

Green Synthesis Of Embelia Ribes Mediated Silver Nanoparticles And Its Cytotoxic Effect And Embryogenic Toxicology Evaluation For Future Prospects In Dentistry: In Vitro Study

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Background : Synthesis of green based nanoparticles in an eco-friendly fashion

Aim : To derive green synthesis of Embelia ribes mediated silver nanoparticles and analyze its cytotoxic effect using Brine Shrimp Lethality Assay and Zebrafish Embryogenic Toxicology test

Materials and Methods : 1g of Embelia ribes seed powder was mixed with 100ml of distilled water and was boiled. The extracts was filtered using Whatman filter paper to obtain a clear solution. 10ml of the above extract obtained was added to a solution containing 90 ml of distilled water 1mMol of Silver nitrate. The nanoparticle mixture was further kept in orbital shaker and centrifuged. Brine Shrimp Lethality Assay and Zebrafish Embryogenic test were used to cytotoxic Evaluation.

Results: Toxicity was detected in Zebrafish Embryonic Toxicology test and minimal toxicity noted in Brine Shrimp Lethality test. Toxicity was directly proportional to concentration of Silver nanoparticles are added to it.

Conclusion :Seeds of Embelia ribes mediated silver nanoparticles demonstrated to have considerably low toxicity,which has been proven using Brine Shrimp Lethality Assay and Zebrafish Embryogenic Toxicology test.

Keywords : Cytotoxicity , Embelia ribes,Endodontics, Green synthesis, Nanoparticles.

Introduction

Nanotechnology and its application in the medical field is gaining momentum due to a myriad of therapeutic uses. [1]Nanomaterial is defined as any particle ranging from size 10-100 nm.[2]Factors such as large surface area to mass ratio, high reactivity and small size is what sets them apart in their physical and chemical properties .[3]Drug Delivery with the advent of nanotechnology has a profound impact on many areas of medicine due to its effect at the molecular level.[4]Metal nanoparticles , primarily silver and gold play an important role in medicine and pharmacology .[5]

In the field of Endodontics , Calcium Hydroxide is being used as an intracanal medicament as a gold standard for the property is possess such as low solubility in water ,high pH ranging from 12.5-12.8 and strong alkalinity.[6,7]The antimicrobial activity of Calcium hydroxide is based in the release of hydroxyl ions when in contact with the fluid or periapical secretion.[8] The ions are oxidant free radicals which exhibits significant reactivity .[9]However few drawbacks are noted with calcium hydroxide, that is tunnel defects created and its minimum inhibition on *E.faecalis* which the primary pathogen in periapical infections.[10] This study is being conducted based on previous evidence on therapeutic properties of Embelia ribes and to evaluate its Cytotoxic property and usage in field of endodontics.[11]

Silver nanoparticles are researched due to their applications in biolabelling, filters, antimicrobial properties against various infectious organisms,integrated circuits, sensors ,cell electrodes and many more. [12]Synthesis of metal nanoparticles using plants are more stable ,[13] cost effective, less toxic,eco friendly , an effective and a suitable alternative to conventional methods.[14]The biomolecules in the plants such as alkaloids, polysaccharides, alcoholic extract,vitamins aid in stabilization,bioreduction and formation of metal nanoparticles.[6][15]Plant based metal nanoparticles are in vogue due to their heavy metal detoxification,its capping and reducing abilities, wide range of size and varied application of the formed silver nanoparticles .[16]

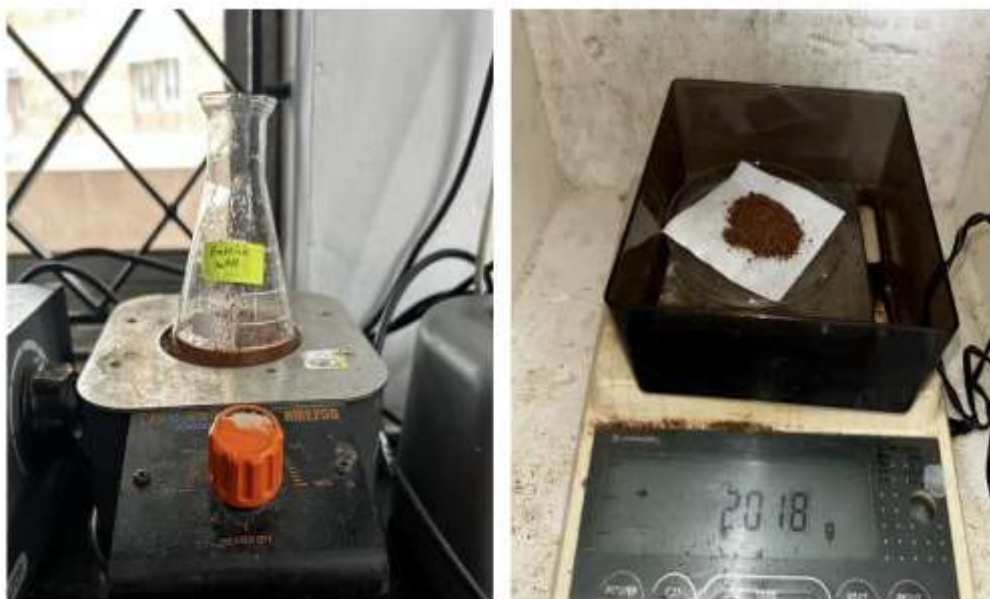
Embelia ribes Burm F a medicinal woody climber belongs to the Myrsinaceae family. It is also commonly known as false black pepper or vidanga .[17]*E. ribes* is one of the 32 medicinal plant species identified by the Medicinal Board, Govt. of India, New Delhi, as being important for large-scale cultivation because of its commercial use and is the most exploited as it is used in 75 ayurvedic preparation.[18,19]The fruits, [20]seeds ,leaves and roots are used to cure various diseases as it contains phenolic compounds such as Embelia . Embelin presented as aqueous and ethanolic extract of the fruit has showed antibacterial, antifertility,antiprotozoal,anti-inflammatory and antioxidant properties. [18]

Embelia ribes have demonstrated promising antioxidant and microbial properties. [21] With incorporation of the silver nanoparticle within the Embelia ribes we focus on enhancing the antimicrobial aspects of silver nanoparticles with reduced side effects.[22] The current research work focuses on discovering the cytotoxicity effect on different concentrations of E.ribes mediated silver nanoparticles using Brine Shrimp Lethality assay and Zebrafish toxicology Assay.[23]

Materials and Methods

Preparation of Plant Extract

The seeds of Embelia ribes was purchased from online herbal store .The seeds procured were refined to powder form.1 gram of E.ribes was mixed with 100 ml of distilled water to the beaker and boiled for 10-20 minutes in heating mantle.The boiled extract was filtered using Whatman filter paper.[5](Figure 1a and 1b)



(Figure 1a : Indicates heating mantle 1b : powdered for of Embelia ribes seed.)

Synthesis of Silver Nanoparticles-

Solution of 1mMol of Silver nitrate in 90 ml water was used to synthesize nanoparticles.To this solution we added 10 ml of filtered E.ribes extract and kept in the orbital shaker with a magnetic stirrer for 2 hours and Color change was observed every 24 hours for 3 days.(Figure 2a and 2b)The synthesized nanoparticles was preliminary analyzed using UV visible spectroscopy.Before the final step,the nanoparticle solution was centrifuged at 8000 rpm (LARK Cooling Centrifuged) removed to prepare nanoparticles pellet powder and followed

by drying in hot air oven at 80 degrees Celsius. The dried powder was sent for characterisation. Finally the left over solution was used to calculate the cytotoxicity effect.[24]



Fig 2a and b : Assessment of toxicity of the Embelia ribes mediated silver nanoparticles

BRINE SHRIMP LETHALITY ASSAY

A setup tank was prepared for hatching of the Brine Shrimp to which 30 grams of iodine free salt was added to one liter of distilled water in the tank. Adequate aeration was maintained in the tank to which one gram of *Artemia salina* eggs were added and kept for a period of 24 hour incubation.

On second Day ,2gm of iodine salt was added to 200ml distilled water and saline water was prepared, which was added qualitatively to a six well enzyme linked immunosorbent assay plates. Newly hatched Brine Shrimp (*Artemis Salina*) larvae (Nauplii) were taken and set 10 of larvae were transferred into each of the 6 wells. The synthesized E.ribes mediated silver nanoparticles were introduced into each of the wells in varying concentrations of 5, 10, 20, 40 and 80 micro litre. One well with live nauplii as control was left in saline water. The samples were left undisturbed for 24 hours. The number of live nauplii after 24 hours was noted and the data was plotted in the graph.

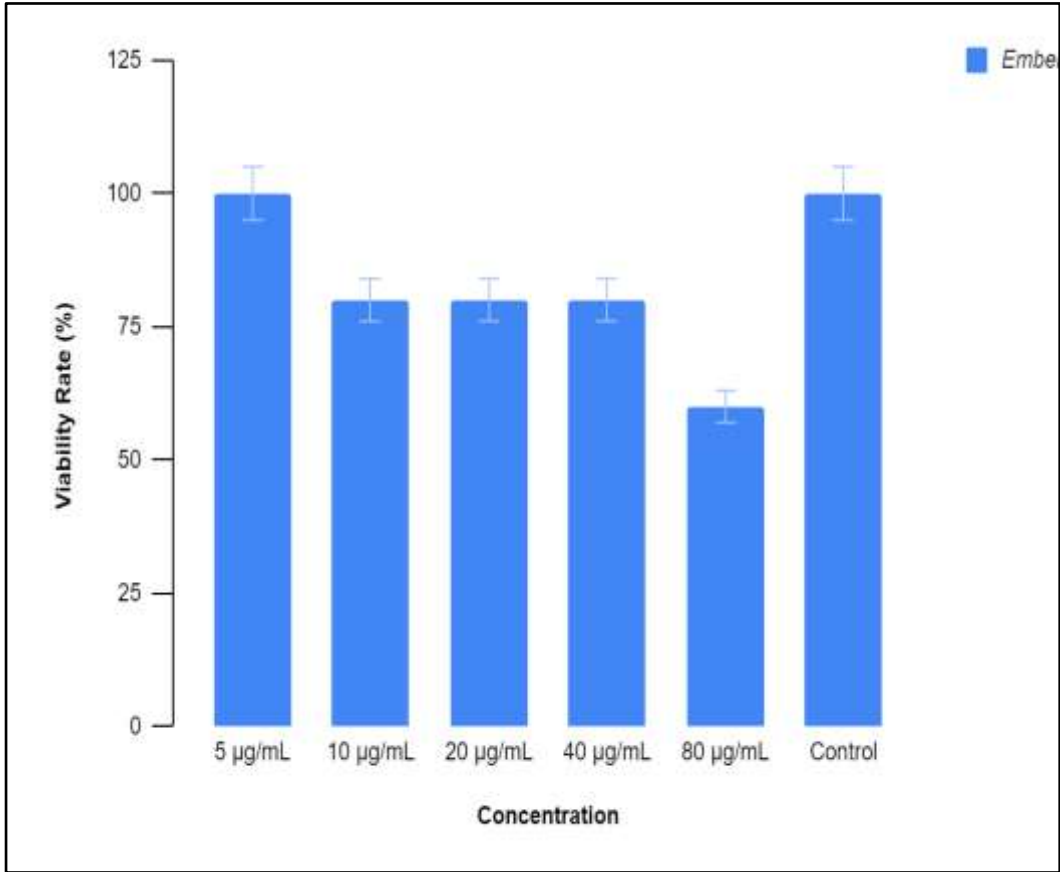
$$(\text{Number of dead nauplii} / \text{Number of dead nauplii} + \text{number of live nauplii}) \times 100$$

ZEBRAFISH EMBRYONIC TOXICOLOGY TEST

The Danio rerio, commonly known as the wild-type zebrafish, were procured from local vendors in India and were housed in distinct tanks under regulated environmental conditions. The experimental conditions encompassed specific parameters, namely a temperature of 28.2 °C, a light/dark cycle of 14:10 h, and a pH range spanning from 6.8 to 8.5. The zebrafish were given dried blood worms or the ideal meal twice a day. Both of these foods are commercially accessible. In order to acquire zebrafish embryos, one female and three male zebrafish were put in a breeding tank together. When this had been performed, viable eggs were produced, which were then retrieved and thoroughly washed at least three times using newly made E3 media that did not contain methylene blue. Fertilized eggs were plated out in 6-well tubes containing 10 embryos each. The 6 wells had varying concentrations of Embelia ribes mediated silver nanoparticles, that is 5,10,20,40,80 microliter and a control group.

Results

Brine Shrimp Viability rates after 24 hrs of exposure to different concentrations.

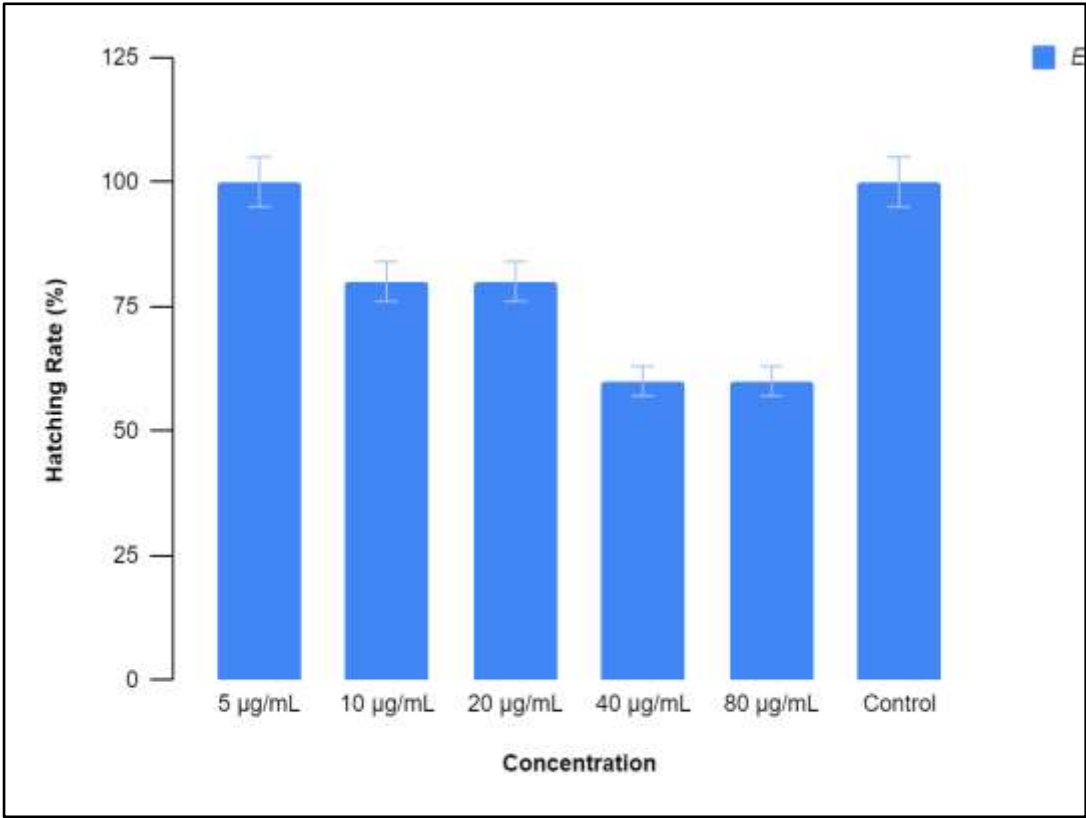


Graph 1 : Showing the cytotoxicity effect of Embelia ribes mediated silver nanoparticles on Brine Shrimp. The X-axis denotes the varying concentrations of E.ribes and the Y axis denotes the viability percentage of Brine Shrimp.

Concentration	Viability Rate (%)
5 µg/mL	100
10 µg/mL	80
20 µg/mL	80
40 µg/mL	80
80 µg/mL	60
control	100

Table 1 : Represents the viability percentage for Brine shrimp at different concentrations.

Zebrafish embryonic Viability rates after 24hrs of exposure to different concentrations.



Graph 2 : Showing the Hatching rate of Embelia ribes mediated silver nanoparticles on the zebrafish embryonic viability .The X-axis denotes the varying concentrations of E.ribes and the Y axis denotes the viability percentage of Brine Shrimp.

Concentration	Hatching Rate (%)
5 µg/mL	100
10 µg/mL	80
20 µg/mL	80
40 µg/mL	60
80 µg/mL	60
Control	100

Table 2 : Represents the hatching percentage for Zebra fish at different concentrations.

Discussion

Cytotoxicity refers to the action of a substance or process that results in the damage of a cell.

Green synthesis was done for this study using Embelia ribes seed and silver nanoparticles as it has reduced toxicity, effective biomolecular compounds and more stable.[25] The formation of Silver nanoparticles was confirmed by UV spectroscopy and notable color changes on the third day.[26–28] 1mMol of Silver nitrate was diluted in 90 ml of water to which Embelia ribes were added. The cytotoxicity was evaluated for its application in the field of medicine. The evaluation was done using two assays discussed below.

At the highest concentration of 80, 60% viability was observed for both Brine Shrimp and Zebra fish. (As noted in Table 1 and 2) Brine Shrimp lethality assay is an important tool for the preliminary cytotoxicity assessment and is applied to detect plant based toxins, [29] fungal toxins, heavy metals, as cytotoxicity test for dental materials, pesticides and is hinged on its ability to kill a laboratory cultured larva (nauplii).[30] It is a simple, cost-effective method and requires a minimum amount of test material. Brine Shrimp Assay The most commonly used organism in Brine Shrimp Lethality Assay is *Artemia salina*.¹⁹ In the presence of salt solution (2%-4%), the shrimp eggs hatch into larvae (nauplii). Seawater is recommended for this purpose. If not available, distilled water with sea salt can be used. Tap water is not recommended for this as it contains chlorine. The ideal pH is 8.0+/- 0.5 which is adjusted using Sodium hydroxide or Sodium carbonate. At room temperature, the nauplii hatch within 20-30 hours. During the study period, the nauplii do not receive any food. The death of the nauplii may be due to the effect of the inoculated substance of starvation. To ensure the mortality effect of the subject under study, a control sample containing nauplii without inoculation of the study substance is used. The nauplii can survive for up to 48 hours without food because they are still fed on their yolk sac.

Brine Shrimp Lethality Assay concluded that the number of live Shrimp larvae at higher concentrations was lesser when compared to lower concentrations, and as the concentration of the nanoparticles increased, the cytotoxicity was also increased which leading to lesser number shrimp larvae which were still alive after 24 hours.

Zebra fish is an accentuated and more sensitive cytotoxicity test followed after the brine shrimp Assay. Graph 1 and Graph 2 shows the cytotoxicity levels of Er-AgNps when added in different concentrations. At 5µg/mL concentration 100 % viability rate was noted for both Brine Shrimp and Zebra fish. At the highest concentration of 80 µg/mL, 60% viability was observed for both Brine Shrimp and Zebra fish. In graph 1 and Table 1, we noted that at the highest concentration, that is 80µg/mL 60 % of viability rate was noted for Brine shrimp. Similarly Table 2 and Graph 2 interpreted similar results, indicating that even at higher concentrations low levels of toxicity was noted.

Conclusion

The above study concludes that *Embelia ribes* mediated silver nanoparticles have shown to have least cytotoxic activity. Studies done previously also have the same affirmation that *Embelia ribes* can be employed as a therapeutic agent in the field of biotechnology and medicine. Future research needs to be conducted in animal findings and clinical trials to substantiate the current data and use in combination for its therapeutic use.

Clinical Significance : This study opens possibilities for *Embelia ribes* mediated nanoparticles to be used in the field of dentistry with least cytotoxicity. As it exhibits significant antioxidant properties as per the literature, it can be used in combination to be served as intracanal therapeutic paste.

References

- [1] Tharani M, Rajeshkumar S, Al-Ghanim KA, Nicoletti M, Sachivkina N, Govindarajan M. Terminalia chebula-Assisted Silver Nanoparticles: Biological Potential, Synthesis, Characterization, and Ecotoxicity. *Biomedicines* 2023;11:1472.
- [2] Joseph TM, Kar Mahapatra D, Esmacili A, Piszczyk Ł, Hasanin MS, Kattali M, et al. Nanoparticles: Taking a Unique Position in Medicine. *Nanomaterials (Basel)* 2023;13. <https://doi.org/10.3390/nano13030574>.
- [3] Amrithaa B, Balaji Ganesh S, Rajesh Kumar S. Cytotoxic Effect of Red Sandal Mediated Silver Nanoparticles Mouthwash Using Brine Shrimp Lethality Assay: An In-vitro Study. *J Pharm Res Int* 2021;433–40.
- [4] Farokhzad OC. Nanotechnology for drug delivery: the perfect partnership. *Expert Opin Drug Deliv* 2008;5:927–9.
- [5] Thiurunavukkarau R, Shanmugam S, Subramanian K, Pandi P, Muralitharan G, Arokiaarajan M, et al. Silver nanoparticles synthesized from the seaweed *Sargassum polycystum* and screening for their biological potential. *Sci Rep* 2022;12:14757.
- [6] Mittal AK, Bhaumik J, Kumar S, Banerjee UC. Biosynthesis of silver nanoparticles: Elucidation of prospective mechanism and therapeutic potential. *J Colloid Interface Sci* 2014;415:39–47.
- [7] Foreman PC, Barnes IE. Review of calcium hydroxide. *Int Endod J* 1990;23:283–97.
- [8] Mohammadi Z, Shalavi S, Yazdizadeh M. Antimicrobial activity of calcium hydroxide in endodontics: a review. *Chonnam Med J* 2012;48:133–40.
- [9] Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. *Aust Dent J* 2007;52:S64–82.
- [10] Cox CF, Sübay RK, Ostro E, Suzuki S, Suzuki SH. Tunnel defects in dentin bridges: their formation following direct pulp capping. *Oper Dent* 1996;21:4–11.
- [11] Dhayalan M, Denison MIJ, L AJ, Krishnan K, N NG. In vitro antioxidant, antimicrobial, cytotoxic potential of gold and silver nanoparticles prepared using *Embelia ribes*. *Nat Prod Res* 2017;31:465–8.
- [12] Kłębowski B, Depciuch J, Parlińska-Wojtan M, Baran J. Applications of Noble Metal-Based Nanoparticles in Medicine. *Int J Mol Sci* 2018;19. <https://doi.org/10.3390/ijms19124031>.
- [13] Agaricus bisporus mediated biosynthesis of copper nanoparticles and its biological effects: An in-vitro study. *Colloid and Interface Science Communications* 2020;35:100254.
- [14] Yan A, Chen Z. Impacts of Silver Nanoparticles on Plants: A Focus on the Phytotoxicity and Underlying Mechanism. *Int J Mol Sci* 2019;20. <https://doi.org/10.3390/ijms20051003>.
- [15] Ansar S, Alshehri SM, Abudawood M, Hamed SS, Ahamad T. Antioxidant and hepatoprotective role of selenium against silver nanoparticles. *Int J Nanomedicine* 2017;12:7789–97.
- [16] Kathiravan A, Udayan E, Rajeshkumar S, Gnanadoss JJ. Unveiling the Biological Potential of Mycosynthesized Selenium Nanoparticles from Endophytic Fungus *Curvularia* sp. LCJ413.

Bionanoscience 2023;13:2232–51.

- [17] Sharma V, Gautam DNS, Radu A-F, Behl T, Bungau SG, Vesa CM. Reviewing the Traditional/Modern Uses, Phytochemistry, Essential Oils/Extracts and Pharmacology of Burm. Antioxidants (Basel) 2022;11. <https://doi.org/10.3390/antiox11071359>.
- [18] Joy B, Nishanth Kumar S, Soumya MS, Radhika AR, Vibin M, Abraham A. Embelin (2,5-dihydroxy-3-undecyl-p-benzoquinone): a bioactive molecule isolated from Embelia ribes as an effective photodynamic therapeutic candidate against tumor in vivo. Phytomedicine 2014;21:1292–7.
- [19] Kumara Swamy HM, Krishna V, Shankarmurthy K, Abdul Rahiman B, Mankani KL, Mahadevan KM, et al. Wound healing activity of embelin isolated from the ethanol extract of leaves of Embelia ribes Burm. J Ethnopharmacol 2007;109:529–34.
- [20] Bhandari U, Ansari MN. Protective effect of aqueous extract of Embelia ribes Burm fruits in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Indian J Pharmacol 2008;40:215–20.
- [21] Othman SNN, Sekar M. In-vitro antioxidant and cytotoxic activities of silver nanoparticles of embelin isolated from Embelia ribes. J Adv Pharm Technol Res 2019;12:4080.
- [22] Nazam Ansari M, Bhandari U, Islam F, Tripathi CD. Evaluation of antioxidant and neuroprotective effect of ethanolic extract of Embelia ribes Burm in focal cerebral ischemia/reperfusion-induced oxidative stress in rats. Fundam Clin Pharmacol 2008;22:305–14.
- [23] Jaki B, Orjala J, Bürgi H-R, Sticher O. Biological screening of Cyanobacteria for antimicrobial and molluscicidal activity, brine shrimp lethality, and cytotoxicity. Pharm Biol 1999;37:138–43.
- [24] Anastas PT, Heine LG, Williamson TC. Green Chemical Syntheses and Processes. 2000.
- [25] Das CGA, Kumar VG, Dhas TS, Karthick V, Govindaraju K, Joselin JM, et al. Antibacterial activity of silver nanoparticles (biosynthesis): A short review on recent advances. Biocatal Agric Biotechnol 2020;27:101593.
- [26] Lewinski N, Colvin V, Drezek R. Cytotoxicity of nanoparticles. Small 2008;4:26–49.
- [27] Weyermann J, Lochmann D, Zimmer A. A practical note on the use of cytotoxicity assays. Int J Pharm 2005;288:369–76.
- [28] Desai R, Mankad V, Gupta S, Jha P. Size distribution of silver nanoparticles: UV-visible spectroscopic assessment. Nanosci Nanotechnol Lett 2012;4:30–4.
- [29] Ruebhart DR, Cock IE, Shaw GR. Brine shrimp bioassay: importance of correct taxonomic identification of Artemia (Anostraca) species. Environ Toxicol 2008;23:555–60.
- [30] Padmaja R, Arun PC, Prashanth D, Deepak M, Amit A, Anjana M. Brine shrimp lethality bioassay of selected Indian medicinal plants. Fitoterapia 2002;73:508–10.