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Biogenic bismuth nanoparticles from *Eclipta prostrata* Leaves: A natural source of antioxidant, antimicrobial and cytotoxic agents

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Abstract

Eclipta prostrata (L.) L., often referred to as Eclipta alba or False daisy, is a traditional herbal remedy from the Asteraceae family, prevalent in tropical and subtropical areas. It has been utilized for generations in Indian, Nepalese, and Bangladeshi medicine to address skin problems, liver conditions, and digestive issues. This research investigates the antioxidant, antimicrobial, and cytotoxic properties of biogenically produced Bismuth Nanoparticles (Bi NPs). The activity of scavenging free radicals was assessed using several in *vitro* methods, such as DPPH, ABTS, FRAP, and radical scavenging assays, all indicating significant free radical neutralization and reducing capacity, highlighting the potential of biogenically synthesized bismuth nanoparticles to mitigate oxidative stress—a key factor in various chronic illnesses. The antibacterial and antifungal effects were assessed against bacterial and fungal strains, revealing notable zones of inhibition that corroborate its traditional application in infection treatment. The Brine shrimp lethality assay (Artemia bioassay) was utilized to evaluate cytotoxicity, showing a dose-dependent rise in mortality, indicating possible anticancer effects. In summary, these results confirm the traditional medicinal applications of biogenically produced Bismuth nanoparticles from the aqueous extract of *Eclipta prostrata* leaves and highlight its potential as a natural source of free radical scavengers, antimicrobial, and cytotoxic agents for therapeutic advancement.

Keywords: *Eclipta prostrata*, Bismuth nanoparticles, Medicinal Herb, Antimicrobial activity, Antioxidant, Brine shrimp, Ferric reducing power, DPPH

Introduction

India is a worldwide leader in the production of medicinal herbs - aptly referred to as the “botanical garden of the world” - due to its abundant biodiversity and longstanding traditional herbal wisdom (Vidyashree and Kumar, 2022) [28]. Among the roughly 17,000 plant species in India, close to 7,500 higher plants possess medicinal traits, which represent 7–13% of the globe's pharmacologically significant flora (Vidyashree and Kumar, 2022) [28]. India's vast botanical resources, combined with its long-standing indigenous healthcare practices, create a distinctive global position in herbal medicine. The need for plant-based treatments - efficient, culturally recognized, and cost-effective - is increasing in both rural and urban settings, driving continuous searches for new medicinal compounds from indigenous plants (Vidyashree & Kumar, 2022) [28]. Bioactive substances obtained from these plants remain essential in contemporary drug development (Timalsina and Devkota, 2021) [26]

A notable instance is *Eclipta prostrata* (L.) L., commonly referred to as Eclipta alba, or False daisy/Ink plant in English, and known as Bhringraj in several Indian languages (Timalsina and Devkota, 2021; Yang *et al*., 2023) [26, 31]. This annual herb of medium size, featuring white flowers, flourishes in wet environments such as rice paddies and riverbanks, found across tropical and subtropical areas of Asia, Africa, and South America (Timalsina and Devkota, 2021; Silalahi, 2022) [24, 26]. Highly respected in ethno medicine, it is utilized for skin conditions, hair thinning, injuries, snakebites, infant catarrh, and to boost liver and digestive function; leaf extract is additionally given to promote and improve hair growth (Timalsina and Devkota, 2021; Yang *et al*., 2023; Silalahi, 2022) [24, 26, 31].

Multiple recent investigations have examined the diverse chemical makeup of *Eclipta prostrata*, uncovering biologically potent substances including flavonoids, phenolics, alkaloids, proteins, lipids, and phytosterols (Chung *et al*., 2017) [8]. Among these, flavonoids—natural pigments from plants usually present in stems, leaves, flowers, and fruits—are particularly important for their antioxidant properties and various biological functions (Wang *et al*., 2021) [29]. These substances aid in plant growth and color while also demonstrating various health benefits, such as anti-inflammatory, anticancer, and anti-amoebic properties (Gao *et al*., 2018; Duan *et al*., 2017; Albuquerque *et al*., 2019) [1, 10, 11]. This has positioned flavonoids as a central interest in areas such as pharmacology, food science, and medical studies. Kim *et al*. (2015) [14] found that E. prostrata has significant amounts of flavonoids featuring ortho-diphenolic structures, which bolster their capacity to counteract free radicals.

Redox imbalance occurs due to a disturbance in the equilibrium between the overproduction of ROS and the effectiveness of the body's antioxidant defense mechanisms, which can result in cellular harm (Ardestani and Yazdanparast, 2007; Singh *et al*., 2020) [3, 25]. These reactive oxygen species can damage essential cellular macromolecules, including DNA, lipids, and proteins, leading to the development of conditions such as cancer, Alzheimer's disease, and multiple sclerosis (Bakoyiannis *et al*., 2019; Wang *et al*., 2021) [4, 29]. Despite the widespread use of industrial antioxidants such as BHA and BHT in the food industry, worries regarding their possible negative impacts—like hepatotoxicity and carcinogenicity—have prompted a move towards natural, plant-based substitutes (Lei *et al*., 2008) [15]. Medicinal herbs such as *Eclipta prostrata* have demonstrated potential as safer and more economical alternatives with wide-ranging therapeutic uses (Jin & Yin, 2012) [13]

Bismuth nanoparticles (BiNPs) have recently gained attention in nanobiotechnology because they are less toxic, have unique properties, and offer several biological benefits. For example, BiNPs made by Delftia sp. show strong antioxidant and cytotoxic effects (Shakibaie *et al*., 2018) [23], which could help manage oxidative stress. PVP-coated BiNPs made through a simple chemical reduction process also show strong antimicrobial effects against Staphylococcus aureus and Candida albicans, both in free-floating and biofilm states (Vazquez-Munoz *et al*., 2020) [27]. When bismuth lipophilic nanoparticles are added to chitosan-based membranes, they nearly stop biofilm formation and reduce the growth of oral pathogens by 90–98%, while still being safe for human gingival fibroblasts (Martínez-Martínez *et al*., 2018) [18]. While there are not many direct studies of BiNPs using brine shrimp lethality tests, this assay is a common first step to check nanoparticle toxicity and can give LC_{50} values before moving to animal studies (Meyer *et al*., 1982) [19]. Overall, these results suggest that bismuth nanoparticles offer both antioxidant and antimicrobial benefits with acceptable toxicity, making them promising for biomedical use, though more testing in models like the brine shrimp assay is needed.

The present study examines on evaluating the antioxidant, antimicrobial and cytotoxic properties of biogenically synthesized Bismuth nanoparticles using the aqueous extract of *Eclipta prostrata leaves*, aiming to provide meaningful insights into their medicinal value and their potential in combating antibiotic resistance.

Materials and Methods

Procurement of medicinal herb *Eclipta prostrata*

Fresh leaves of *Eclipta prostrata* (L.) L. was collected from the Vellore region in Tamil Nadu, India. Once collected, the fresh leaves of Eclipta were rinsed with running tap water to eliminate dust and debris, subsequently rinsed with deionised water to ensure cleanliness. The leaves were then air dried in the shade at ambient temperature for 7–10 days, avoiding direct sunlight to preserve their phytochemical integrity, as recommended by Bimakr *et al*. (2011) [7]. After complete drying, the leaves were coarsely powdered using a mechanical grinder and stored in airtight containers until further extraction.

Preparation of aqueous extract of *Eclipta prostrata* medicinal herb

For aqueous extraction, 100 g of powdered leaf of *Eclipta prostrata* was macerated in 300 mL of deionized and sustained at standard ambient temperature for 24-48 hours subjected intermittent agitation. The extract was subjected to filtration and lyophilized or evaporated under reduced pressure to obtain a dry aqueous extract, as described in the methods by Do *et al*. (2014) and Panda and Padhi (2020) [21]. The dried extract was stored at 4°C in amber-colored bottles to protect it from light and degradation.

Biosynthesis of Bismuth Nanoparticles Using the Aqueous Extract of *Eclipta prostrata*

In the typical synthesis of Bismuth Nanoparticles, 2 grams of BiNH3 was added to 10 milliliters of *Eclipta prostrata* leaf extract and 90 mL of distilled water. The combination remained at 60-80 °C for 20 minutes.' Using a magnetic stirrer, the solution was continuously mixed for 30 minutes at 1000 RPM until it was left to steep overnight. A golden-yellow color was observed, which was indicative of the formation of nanoparticles called bismuth. To remove impurities, the nanoparticles were centrifuged at 5000 rpm for 20 minutes and then rinsed with ethanol and distilled water (2-3 times). Finally, the nanoparticles were dried in a hot air oven at 110 °C for 1 hour and stored in a sealed container for further use.

Antioxidant activity of the biogenically synthesized Bismuth Nanoparticle

DPPH Free Radical Neutralization Test

The antioxidant capability of biogenically produced Bismuth Nanoparticles was first evaluated through the DPPH radical scavenging assay based on the procedure outlined by Williams *et al*. (1995) [30]. A methanol solution of DPPH at 0.1 mM was created. Various concentrations of the biogenically produced Bismuth Nanoparticles were also created in methanol. 1 mL of the extract was added to 1 mL of the DPPH solution in each test tube. The combination was allowed to sit in the dark at room temperature for 30 minutes, and the absorbance was recorded at 517 nm with a UV-visible spectrophotometer. The scavenging activity was determined using: DPPH Scavenging (%) = $[(A_0 - A_s) / A_0] \times 100$, where A_0 represents the absorbance of the control and A_s denotes the absorbance of the sample.

Ferric Reducing Antioxidant Power (FRAP) Test

The FRAP assay was performed to assess the reducing capability of the biogenically produced Bismuth Nanoparticles, adhering to the procedure outlined by Benzie and Strain (1996) [6]. The FRAP reagent was created by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1. 100 µL of the extract was combined with 900 µL of the newly made FRAP reagent and incubated at 37°C for 30 minutes. The absorbance was recorded at 593 nm.

ABTS Radical Cation Color Removal Test

This test was conducted following the method outlined by Re *et al*. (1999). The ABTS radical cation ($ABTS^+$) was produced by combining 7 mM ABTS with 2.45 mM potassium persulfate and letting the solution sit in the dark for 12 to 16 hours. A 10 µL sample of the biogenically produced Bismuth Nanoparticles was mixed with 1 mL of the $ABTS^+$ solution, and after 6 minutes, the absorbance reduction was recorded at 734 nm

Superoxide Radical Scavenging Assay

Superoxide radical scavenging activity was measured according to Nishikimi *et al*. (1972) [20]. The reaction mixture contained 0.1 mM EDTA, 0.1 mM xanthine, 0.1 mM NBT, and xanthine oxidase (0.1 units/mL) in phosphate buffer (pH 7.4). The reaction was initiated by adding xanthine oxidase to the mixture containing biogenically synthesized Bismuth Nanoparticles and reagents, and incubated for 30 minutes. Absorbance was recorded at 560 nm.

Nitric Oxide Capture Test

The activity of scavenging nitric oxide was assessed according to Marcocci *et al.* (1994) [17]. Sodium nitroprusside (10 mM) in phosphate buffer (pH 7.4) was combined with the biogenically produced Bismuth Nanoparticles and left to incubate at room temperature for 15 minutes. Subsequently, 1 mL of Griess reagent was introduced, and the absorbance was measured at 546 nm. The scavenging rate was determined in a manner akin to DPPH.

Assay for Hydroxyl Radical Scavenging

The ability to scavenge hydroxyl radicals was assessed using the method established by Halliwell *et al.* (1987) [12]. The mixture for the Fenton reaction contained EDTA, $FeCl_3$, H_2O_2, deoxyribose, ascorbic acid, and phosphate buffer. The extract was included in this mixture and incubated at 37°C for one hour. After incubation, TCA and TBA were incorporated, and the blend was heated for 10 minutes. Absorbance was measured at 532 nm.

Total Phenolic Concentration (Folin–Ciocalteu Technique)

The overall phenolic content was evaluated utilizing the Folin–Ciocalteu reagent as outlined by Makkar *et al.* (1993) [16]. A 0.1 mL sample of biogenically produced Bismuth Nanoparticles was combined with 0.1 mL of 2N Folin–Ciocalteu reagent and 2.8 mL of 10% sodium carbonate. The mixture was incubated for 40 minutes at room temperature, and the absorbance was recorded at 725 nm. Gallic acid served as a standard, and the findings were reported in mg gallic acid equivalents (EPL/G) for biogenically synthesized Bismuth Nanoparticles.

Antimicrobial Activity of the biogenically synthesized Bismuth Nanoparticles

The antimicrobial properties of biogenically produced Bismuth Nanoparticles from the aqueous extract of *Eclipta prostrata* were assessed through the agar well diffusion method against three bacterial strains (Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa) and three fungal strains (Candida albicans, Aspergillus niger, and Fusarium oxysporum), in accordance with the methodology outlined by Balouiri *et al.* (2016) [5]. Bi NPs was made in sterile distilled water at concentrations of 100, 250, and 500 mg/mL. Microorganisms were cultivated in nutrient broth (for bacteria) and Sabouraud dextrose broth (for fungi), and incubated at suitable temperatures (37°C for bacteria and 28°C for fungi) until they attained the log phase of growth. The microbial inoculum was adjusted to align with the 0.5 McFarland standard. Sterile Mueller-Hinton agar (MHA) plates for bacteria and Sabouraud dextrose agar (SDA) plates for fungi were uniformly swabbed with the inoculum, and wells of 6 mm diameter were created using a sterile cork borer. Every well received 100 µL of the Bi NPs, then underwent pre-diffusion for 30 minutes at room temperature. The plates were subsequently incubated (24 hours for bacteria at 37°C and 48 hours for fungi at 28°C), and the inhibition zones were measured in millimeters. Ciprofloxacin (10 µg/mL) and fluconazole (10 µg/mL) were utilized as positive controls for bacteria and fungi, respectively, whereas sterile distilled water acted as a negative control.

Toxicity analysis of the biogenically synthesized Bismuth nanoparticles using the aqueous extract of *Eclipta prostrata*

Brine Shrimp Hatching Assay

Brine shrimp (*Artemia salina*) eggs were incubated in a prepared seawater solution for hatching (35 g NaCl/L) and kept under continuous light at 25–30°C for 24 to 48 hours (Ameen *et al.*, 2011) [2]. The hatching success rate was evaluated using the following equation:

Hatching (%) = [Number of Nauplii / (Nauplii + Unhatched Eggs)] × 100

Brine Shrimp (*Artemia salina*) Lethality Assay

Artemia salina bioassay was utilized to screen for cytotoxic effect of Bi NPs by the methodology described by Ameen *et al.* (2011) [2]. Test solutions were prepared in seawater containing 1 % DMSO. Thirty brine shrimp nauplii were inoculated to each well of a 24-well microplate along with 1 mL of the test solution. A negative control (seawater containing 1 % DMSO) and a positive control (100 µg/mL Potassium dichromate) were used in the experiment. The plates were maintained at a temperature of 25–30°C for 24 hours under continuous illumination. To determine mortality, immobile nauplii were counted under a microscope. The mortality percentage was estimated using the formula:

Mortality (%) = (Number of Dead Nauplii / Total Nauplii) × 100

Results

Preparation of aqueous extract of *Eclipta prostrata* medicinal herb

Water – based extraction of *Eclipta prostrata* yielded a dark brown, semi-solid extract after soaking 100 g of powdered leaf material in 300 mL of deionized water at ambient temperature for 24–48 hours with intermittent agitation. Following filtration, the extract was lyophilized (or evaporated under reduced pressure), resulting in a dry aqueous extract. The final yield was stored at 4 °C in amber-coloured bottles to prevent photodegradation and maintain extract stability. The extraction process followed the methods outlined by Do *et al.* (2014) and Panda & Padhi (2020) [21]. (Figure 1)

Biogenic synthesis of Bismuth Nanoparticles using the aqueous extract of the *Eclipta prostrata*

Aqueous extract of *Eclipta prostrata* were used to reduce bismuth nitrate. After heating and stirring, the solution turned golden-yellow, showing that bismuth nanoparticles had formed. We collected the particles by centrifugation and washed them several times with ethanol and distilled water to get a clean sample. After drying at 110 °C, we obtained stable bismuth nanoparticles in the golden yellow colour, which we stored for later analysis and use. (Figure 2)

Antioxidant potential of thebiogenically synthesized Bismuth Nanoparticles

The Biogenic Bi NPs demonstrated notable antioxidant potential across several in *vitro* assays, with activity increasing consistently with concentration. In the DPPH radical scavenging assay, a steady enhancement in free radical neutralization was observed, starting at 20 % inhibition at 100µg/mL and reaching up to 80 % at 500µg/mL. Similarly, the FRAP assay showed a proportional rise in reducing power, with values increasing from 150 µmol Fe(II)/g extract at the lowest concentration to 750 µmol Fe(II)/g extract at the highest. The ABTS assay supported these findings, indicating scavenging activity that escalated from 25 % to 90 % across the same concentration range. Superoxide and nitric oxide radical scavenging assays also followed this trend, with inhibition rates rising from 15 % and 10 % at 100µg/mL to 75 % and 70 % at 500µg/mL, respectively. Although hydroxyl radical quenching activity was generally reduced compare to the other assays, it still showed a significant increase from 5% to 65% as the concentration increased. Total phenolic content also reflected the extract's antioxidant capability, ranging from 50 µg GAE/g extract at 100µg/mL to 250 µg GAE/g at 500µg/mL, confirming the phenolic compound's role in contributing to the observed antioxidant effects. (Table 1 & Figure 3)

Antimicrobial efficacy of the water - based extract of *Eclipta prostrata* medicinal herb

The biogenic Bi NPs displayed strong antimicrobial activity, effectively acting against both Gram-positive and negative bacteria, along with several fungal species. Through the agar well diffusion assay, the extract produced zones of inhibition measuring 19 mm for *Staphylococcus aureus*, 17 mm for *Escherichia coli*, and 14 mm for *Pseudomonas aeruginosa* at a concentration of 500 mg/mL. These outcomes reflect the extract's potential as a broad-spectrum antibacterial agent. Antifungal efficacy was also noted against *Candida albicans, Aspergillus niger*, and *Fusarium oxysporum*, with inhibition zones of 18 mm, 15 mm, and 13 mm, respectively. Overall, the findings substantiate the traditional medicinal application of *Eclipta prostrata* in managing microbial infections and underscore its promise as a natural antimicrobial source. (Table 2 & Figure 4)

Artemia salina assay of the water-based extract of *Eclipta prostrata* medicinal herb

Toxic properties of thebiogenic Bi NPs were evaluated using the Brine Shrimp Lethality Assay, which revealed a concentration-responsive increase in lethality. At 20µg/mL, the extract caused a mortality rate of 17%, which gradually increased to 35 %, 39.6 %, and 46 % at 40, 60, and 80µg/mL, respectively. The highest test concentrations of 100µg/mL and 120 µg/mL demonstrated the most notable toxic effects, with mortality rates of 53.3 % and 66.6 %. These findings suggest that theBi NPs contains bioactive compounds with potential cytotoxic or antitumor activity. Furthermore, the hatching percentage of Artemia salina nauplii was inversely proportional to Bismuth concentration. The control group showed over 70 % hatching, while at 20µg/mL, the hatching percentage dropped to 57 %. This further declined to 32.6 % and 22.6 % at 100µg/mL and 120µg/mL, respectively. The decrease in hatching success indicates the Bismuth Nanoparticles possible toxicity on embryonic development at higher doses. (Table 3 & Figure 5, Figure 6)

Discussion

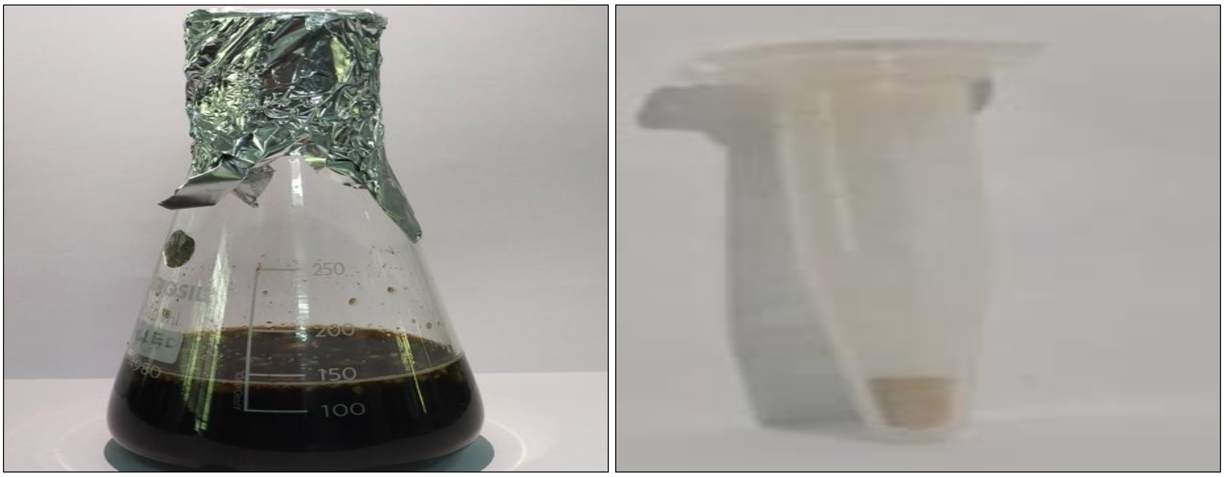
Bismuth nanoparticles (BiNPs) synthesized with aqueous *Eclipta prostrata* extract exhibit significant antimicrobial activity against multiple bacterial and fungal species. The observed golden-yellow coloration during synthesis indicates the reduction of Bi^{3+} ions to elemental bismuth, facilitated by plant-derived phytochemicals such as flavonoids, tannins, alkaloids, and phenolics, which serve as reducing and stabilizing agents. Measured zones of inhibition for BiNPs, including 19 mm for *Staphylococcus aureus*, 17 mm for *Escherichia coli*, and 14 mm for *Pseudomonas aeruginosa*, demonstrate broad-spectrum antibacterial efficacy that surpasses the activity of the plant extract alone. The antimicrobial properties of these green-synthesized BiNPs likely result from the combined effects of bismuth toxicity toward microbial enzymes and the bioactive phytochemical coating from *Eclipta prostrata*, which damages microbial cell walls, disrupts enzymatic processes, and impairs nucleic acid synthesis.

The BiNPs also displayed promising antifungal activity, showing clear inhibition against *Candida albicans, Aspergillus niger* and *Fusarium oxysporum*, aligning with previous reports on plant-mediated metallic nanoparticles rich in phytoconstituents (Albuquerque *et al*., 2019) [1]. Notably, C. albicans, a common opportunistic fungal pathogen, was found to be particularly susceptible, suggesting potential applications of these BiNPs in treating mucosal and dermal fungal infections.

The inhibitory effect against a broad spectrum of Gram-positive and Gram-negative bacteria indicates that the BiNPs can overcome the outer lipopolysaccharide membrane of Gram-negative organisms. Gram-negative bacteria are typically more tolerant to antimicrobials; hence, their inhibition highlights the potency of these nanoparticles (Duan *et al*., 2017) [10]. The mechanism of action may involve nanoparticle-induced membrane disruption, enzyme inhibition, ROS generation, and synergistic antioxidant activity from *Eclipta prostrata* phytochemicals (Gao *et al*., 2018; Wang *et al*., 2021) [11, 29]. Collectively, these findings validate the ethnomedicinal relevance of *Eclipta prostrata* and underscore the potential of its green-synthesised bismuth nanoparticles as effective natural antimicrobial agents for future biomedical applications.

List of Figures & Tables

Fig 1: Fresh leaves of Medicinal herb *Eclipta prostrata*



(a) (b)

Fig 2: (a) Aqueous extract of *Eclipta prostrata* 2(b) Biogenic Bismuth Nanoparticles

Table 1: Antioxidant Activities of Aqueous *Eclipta prostrata* Extract

Conc. (µg/mL)	DPPH (%)	FRAP (µmol Fe/g)	ABTS (%)	Superoxide (%)	Nitric Oxide (%)	Hydroxyl (%)	Total Phenolic (µg GAE/g)
100	20	150	25	15	10	5	50
200	40	300	50	35	30	20	100
300	55	450	70	55	50	35	150
400	68	600	85	65	60	50	200
500	80	750	90	75	70	65	250

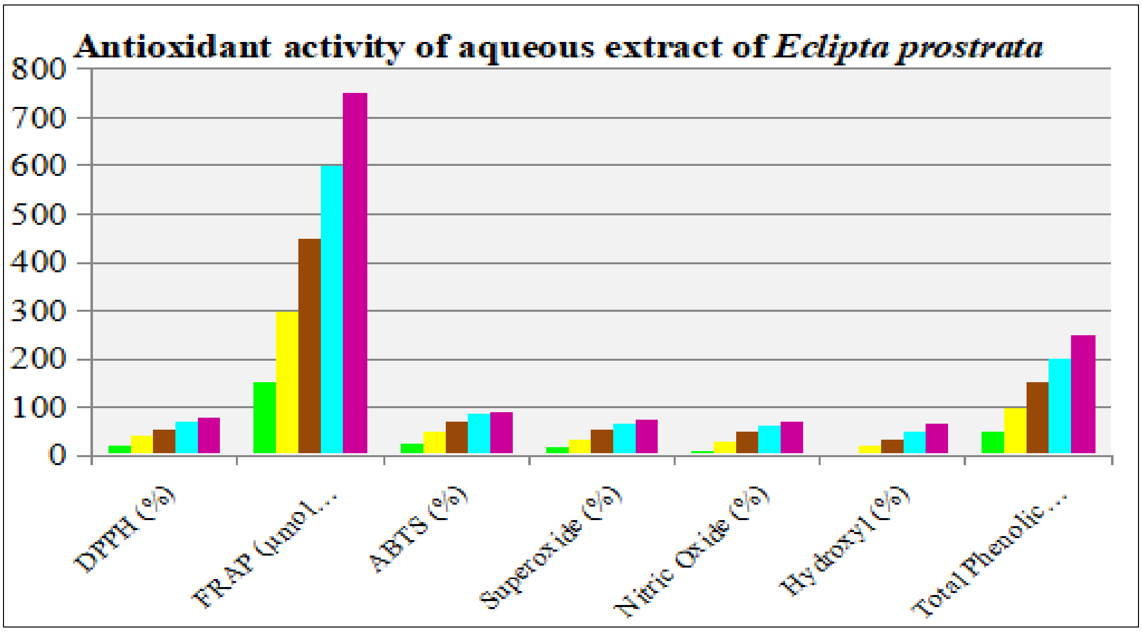


Fig 3: Antioxidant activity of aqueous extract of *Eclipta prostrata*

Table 2: Antimicrobial Activity of aqueous extract of *Eclipta prostrata*

Microorganism Tested	Zone of Inhibition (mm)
Staphylococcus aureus	19
Escherichia coli	17
Pseudomonas aeruginosa	14
Candida albicans	17
Aspergillus niger	15

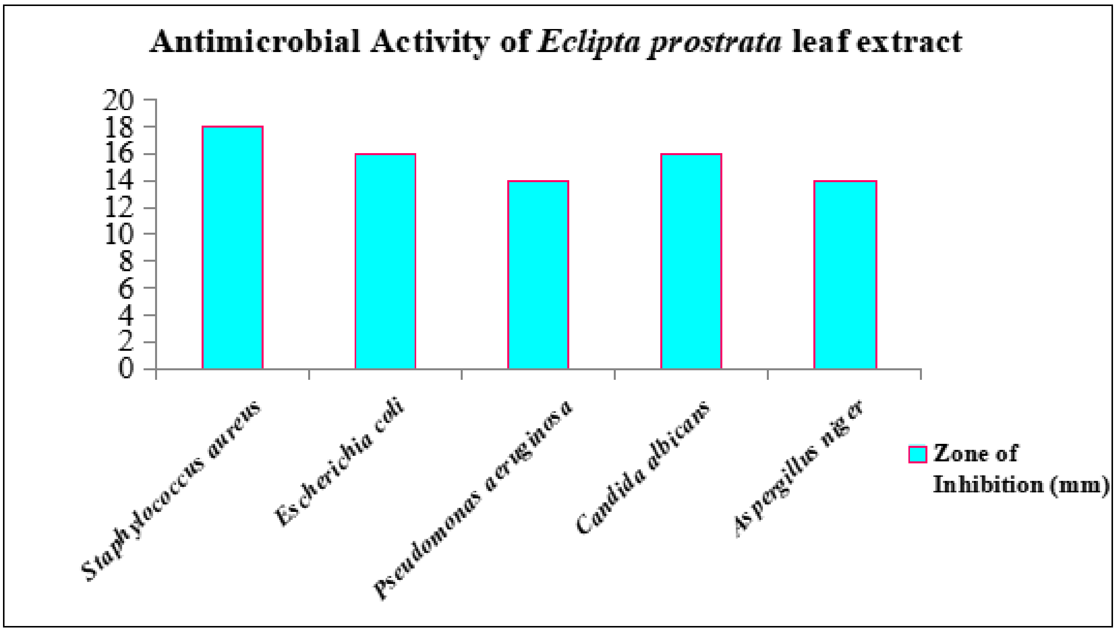


Fig 4: Antimicrobial Activity of *Eclipta prostrata* leaf extract

Table 3: Cytotoxicity Assay (Brine Shrimp Lethality) of *Eclipta prostrata* aqueous leaf extract

Concentration (µg/mL)	Mortality (%)	Hatching (%)
20	16.0	59.0
40	34.0	55.0
60	37.6	44.6
80	44.0	37.0
100	53.3	32.6
120	66.6	22.6

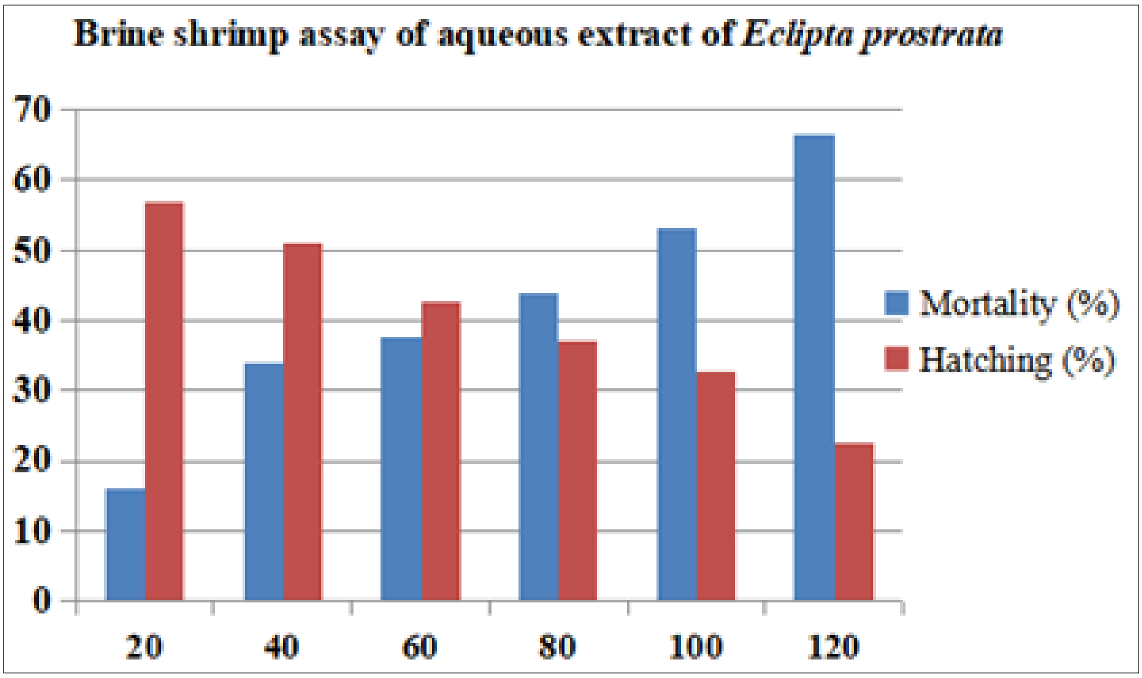


Fig 5: Brine Shrimp assay of aqueous leaf extract of *Eclipta prostrata*

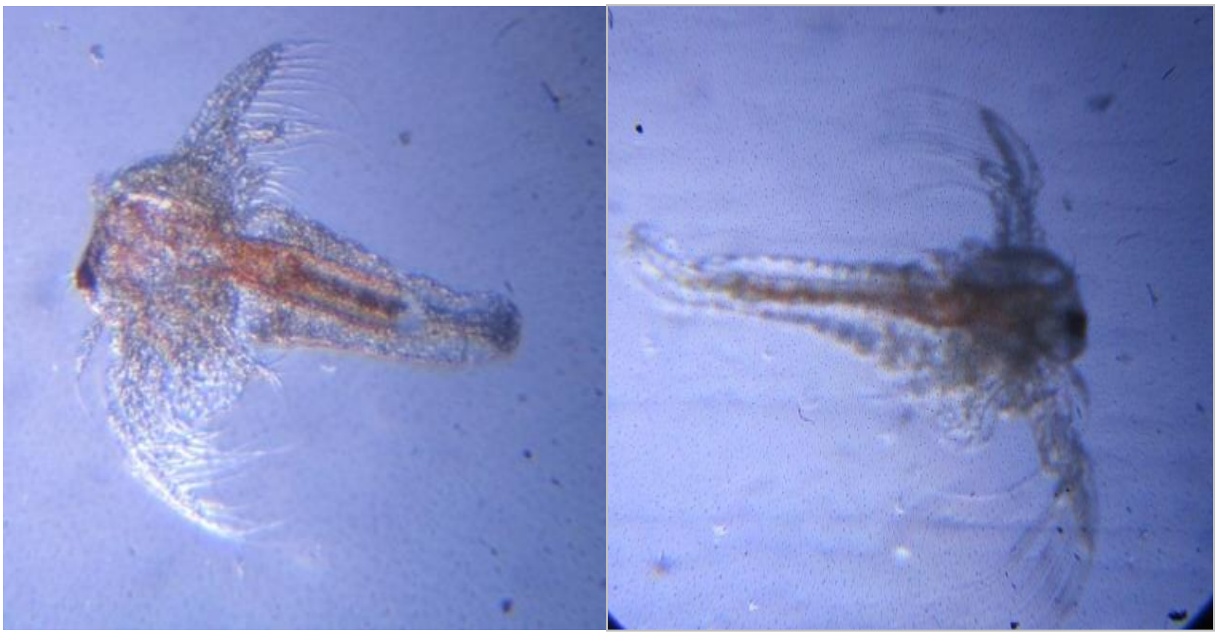


Fig 6: Brine shrimp larvae after exposure to concentrations of Bismuth Nanoparticles

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