



Research Article

NOVEL BIOGENIC SYNTHESIS OF AgNPs FROM SEED EXTRACT OF *EUGENIA UNIFLORA* L.: *IN VITRO* ASSESSMENT OF THEIR ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC POTENTIAL

Dugganaboyana Guru Kumar *, Sharanya Raj NL, Rakshith Kumar, Nagendra KS

Postgraduate Department of Biochemistry, JSS College of Arts, Commerce and Science (Autonomous), B N. Road, Mysuru, Karnataka, India

*Corresponding Author Email: dgurukumar.phd@gmail.com

Article Received on: 27/10/17 Approved for publication: 27/11/17

DOI: 10.7897/2230-8407.0811227

ABSTRACT

Here, we explored the medicinal uses of the novel biogenic silver nanoparticles of *Eugenia uniflora* L. (*E. uniflora*) seed extract as a cost effective, eco-friendly, reducing and stabilizing compounds. This study describes the synthesis of silver nanoparticles from *Eugenia uniflora* L. (*E. uniflora*) seed extract and their antioxidant, antibacterial and cytotoxic potential. Biosynthesis of AgNPs was monitored by UV-visible spectroscopy which revealed intense surface plasmon resonance bands at 447 nm and X-ray diffraction were employed to identify various functional groups and crystalline nature of AgNPs. Scanning electron microscopy studies demonstrated that synthesized particles were crystalline in nature with average size of 78-100nm. *In vitro* antioxidant effects were analyzed by butylated hydroxytoluene (BHT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), which exhibited antioxidant activity there in the particles could scavenges the stable free radical DPPH of 75% to that o positive control BHT. The value of 50% inhibition concentration (IC₅₀) of Standard BHT is 65.55 and *E. uniflora* is 38.63µg/ml. The antibacterial activity of green AgNPs displayed better zone of inhibition against selected human pathogens. The present study also investigated the toxicity effect of biogenic AgNPs against human prostate cancer cells (PC-3) and the inhibitory concentrations (IC₅₀) were found to be 6.25µg/ml, respectively. It could be concluded that *E. uniflora* seed extract AgNPs can be used efficiently for potential antioxidant, antibacterial and cytotoxic potential AgNPs with potent biomedical applications.

Keywords: *Eugenia uniflora*, Silver nanoparticles, X-ray diffraction, Scanning electron microscope, Cytotoxic Activity

INTRODUCTION

Photochemical reduction and heat vaporization are widely used methods for the synthesis of silver nanoparticles^{1, 2}. These processes involve several toxic chemicals as reducing agents. Because of using noble metal nanoparticles in areas of human contact³, there is an emergent need to develop eco-friendly biosynthesis processes that hinders the use of toxic chemicals. Recently, bio-green method using medicinal plant extract has gained the unique importance due to non-toxic and less time requirement in the synthesis of nanoparticles. Nanotechnology is an interdisciplinary approach in biochemical applications and focusing on synthesis of nanoparticles having improved antibacterial and antioxidant properties against the degenerative diseases and cancer⁴. Currently, research trends in natural and, synthetic antioxidant led the screening and identification of new antioxidants from the plant sources. Synthetic antioxidant is reported to have various properties such as antiallergicity, anticarcinogenicity, antiaging activity and anti-mutagenicity⁵. Antioxidant activity in plant extract is due to the redox potential of phytochemicals⁶, which can play an important role in quenching singlet and triplet oxygen, decomposing the peroxides or neutralizing the free radicals. Therefore, it is assumed that higher antioxidant activity of nanoparticles is might be due to the preferential adsorption of the antioxidant material from the extract onto the surface of the nanoparticles.

Eugenia uniflora L. is a well-known medicinal plant belongs to the family *Myrtaceae*, popularly known as surinam cherry was useful herbal drug in India and it is used as reductant and stabilizer for silver nanoparticles⁷. The use of *E. uniflora* has a

long history in folk medicine of many countries. *E. uniflora* seeds and leaves are used as an antioxidant, hypertensive, anti-inflammatory and hypoglycemic agent⁸. It may also reduce weight, blood pressure and serve as a diuretic⁹. With these evidences, this study was designed to synthesize AgNPs using *E. uniflora* seed extract and *in vitro* assessment of their antioxidant, antibacterial and cytotoxic potential.

MATERIALS AND METHODS

Plant collection and Authentication

The seeds of *Eugenia uniflora* L. were collected from College of Horticulture, Horticulture form Yelawala, Mysuru, Karnataka, India. It was authenticated by Dr. G.V.S. Murthy, Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in the laboratory for future reference (BSI/SRC/5/23/2016Tech./882).

Sample preparation and phytochemical screening

Fresh seed *Eugenia uniflora* L. extract was used for the bio-reduction of AgNO₃ to Ag. 10 g of fresh seeds were washed thoroughly and ground into a fine powder in a 500 ml Erlenmeyer flask along with 100 ml of double distilled water. Further, the pure seed extract was separated by reiterated vacuum filtration and then stored at 4°C and used for further experiments. The phytochemical screening of fresh *E. uniflora* seed extract was performed as per previous procedures^{10, 11}.

Chemicals and preparation of AgNO₃ solution

AR-grade silver nitrate (AgNO₃) was purchased from Finar Chemicals and fresh 0.01697 g of AgNO₃ was dissolved in 100 ml double distilled water (Millipore) to produce 1 mM solution of AgNO₃.

Synthesis of silver nanoparticles (AgNPs)

Synthesis of AgNPs methodology was developed according to with minor modifications¹². 30 g of fresh unripe seeds in 100 ml distilled water (Millipore) are crushed and filtered by using Whatman No.1 filter paper. 1 mM of 100 ml Silver nitrate solution was prepared in a 250 ml beaker covered with aluminium foil, and kept in a magnetic stirrer. With vigorous stirring, 10 ml of seed extract was added drop wise to the silver nitrate solution. With vigorous stirring, the extract was added drop wise to the AgNO₃ solution and the total volume was made up to 100 ml by addition of double distilled water. The colour changed from light yellow to dark brown after continuous stirring for 4hrs. The AgNPs synthesis was confirmed by UV/visible spectra at 350-700 nm and λ_{max} was noted.

Characterization of AgNPs

Characterization of nanoparticles is important to understand and control nanoparticle synthesis and applications¹³. The formation of AgNPs was confirmed by sampling the reaction mixture at regular intervals and the absorption maximum was scanned by UV-Visible spectra, in a range of wavelength between 350 and 700 nm using HITACHI U-2900 Double beam spectrometer. The X-ray diffraction (XRD) patterns of the silver nanoparticles were recorded using Smart Lab 3kW, Item (C/N) 2080B211, Rigaku Corporation Made in Japan. DLS measurements were carried out with a DLS particle size analyzer (Microtrac.INC W3231 Made in USA) to estimate the average size distribution of the prepared particles. Scanning electron microscopy (SEM) analysis was performed (HITACHI S-3400N) to study the morphology of the AgNPs.

IN VITRO ANTIOXIDANT ASSAY

DPPH free radical scavenging assay

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging potential of the AgNPs was determined using the method by¹⁴. Various concentrations (20, 40, 60, 80 and 100 µg/ml) of AgNPs and standard butylated hydroxytoluene (BHT) were taken in different test tubes. In the above samples, 1 mL of freshly prepared DPPH (0.1 mM) dissolved in methanol was added and vortexed thoroughly. Finally, the solution was incubated in dark place for 30 min. The absorbance of stable DPPH was recorded at 517 nm. The DPPH (containing no sample) was used as a control prepared using the same procedure. The free radical scavenging activity was expressed as the inhibition percentage. The inhibition percentage was calculated using the following formula

$$\text{Percentage of radical scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

BHT was taken as reference standard. The percentage inhibition vs. concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC₅₀ value.

Evaluation of the bactericidal activity

The bactericidal activity of the AgNPs was evaluated against the clinical isolates *Staphylococcus aureus*, *Bacillus subtilis*,

Micrococcus luteus (Gram-positive) and *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative) obtained from the Postgraduate Department of Biochemistry, JSS College of Arts, Commerce and Science (Autonomous), Mysuru-570025, Karnataka, India. The bactericidal activity was carried out with 24 h active cultures by employing the disc diffusion method¹⁵. About 150 CFU/mL of inoculums was swabbed onto nutrient agar plates uniformly and allowed to dry in a sterile environment. Sterile disc of 6 mm (HIMEDIA) was loaded with different concentration of AgNPs (5, 10 and 15 µg/ml) solutions, and another disc was dipped in 1 µg/ml of antibiotic ampicillin was used as positive control. The plates were incubated at 26°C for 2 days to measure the zone of inhibition. The mean was calculated by performing the experiments in triplicates.

CYTOTOXIC ACTIVITY

MTT Assay

The cytotoxic activity of on PC-3 cells (Prostate cancer cells) was determined by the MTT assay¹⁶. Cells (1 × 10⁵/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO₂ incubator for 72 hours. Then, add various concentrations of the samples in 0.1% DMSO for 48hrs at 5 % CO₂ incubator. After removal of the sample solution and 20µl/well (5mg/ml) of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically. The effects of the samples on the proliferation of PC-3 cells were expressed as the % cell viability, using the following formula

$$\% \text{ of Cell viability} = \frac{\text{PC-3 of treated cells}}{\text{PC-3 of control cells}} \times 100$$

Statistical analysis

The results were expressed as mean ± SD of three independent experiments (P<0.01). IC₅₀ values were calculated from DPPH assay and subjected to statistical analysis.

RESULTS

Visual observation and UV-Vis spectroscopy

The addition of *Eugenia uniflora* seed extract to the aqueous AgNO₃ solution resulted in the pale yellow to reddish brown colour surface plasmon resonance (SPR) is shown in (Fig.1). The reduction of aqueous Ag⁺ ions to Ag⁰ by the *Eugenia uniflora* seed extract was simply analyzed by UV-visible spectroscopy. The UV-Vis spectra of the AgNPs band occur near 420 nm is shown in (Fig.2) indicating the formation of AgNPs due to reduction of silver ions by active molecules present in the seed extract. In accordance with previous literature studies were also reported by many researchers^{17, 18}. The brown color confirms that it was due to the reduction of Ag⁺ which indicates the formation of AgNPs.

X-ray diffraction (XRD) measurement

The X-ray diffraction pattern of the biosynthesised AgNPs from the seed extract is shown in (Fig.3). The intensity data were collected over a 2 theta range of 20°- 80°. The strong peak was located at 11.24°, 7.86°, 21.40°. The three diffraction peaks located at 11.24°, 7.86°, 21.40°. A sharp and strong diffraction peak centered at 11.24° was appeared and indicated the reflections of metallic silver. The sharp peaks clearly indicate

the synthesized AgNPs are crystalline in nature, with a face-centered cubic (fcc) structure.

Scanning electron microscopy (SEM) measurement

The SEM analysis was used to determine the structure of the reaction products that were formed. The morphology of the AgNPs was predominantly showed in (fig.4) individual silver particles as well as a number of aggregates. SEM images of AgNPs derived from the seed extract of *E. uniflora* showed spherical shaped with the average range of particle size distribution from 78 nm.

Antioxidant activity of AgNPs

Antioxidants are micro-constituents that can be act as a scavenger of reactive oxygen species (ROS) by terminating the oxidizing chain reaction. ROS play a fundamental role in the pathogenesis of a variety of degenerative conditions including cardiovascular diseases and carcinogenesis. DPPH assay are widely used to evaluate the radical-scavenging ability of green synthesized nanoparticles. In the present study, DPPH, a stable free radical with a characteristic absorption at 517nm was used to study the radical-scavenging effects¹⁹. The DPPH scavenging assay exhibited effective inhibition activity of AgNPs when compared with the standard BHT (butylated hydroxytoluene). The antioxidant potential of AgNPs could be attributed to functional groups adhered to them which were originated from the seed extract. The antioxidant activity (DPPH approach) suggest that, *E. uniflora* seed extract of IC₅₀ value was 38±63 µg/ml which was compared with standard Ascorbic acid (65.55 ±1.02 µg/ml) was shown in (Fig.5). The *E. uniflora* seed extract percent of inhibition was near to the standard. The previous study reveals that, *Helicteres isora* root extract AgNPs showed good antioxidant activity as compared to standard butylated hydroxytoluene by²⁰.

Evaluation of the bacterial activity

The bactericidal activity of the green synthesized *E. uniflora* seed extract AgNPs has potent antibacterial activity against both Gram-negative and Gram-positive human pathogens is shown in (Fig. 6 & Table. 1). AgNPs displayed antibacterial activity against Gram positive and Gram negative bacteria, with varying degrees, as suggested by the diameter of inhibition zone, while synthesized *E. uniflora* seed extract AgNPs show high antibacterial activity compare to AgNO₃. The results showed that AgNPs are effective antibacterial activity against *Staphylococcus aureus* (Gram positive), *Escherichia coli* (Gram negative) and *Pseudomonas aeruginosa* (Gram negative) than *Bacillus subtilis* (Gram positive).

Cytotoxic Activity against PC-3 cell line

Silver nanoparticles (AgNPs) have encouraging application in curative efficacy like wound care, skin cancer and breast cancer²¹. Moreover, these are being used as bone cementing and prosthetic materials for fast recovery. In order to support the development of anti-cancer therapy, the biosynthesized silver nanoparticles were taken for cytotoxic study against prostate cancer (PC-3) cell line. The cell viability (%) of PC-3 cell line post-treatment with biosynthesized silver nanoparticles was determined by studying the MTT assay. The viability of the cells (%) treated with various concentrations of AgNPs has shown in (Fig. 7 & 8). The IC₅₀ value was determined to be 6.25µg/ml for seed extract of AgNPs. The silver nanoparticles showed excellent anticancer activity against PC-3 cell line with similar IC₅₀ values as reported earlier²². This study strongly revealed the significant antiproliferative activity of biosynthesized silver nanoparticles. However, such activity may be due to the synergetic effect of both nano sized silver and the bioactive phytocompounds attached on the surface of the nanoparticles. In depth study will be required to understand the real mechanism behind anticancer activity of the synthesized silver nanoparticles.

Table 1: Effect of synthesized AgNPs against various pathogenic bacterial strains

Sl. No	Name of the organism	Zone of inhibition (mm)				
		Ampicillin (1µg/ml)	AgNO ₃ (1µg/ml)	Seed extract (5µg/ml)	AgNPs (5µg/ml)	AgNPs (10µg/ml)
1	<i>P.aeruginosa</i>	26.4±0.60	12.5±0.34	18.44±0.64	10.2±0.32	14.4±0.42
2	<i>B.subtilis</i>	29.3±0.32	10.6±0.24	15.52±0.42	10.3±0.33	12.6±0.58
3	<i>S.aureus</i>	30.7±0.64	14.8±0.26	20.52±0.46	12.6±0.96	13.4±0.58
4	<i>E.coli</i>	32.2±0.26	15.4±0.54	17.94±0.40	14.4±0.52	16.8±0.96

Values are average of triplicates, ± indicates standard error

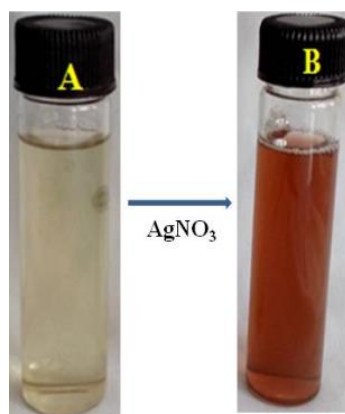


Figure 1: Visual observation of the solution before bio-reduction (A) and after bio-reduction (B)

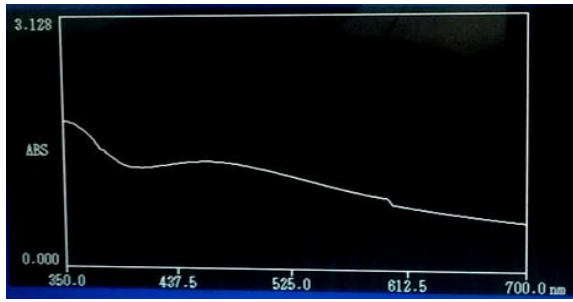


Figure 2: UV-Vis absorption spectrum of synthesized AgNPs

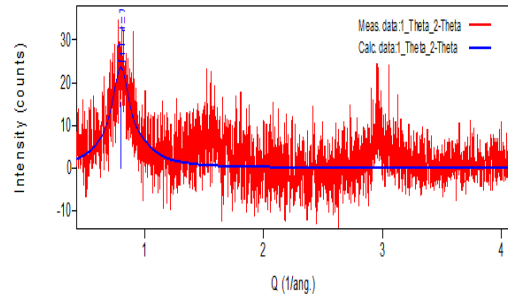


Figure 3: XRD pattern of biosynthesized silver nanoparticles using seed extract

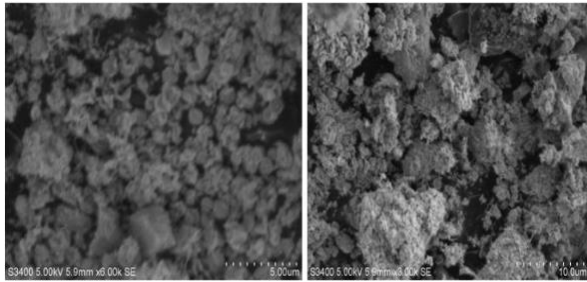


Figure 4: SEM image of synthesized AgNPs using *Eugenia uniflora* seed extract

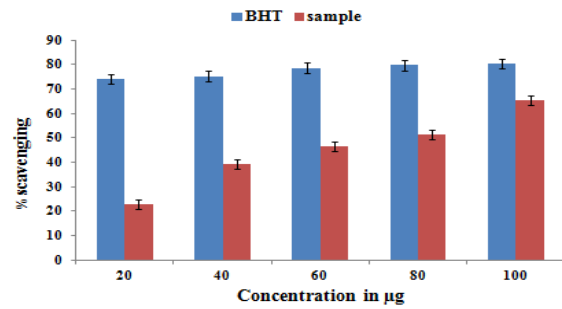


Figure 5: DPPH radical scavenging assay

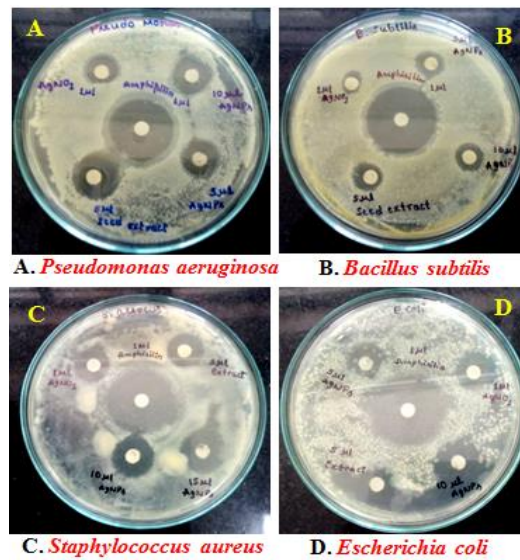


Figure 6: Antibacterial activity of green synthesized AgNPs from *Eugenia uniflora* seed extract

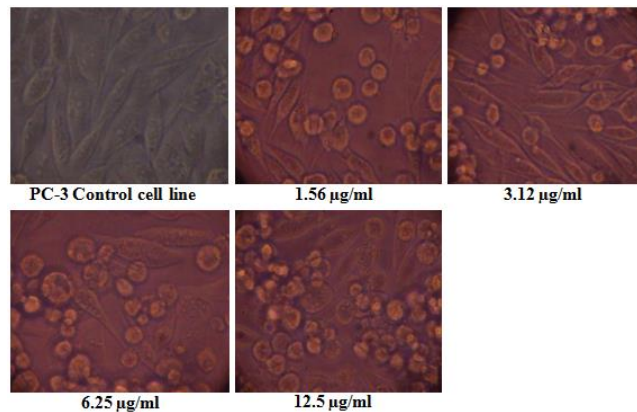


Figure 7: Cytotoxic Activity of Synthesized AgNPs from *Eugenia uniflora* seed extract against prostate cancer cell line (PC-3)

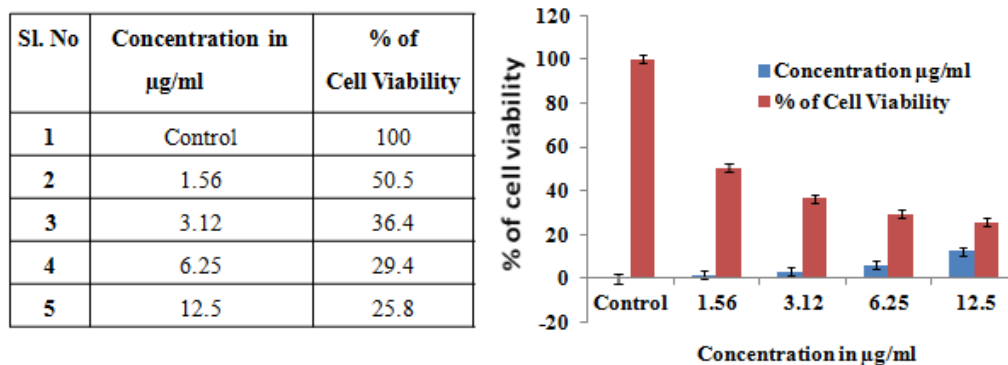


Figure 8: Cytotoxic Activity of Synthesized AgNPs from *Eugenia uniflora* seed extract against prostate cancer cell line (PC-3)

DISCUSSION

To the best of existing information, the present study is the first report green synthesis of silver nanoparticles by using *Eugenia uniflora* L. seed extract and their antioxidant, antibacterial and cytotoxic potential. Herbal plants are very ancient and true natural medicines which are useful for the treatment of different diseases. The addition of *E. uniflora* seed extract to the aqueous AgNO_3 solution resulted in the pale yellow to reddish brown color surface plasmon resonance (SPR). Noble metals are known to exhibit unique optical properties due to the property of surface plasmon resonance²³. They can be used directly or in extracted forms for the management of various ailments due to the presence of various secondary metabolites and have found very important applications in the fields of agriculture, human and medicine²⁴. A *Eugenia uniflora* seed has been to synthesize silver nanoparticles and it has shown potent antioxidant activity and antibacterial activities. It is generally assumed that use of plant derived phytoconstituents may contribute to the stability in the direction of a sufficient antioxidant status. Nanomedicine is a rapidly developing and promising field that makes best use of inert metals like silver, gold and platinum to synthesize metallic nanoparticles with high therapeutic potential for various biomedical applications. Silver with its potent antimicrobial activity has been used in the synthesis of silver nanoparticles which finds extensive use in the preparation of food processing, topical ointments and medical implants²⁵. Hence, the antibacterial activity of *Eugenia uniflora* seed extract can be attributed to the secondary metabolites present in the seed extract. The silver nanoparticles showed excellent anticancer activity against PC-3 cell line with similar IC_{50} values as reported earlier²². This study strongly revealed the significant antiproliferative activity. However, such activity may be due to the synergetic effect of both nano sized silver and the bioactive phytocompounds attached on the surface of the nanoparticles.

CONCLUSION

Biogenic synthesis is an effective way to synthesize silver nanoparticles due to its eco-friendly, simple, cost-effective and efficient protocol. Here, we explored the medicinal uses of the novel biogenic silver nanoparticles of seed extract *E. uniflora* as a cost effective, eco-friendly, reducing and stabilizing compounds. It is concluded from our results that novel biogenic synthesis of AgNPs was carried successfully by using *E. uniflora* seed extract. Presence of active phytoconstituents such as phenolic and flavonoid compounds is added advantage of synthesized nanoparticles. Further, these nanoparticles were evaluated for their activities and showed potent antioxidant, antibacterial and cytotoxic potentials. The significances of this

study demonstrate a wide range of medicinal and technological applications of synthesized nanoparticles.

ACKNOWLEDGMENT

The author sincerely thank to the authorities of JSS Mahavidyapeetha, Mysuru and Principal of JSS College of Arts, Commerce and Science (Autonomous), B N. Road Mysuru-25, Karnataka and India for providing laboratory facilities and constant support.

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Cite this article as:

Dugganaboyana Guru Kumar et al. Novel biogenic synthesis of AgNps from seed extract of *Eugenia uniflora* L.: *In vitro* assessment of their antioxidant, antimicrobial and cytotoxic potential. Int. Res. J. Pharm. 2017;8(11):109-114 <http://dx.doi.org/10.7897/2230-8407.0811227>

Source of support: Nil, Conflict of interest: None Declared

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