plates were incubated overnight at 35±2°C for 24 h and the zone of inhibition was measured[21]. Similarly, the antifungal assays were done with different fungi *viz*., *Rhizopus arrizhus, Aspergillus nidulans, Fusarium dimerum* and *Candida albicans.* Potato dextrose agar medium was inoculated with fresh fungal cultures (100 µL). About 20 µg/mL of extract were loaded on sterile 5 mm disc and allowed to dry which was then place on the inoculated plate. Amphotericin antibiotic disc (20 µg/disc) was used as reference drug. The plates were incubated at 35±2 °C for 24-48 h. After incubation, the different levels of zone of inhibition were measured using the Hi antibiotic zone scale[22].

#### *Antioxidant activity*

The antioxidant activity of root extract was measured by DPPH method[23]. Briefly, different concentrations of the root extract (5-100 µg/mL) were mixed with 50 µL of DPPH (0.004% in methanol) in labelled tubes. The tubes were incubated in dark for 30 min, and absorbance was noted at 517 nm (Cary 100 Perkin - Elmer UV-visible spectrophotometer, USA). Ascorbic acid was used as standard reference. All the tests were performed in triplicates. The antioxidant activity percentage was calculated using the formula:

$$\text{Antioxidant activity (\%)} = [(AC - AE)/AC] \times 100$$

[Where, AC is the absorbance of a DPPH solution without extract, AE is the absorbance of the test extract. The $IC_{50}$ value for root extract was calculated. $IC_{50}$ denotes the concentration of extract required to scavenge 50 % of DPPH free radicals]

### In vitro cytotoxicity assay (MTT)

HEp-2 cell lines were obtained from National Centre for Cell Science, Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10 % FBS, Penicillin (100 U/mL), and Streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL $CO_2$ at 37°C. The MTT

(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to determine the cytotoxic effect of the root extract on HEp-2 cells[24]. The assay depends on the reduction of MTT to a purple formazan product by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells. Cells ($1\times10^5$/well) were seeded in 1 mL of fresh culture medium/well in 24-well plates. After 48 h incubation, the cells reached confluence, which were then incubated in the presence of various concentrations of the root extract dissolved in 0.1% DMSO for 48 h at 37ºC. After removal of the sample solution and washing with phosphate buffered saline (pH 7.4), 200 µL/ well (5 mg/mL) of 0.5 % MTT was added and the plates were incubated for 4 h at 37 ºC. The purple formazan product was dissolved by addition of 100 µL of 0.1% DMSO. Optical density (OD) was read at 570 nm using a microplate reader (Thermo Scientific Multiskan Ascent, USA), and each experiment was done in triplicates. The antitumour effect of tested extract was determined by comparing the optical density of the treated cells against optical density of the untreated cells. Cell viability was calculated by using the formula:

% Viability = (OD of test sample / OD of control) × 100.

#### Synthesis of AgNPs using *Bixa* root extract

The washed and dried *Bixa* roots were powdered (5 g) using mixer grinder, and boiled in a 250-mL beaker along with 100 mL of Millipore water for 15 min and filtered through Whatman No.1 filter paper.10 mL of fresh root extract was mixed with 500 mL of 1 mM aqueous silver nitrate solution, and the reaction was allowed to proceed under dark condition at 30±2°C[16]. For purification, the AgNPs obtained from *Bixa* root extract were centrifuged at 10,000 rpm for 15 min, supernatant was discarded and the pellet was re-dispersed in Millipore water to get rid of any uncoordinated biological materials and taken for experimental characterization[25].

#### Characterization of synthesized AgNPs

The synthesized AgNPs were characterized using various instrumental techniques *viz*., UV-Vis Spectroscopy (Cary 100 Perkin- Elmer), FTIR spectroscopy (Nicolet Impact 400 thermo electron) and X-ray Diffraction (Philips PW1830 x-ray diffract meter) with Cu kα radiation (λ = 1.5406 Å) source in the 2θ range of 10–80° with 4/min scanning rate. The average crystallite size of the synthesized AgNPs was calculated using Scherrer′s formula, $D= 0.9\lambda/\beta\cos\theta$. The surface morphology, shape and size of the synthesized AgNPs were observed by SEM (JOEL-JFC 6360).

#### Antimicrobial activity of the synthesized AgNPs

The AgNPs synthesized from *Bixa* root extract were tested for their antimicrobial activity as mentioned in the section above. The only difference was the discs were loaded with synthesized AgNPs instead of extract.

## Results

*Bixa* root extracts were prepared using various solvents using Soxhlet extraction. Among all the solvents used methanol gave a higher yield towards the extraction of constituents from *Bixa* roots and hence, was used for further analysis (Table 1). The phytochemical investigation of the methanolic root extract revealed the presence of tannins, flavonoids, terpenoids, steroids, alkaloids, phenolics and saponins (Table 2).

#### Bioactivity of root extract

##### *Determination of antibacterial and antifungal activity*

The antimicrobial activity of the *Bixa* root extract is shown in Table 3. Among all bacterial strains tested, maximum zone of inhibition was observed against *S. aureus* (16 mm) and least activity was recorded for *S. typhi* measuring 11 mm. Streptomycin was used as a control which showed activity against all tested organisms as should be the case. Notably the root extract showed greater inhibition (13.9 mm) in

Table 1 — Percent yield of *Bixa* extract

| Solvents | Yield (%) |
|---|---|
| Methanol | 35.48 |
| Ethyl acetate | 20.08 |
| Petroleum ether | 18.67 |
| Water | 14.51 |
| Chloroform | 8.6 |

Table 2 — Qualitative phytochemical analysis of methanolic root extract of *Bixa orellana*

| Chemical constituents | Result |
|---|---|
| Tannins | + |
| Saponins | + |
| Flavonoids | + |
| Alkaloids | + |
| Phenolics | + |
| Steroid | + |
| Terpenoid | + |
| Glycosides | - |

[+, Positive; -, Negative]

comparison to Streptomycin (11.9 mm) against *B. subtilis*. The root extract showed significant antifungal activity against all tested fungal strain, however, the activity against *R. arrizhus* (24 mm) was highest and least activity against *C. albicans* (12 mm).

***Antioxidant activity of root extract***

The various concentrations of *Bixa* root extract were tested against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) to assess the free radical scavenging activity which was found to increase with increase in concentration of extracts[26]. *Bixa* extract showed 88% DPPH free radical scavenging activity at 125 µg/mL. The $IC_{50}$ value of the extract was found to be 25.86 µg/mL, which is much lower than the $IC_{50}$ value of ascorbic acid (46.74 µg/mL). This indicates that the extract has significantly higher antioxidant activity than ascorbic acid (Fig. 1). A comparison between the DPPH radical scavenging ability of *Bixa* extract with other traditional medicinal plants indicated that the former was more potent whereby its $IC_{50}$ values were much lower than other medicinal plants suggesting its potential in exerting its radical scavenging effects at a much lower concentration. A recent study with *Bixa orellana* extract reported antioxidant activity of 59.74% inhibition at 100 µg/mL[27].

Table 3 — Activity of root extract of *Bixa* against bacteria and fungi

| Test organism | Zone of inhibition in diameter (mm) | |
|---|---|---|
| *Bacillus subtilis* | 13.9 ± 0.52 | 11.9 ± 0.58 |
| *Proteus vulgaris* | 14.2 ± 0.48 | 18.5 ± 0.40 |
| *Salmonella typhi* | 11 ± 0.85 | 22 ± 0.78 |
| *Staphylococcus aureus* | 16 ± 0.55 | 28.12 ± 0.58 |
| *Escherichia coli* | 13.9± 0.44 | 20.1± 0.42 |
| *Klebsiella pneumonia* | 14.1 ± 0.85 | 21 ± 0.78 |
| *Pseudomonas aeruginosa* | 14 ± 0.68 | 18.12 ± 0.61 |
| *Aspergillus nidulans* | 18.4 ± 1.44 | 17.6±1.15 |
| *Fusarium dimerum* | 18.0 ± 1.50 | 21± 0.57 |
| *Rhizopus arrhizus* | 24.0 ± 1.75 | 28± 1.52 |
| *Candida albicans* | 12.0 ± 0.46 | 12 ± 0.62 |

***In vitro* cytotoxicity**

*In vitro* antitumour activity of the root extract was assessed by MTT assay at different concentrations. The cytotoxicity was increased at higher concentrations of root extract. The $IC_{50}$ value of the methanolic extract was found to be 2.6 mg/mL. At a concentration of 10 mg/mL, cell mortality was found to be 89.8%. The proliferation of HEp-2 cells was inhibited by *Bixa* root extract at lowest concentration (0.15 mg/mL) (Fig 2A and Fig 2B). *Bixa* root extract had the ability to inhibit the growth of the HEp-2 cells by less than 15% at a concentration of 10 mg/mL. Morphological changes observed include cell shrinkage and reduction of MTT to purple colour formazon. The plant-derived products are more likely to have fewer side effects than synthetic anticancer drugs, and they can also be produced at a lower cost.

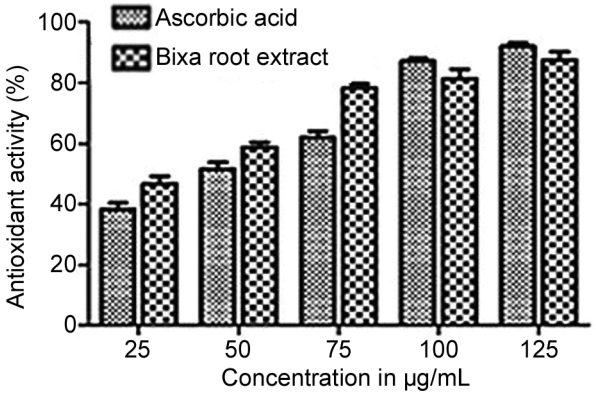


Fig.1 — DPPH radical scavenging activity of *Bixa* root extract compared with standard.

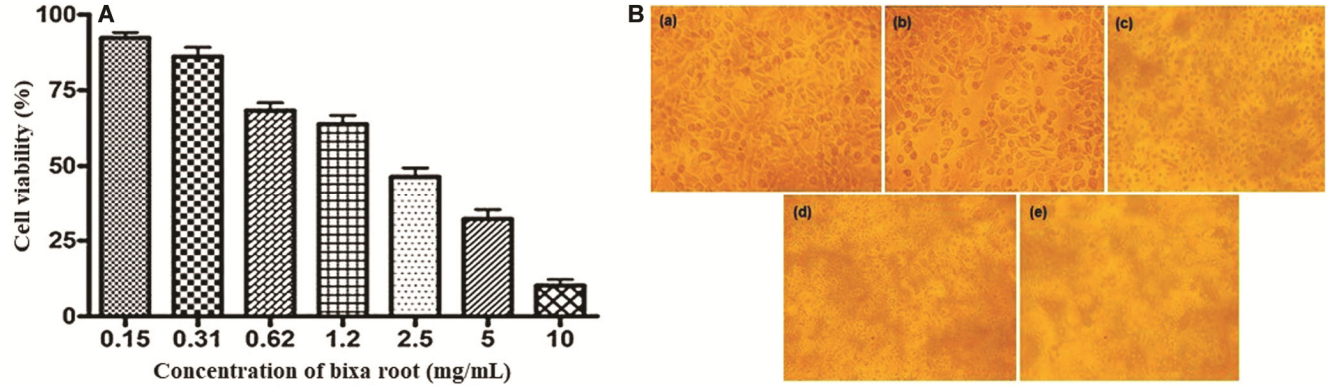


Fig. 2 — (A) Cytotoxicity of *Bixa* root extract on Hep-2 cell lines and (B) Antitumour activity of *Bixa* root sample on HEp-2 cell lines (a) Normal HEp-2 cell line (b) Toxicity- 0.625 mg/mL (c) Toxicity-1.25 mg/mL (d) Toxicity-2.5 mg/mL (e) Toxicity-10 mg/mL.

**Synthesis of AgNPs using *Bixa* root extract**

The reaction of *Bixa* root extract with aqueous solution of silver nitrate changed its colour from pale yellow to reddish brown indicative of the formation of AgNPs and exhibited a plasmon absorption band at 425 nm (Fig. 3). The bio-reduction rate of the silver ions occurs very quickly and colour arises due to excitation of surface plasmon vibrations in the AgNPs [28]. Further, broadening of peak indicated that the nanoparticles are polydispersed. The frequency and width of the surface plasmon absorption depends on the size and shape of the metal nanoparticles as well as on the dielectric constant of the AgNPs. Three different routes for the reduction of silver in plant extract have been proposed which are the secondary metabolites present in plant systems may be responsible for the reduction of silver and synthesis of nanoparticles, the second biogenic route is the energy (or) electron released during glycolysis (photosynthesis) for conversion of NAD to NADH led to transformation of silver nitrate to form nanoparticles and another mechanism is releasing of an electron when formation of ascorbate radicals from ascorbate reduces the silver ions and the reduction of silver ions and resulting formation of AgNPs might have been driven by some active components in root extract[29].

**Characterization of AgNPs synthesized from *Bixa* root extract**

***Scanning electron microscopy (SEM)***

The SEM image revealed high density AgNPs synthesized by *Bixa* root extract. It was observed that relatively spherical shaped AgNPs were formed with diameter ranging from 33 to 53 nm (Fig. 4). The AgNPs assembled due to the hydrogen bond and electrostatic interactions between the bio-organic capping molecules bound to the AgNPs. The large sized observed nanoparticles may be due to the self-assembly of smaller nanoparticles.

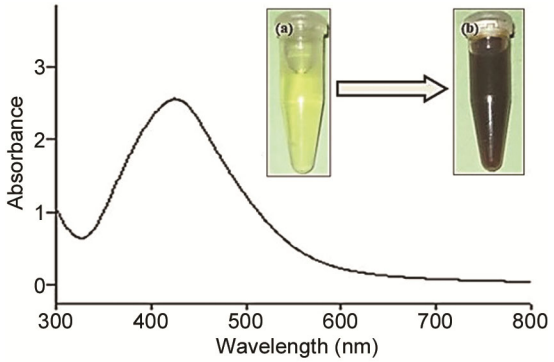


Fig. 3 — UV- Visible absorption spectra of the AgNPs synthesized from *Bixa* root extract. Observation of colour change (a) Control (*Bixa* root extract) (b) *Bixa* root extract and silver nitrate.

***X-Ray diffraction studies***

The synthesized AgNPs were further confirmed by the characteristic peaks observed in XRD image (Fig. 5). The XRD pattern showed five intense peaks at 27.82, 32.2, 38.05, 44.30, and 64.12 corresponding to AgNPs. Several bragg reflections corresponding to the (110), (112), (111), (200), (226) and (220) sets of lattice planes are observed which may be indexed based on the face centered cubic (fcc) crystalline structure of metallic silver.

***Fourier Transform Infra-Red (FTIR) spectroscopy***

FTIR spectrum of *Bixa* root (Fig. 6A) extract and AgNPs synthesized are shown in (Fig. 6B). Results of

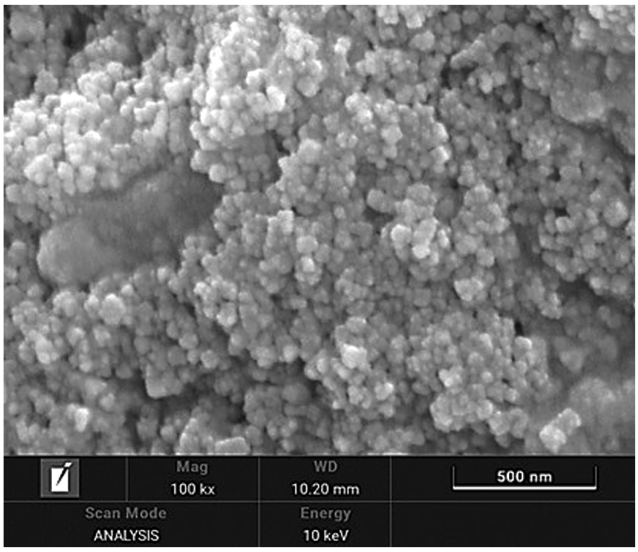


Fig. 4 — SEM image of AgNPs synthesized from *Bixa* root extract.

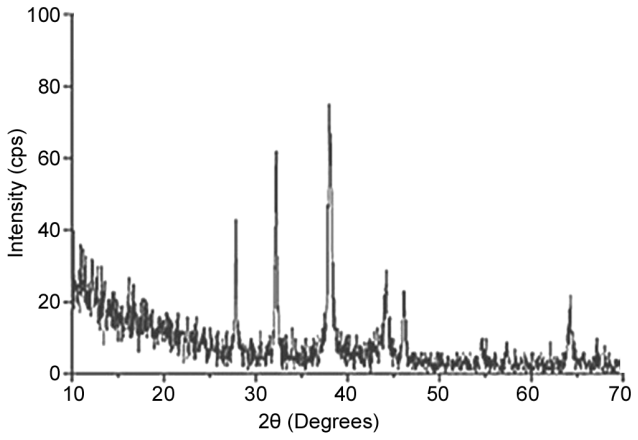


Fig. 5 — XRD pattern of AgNPs synthesized from *Bixa* root extract.

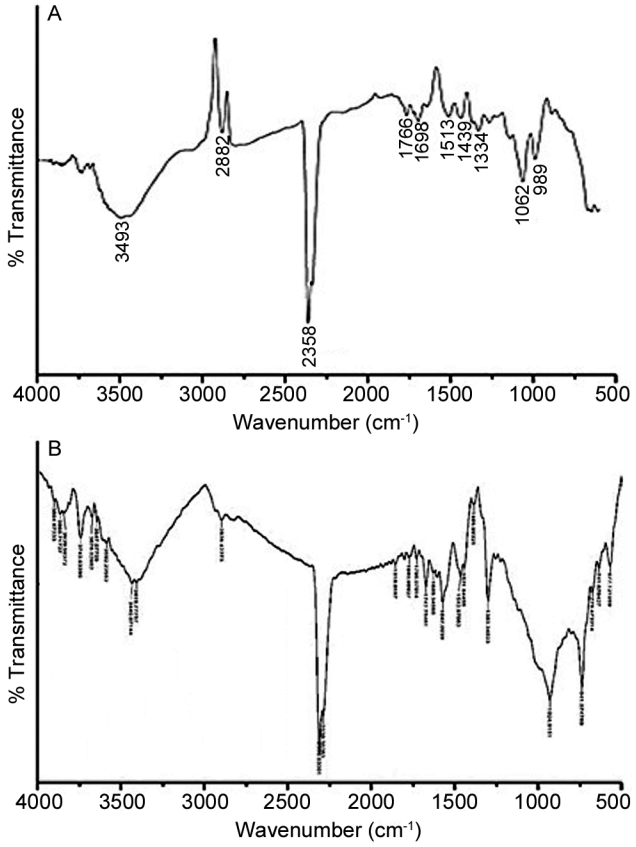


Fig. 6 — FTIR spectrum of (A) *Bixa* root extract (B) AgNPs synthesized by *Bixa* root extract.

FTIR showed sharp absorption peak at 3417 $cm^{-1}$which can be assigned to OH stretching in alcohols and phenolic compounds present in the extract. The peak at 2360 $cm^{-1}$ and 1650 $cm^{-1}$ corresponds to phosphine and amides. The peak at 1442 $cm^{-1}$ and 1018 $cm^{-1}$ corresponds to aromatics and C-N stretching vibrations of aliphatic amines which is present only in plant extracts. The total disappearance of this 1766 $cm^{-1}$ band after the bioreduction may be due to the fact that the carboxyl groups are mainly responsible for the reduction of silver ions, whereby they themselves get reduced to amide leading to a broad peak at 1650 $cm^{-1}$ (for reduction of silver).

**Antimicrobial activity of synthesized AgNPs**

AgNPs was tested against pathogenic bacteria and the antibacterial activity is as depicted in Fig. 7. It was shown to display antibacterial activity against all tested bacteria ranging between 15 to 26 mm. The standard Streptomycin showed highest zone of inhibition which measured 26 mm. The synthesized AgNPs showed strong activity against *Proteus vulgaris*, which measured a zone of 25 mm and least

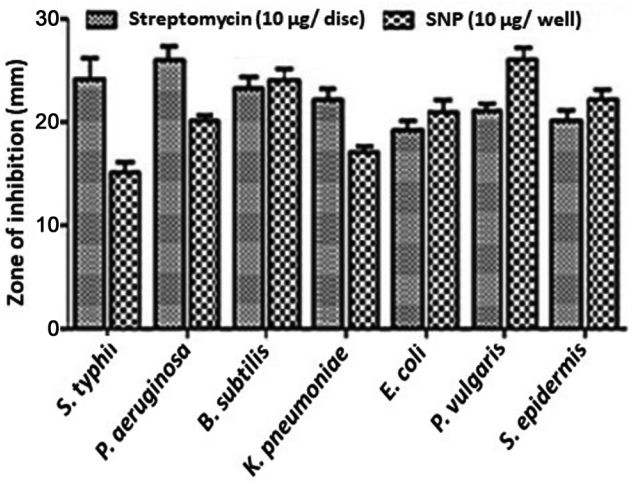


Fig. 7 — Antibacterial activity of AgNPs obtained from *Bixa* root extract.

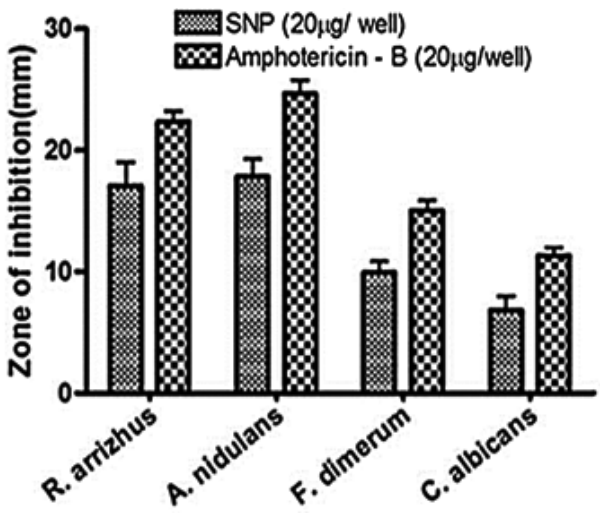


Fig. 8 — Antifungal activity of AgNPs obtained from *Bixa* root extract.

activity against *S. typhii* which measured 15 mm compared to Streptomycin (Fig. 7). Some studies have reported that the antimicrobial activity of the AgNPs is due to the electrostatic attraction between positive charged AgNPs and negative charged cell membrane of microorganism[22]. It is also believed that AgNPs after penetration into the bacteria activated their enzymes, generating hydrogen peroxide and eventually leading to cell death[30].

The synthesized AgNPs were also tested against fungi and the antifungal activity is as shown in Fig. 8. The synthesized AgNPs revealed strong activity against *R. arrizhus* (17 mm) *A. nidulans* (18 mm) and least activity against *Candida* species which measured 7 mm. As standard, Amphotericin showed highest

zone of inhibition against *A. nidulans* which measured 24 mm. The AgNPs may also penetrate inside fungi, causing damage by interacting with phosphorus and sulphur-containing compound, DNA. As compared to the native root extract the AgNPs synthesized from the same exhibited superior activity against both bacteria and fungi.

**Discussion**

This study demonstrates efficient and rapid biosynthesis of AgNPs using root extract of *Bixa orellana* L. The antimicrobial, antioxidant and anticancer activity of native root extract exhibited good activity against gram-positive and gram-negative bacteria and fungi. The characterization of the green synthesized AgNPs was successfully carried out by using UV-visible spectrophotometer, FTIR, SEM and XRD. Antimicrobial activity may be attributed to the different phytoconstituents present in the root extract. Flavonoids have been shown to possess anti-mutagenic and anti-malignant effects. The activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls eventually disrupting the bacterial membranes[31]. The antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell[32]. The actual mechanism of the antifungal activity of the hydrolysable tannin can be postulated to be disruption of the structure of the cell membrane and subsequent inhibition of the normal growth process due to the destruction of membrane integrity[33]. The results of this study provide sufficient scientific backing for the use of *Bixa* root in traditional cures and pave the way for its use as a potential antibacterial and antifungal compound. Moreover, flavonoids have a chemo-preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis[34]. However, the antitumour activity may be due to phenolic contents in the fractions. It was reported that along with antioxidant activity, this group of compounds also possesses a wide variety of biological functions which are mainly related to modulation of carcinogenesis[35]. Taken together, these results suggest that the root extract has good cytotoxicity against HEp-2 cancer cell lines. Moreover, when nanoparticles are synthesized it is known that heavy metals are toxic and react with proteins, as a result of which cellular metabolism is inhibited causing death of microorganism[36]. There is another possible mechanism that metal depletion may cause the formation of irregularly shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins. Experimental evidence indicates that DNA loses its replication ability once the bacteria have been treated with silver[37]. The biosynthesis of metal nanoparticles using the plant derivates has been widely studied in the last few decades. The metabolites present in the plants persuade the production of metallic silver nanoparticles in eco-friendly manner widening their prospective in healthcare, commercial products, pharmaceuticals and therapeutics *etc*.

**Conclusion**

Natural products discovered from medicinal plants have played an important role in drug development. The results of the present study demonstrate that *Bixa* root extract contains various phytochemicals which possess noticeable antioxidant, antimicrobial activity and cytotoxic activity against HEp-2 cell lines. The AgNPs prepared from the root extract ranged between 33-53 nm, the presence of various functional groups as evidenced by FTIR, may be responsible for the enhanced antimicrobial activity. This opens the avenue for using the root extract as a precursor for designing effective antimicrobial and anticancer drug. In addition to this, the rapid green synthesis of AgNPs using *Bixa* root extract provides an environmentally friendly, simple and efficient route for synthesis of benign nanoparticles. These reduced AgNPs were surrounded by a faint thin layer of proteins and metabolites such as terpenoids, flavonoids, and alkaloids having functional groups of amines, alcohols, and carboxylic acids. It is efficient in providing high antimicrobial efficacy and hence has great potential in the preparation of drugs against bacterial and fungal diseases.

**Acknowledgements**

The authors thank Director, CSIR-CLRI for granting the permission to carry out this work. The authors also thank CATERS, CSIR-CLRI for instrumentation analysis. CSIR-CLRI communication number 1842

**Conflict of interest**

The authors declare no conflict of interest.

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