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Antimicrobial activity of silver nanoparticles synthesized from fruit epicarp of *Glycosmis pentaphylla*

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Abstract: [a1]Our study aim is to characterize and assess the antimicrobial effect of silver nanoparticles (AgNPs) synthesized from the fruit epicarp of *Glycosmis pentaphylla* against few crops and human pathogens. Our study suggests a novel method for biosynthesis of silver nanoparticles from the epicarp of *Glycosmis pentaphylla.* The study confirms the ability of the fruit epicarp extract of *Glycosmis pentaphylla* for the biosynthesis of silver nanoparticles grown under *in-vitro* conditions. The green synthesis of AgNPs from (Ethyl alcohol) EtOH extracts of *Glycosmis pentaphylla* was performed through standard protocols. The synthesized AgNPs were confirmed by colour changes (green to brown) within <10 minutes and characterized by UV-visible spectral, SEM and TGA analysis (Scanning electron microscope, Thermal gravimetric analysis). Antimicrobial activities of the silver nanoparticles were performed by agar well diffusion method against crops pathogenic fungus and human pathogenic bacteria. The highest antifungal activities of silver nanoparticles were found against *Colletotrichum lindemuthianum* and *Alternaria alternata.* The antibacterial activity was measured through the zone of inhibition against *B. subtilis* (18 mm), *S. typhimurium* (17.33 mm), *S. mutans* (17 mm) and *E. coli* (17 mm). The antimicrobial potential of AgNPs was determined by minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC) tested against human and plant pathogens. In addition, AgNPs displayed the significant synergistic antibacterial effect when it combined with Streptomycin and Ciprofloxacin in the ratio of 1:1. This eco-friendly, biocompatible and sustainable phytofabrication approach of bioactive AgNP synthesis is a progressive step towards various applications to control few crops (Chilli, and Tomato) and human pathogens in near future.

Keywords: *Glycosmis pentaphylla,* fruit epicarp, silver nanoparticles, antimicrobial activity, synergistic effect

Keywords atleast should be 5-6 words (Specific to your study)

1. INTRODUCTION[a2]

The worldwide agricultural production gets compromised over the past few years due to crop pathogens. The harmful effects on fruits and vegetables of these pathogens have compromised the quality of the crops along with economical loss globally. The consumption rate of fruits and vegetables has increased up to 40% during the past few years. Every year, the amount of loss is approximately 20% of all fruits and vegetables[1].The fresh fruits and vegetables are exposed to contamination by microorganisms, especially plant fungi from direct contact with soil, dust, water during harvesting or post harvesting[2]. Now a day's the traditional antibacterial treatment against the growing resistance of human pathogenic bacterial strains has become a big challenge[3]. The microbial infection is a serious problem in the agriculture and healthcare sector worldwide. Therefore, it is needed to develop a new antimicrobial agents including different characteristics, such as eco-friendly, low toxicity, antimicrobial potency, and great compatibility. In this situation, nanoparticles (NPs) have accepted as alternative to chemical pesticides worldwide, due to their electrostatic attraction between positively charged NPs and negatively charged microbial cells, and a large surface to volume ratio, resulting in improved physicochemical properties and enhanced antimicrobial activities of the NPs[4,5]. The antibacterial and antifungal properties of NPs have recently been widely reported [6, 7, 8]. The application of metal nanocomposites enhanced antimicrobial activity against multi drug resistance bacterial infection [9, 10, 11]. The antibiotics could be more effective when combined with metal NPs conditions [12] as, NP-antibiotic conjugate could lower the amount of both dosages, which reduces noxiousness and increases antimicrobial properties. The various research areas like drug discovery, biomedical sciences, cosmetics, luminescence, and renewable energy technologies focus on novel properties of NPs which make them extremely versatile [13]. Among the different types of metallic nanoparticles, silver nanoparticles are widely used because of their unique and remarkable properties, enhanced permeability, retention effect and antimicrobial activity [14, 15, 16]. Therefore, the market value of AgNPs increased day by day due to this inherent property which extends the AgNPs applications as an antimicrobial agent in different arrays of products, such as soaps, plastics, food and textiles [17]. The binding and absorption rate of the drug on patient cells increased due to the existence of protein caps on nanoparticles which also bind to the bacterial cell surface. The mode of action of AgNPs showed that it decreased the rate of bacterial cell permeability, cellular respiration, DNA and protein function inside the cell [18]. Many researchers focus on numerous evidences of synthesis of different AgNPs and their antimicrobial activity but MIC, MBC and Synergistic effect against human pathogenic bacteria are rare. The modern age of nanotechnology, there is an ongoing competition to identify "green" pathways to synthesize metallic nanoparticles using biological resources, mainly plant. There are several scopes for improving the green pathway for a single step rapid synthesis of AgNPs at room temperature by different modifications. In an earlier report, AgNPs were synthesized by the reduction of aqueous Ag^+ ions using unexploited weed resources (*Ipomoea, Enhydra*, and *Ludwigia*) and sunflower (*H. annuus*)[19,20]. There is a scope for improvement in bio-based methods for single step rapid synthesis of metallic Nps at room temperature by modulating their size. The fruit epicarp of *Glycosmis pentaphylla* has many medicinal and reducing properties which have been used for synthesizes of AgNPs by the reduction of $AgNO_3$ by using aqueous extract of the fruit epicarpat room temperature within 72hrs. However, the synthesis of AgNPs using such plant constituents has not yet been fully studied along with their antimicrobial activity. The nanoparticles synthesized from various plant parts used to control different plant and animal disease causing microorganisms. There is numerous evidence of the synthesis of different AgNPs but the fruit epicarp antifungal activity of AgNPs with detailed study (MIC & MFC) against human pathogenic bacteria (MIC & MBC) is rare. In the present work, we have analyzed the antimicrobial activity against a few crop pathogens, antibacterial activity against four Gram-positive bacteria and Gram-negative bacteria with MIC and MBC study. We also investigated the individual and synergistic antibacterial activities of AgNPs with two conventional antibiotics (streptomycin and ciprofloxacin) to evaluate their biomedical applications in minimizing antibiotic dose overexploitation.

2 MATERIALS AND METHODS[a3]

2.1. Experimental Microorganisms

The referred microbial strains of fungus and bacteria were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Reference fungal strains included *Colletotrichum gloeosporioides* (MTCC-4618), *Colletotrichum lindemuthianum* (MTCC-8474), *Fusarium moniliforme* (MTCC-2015), *Fusarium oxysporum* (MTCC 2480), and *Alternaria alternata* (MTCC-8459).The bacterial strains included two Gram-positive (*Bacillus subtilis*- MTCC 121, *Streptococcus mutans*- MTCC 497) and two Gram-negative bacteria (*E. coli*- MTCC 723 and *Salmonella typhimurium*- MTCC 98).

2.2. Plant sample collection and extract preparation

The ripened fruits of *Glycosmis pentaphylla* were collected from the college ground of Sreegopal Banerjee College, Bagati, Mogra, Hooghly, India. Identification and authentication of *Glycosmis pentaphylla* was done by Dr. Monoranjan Chowdhury, Associate Professor,Taxonomy of Angiosperm and Biosystematic Laboratory, Botany Department, University of North Bengal, India, with the voucher number SBCH2017. The fleshy epicarp separates from the fruit and dries in hot air woven at 40°C. The dry fruits epicarp were crushed in dust form and extracted in different solvents to analyze its photochemical[21]. The fruit epicarp extract was prepared by taking 50 g of dry dust fruit epicarp in a 500 ml conical flask with100 ml of 30% ethanol (EtOH) for 24hrs at 30°C room temperature. The crude extracts were filtered through Whatman`s No.-1 filter paper and stored at 4°C for the synthesis of AgNPs.

2.3. Phytochemical analysis of the crude extract

The fruit epicarp extract was dipped in different solvents for extraction of different secondary chemicals. The biochemical analysis was done for various chemicals estimated, such as total phenolics[22], flavonoids [23], tannin [24], saponins[24] and alkaloids [25], phytate[26] and oxalate [27]. Determination of each biochemical analysis was repeated three times and expressed in a percent dry weight basis.

2.4. Synthesis of Silver Nanoparticles (AgNPs)

The aqueous solution of 1mM silver nitrate ($AgNO_3$) [analytical grade (AR), purchased from E. Mark (India)] was prepared and used for the synthesis of AgNPs. The fruit

epicarp extract (5 ml) was added into 50 ml of an aqueous solution of 1 mM $AgNO_3$ for reduction of Ag+ to Ag^0 [28]. The reaction mixture was incubated (15 minutes) at room temperature till the turn up of green to brown colour. The particles were isolated by centrifugation (6,000 rpm up to 15 minutes), repeated washing and drying at 75°C for further characterization.

2.5. Characterization of synthesized AgNPs using UV-visible spectrophotometer

The reduction of Ag^+ to Ag^0 was monitored by measuring the UV-Vis spectrum of each reaction mixture at different time intervals (10, 20, 30, 40, 50, 60, 120, 180, 240, 300 minutes) within the range of 370-500 nm in the UV-Vis spectrophotometer (Shimadzu UV-VIS Spectrophotometer, Japan) because the absorption spectrum of aqueous $AgNO_3$ and green synthesized AgNPs solution exhibited λ_{max} at about 220 nm and 430 nm, respectively [29][a4].

2.6. Characterization of Synthesized AgNPs using Scanning Electron Microscopy

Morphological characterization of AgNPs was done by Scanning electron microscopy [30]. For SEM analysis, the EtOH is used as a blank reference. The isolated dried and powdered AgNPs were used for SEM study. A thin film of each sample was prepared separately on a small glass cover slip (3x3 mm), and set on a copper stab for electron microscopy using Hitachi made Scanning Electron Microscope (SEM) (Model: S530 with IB2 ion cotter, Japan).

2.7. Characterization of synthesized AgNPs using Thermal Gravimetric Analysis (TGA)

TGA analysis of the synthesized AgNPs was also observed with an increasing temperature range of 160˚–550˚C [19].

2.8. In vitro antifungal activity of green synthesized AgNPs

The antifungal activity of bio–synthesized AgNPs was tested against various crop pathogens according to Loo et al, 2018, agar well diffusion method [31]. To examine the antifungal activity of biosynthesized AgNPs, Potato Dextrose agar plates were sterilized and allowed to solidify. After solidification, 30 µl of each fungal spore's suspension containing 1×10^6 CFU/ml was inoculated on the Petri plates by a sterile glass rod and 8 mm cup was cut with the help of a sterile cork borer in each inoculated plates. The well filled with 10 µl antifungal AgNPs solution and incubated at 28°C for 5 days. Controls of silver-free plates were incubated under the same conditions.

2.9. *Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)*

The minimum inhibitory concentrations (MIC) of synthesized AgNPs were determined according to the standard protocol[32]. The stock AgNPs solution (50 µg/ml,) was serially diluted up to 5.26 µg/m using sterile 30% ethanol and 30% ethanol was serving as control. The spore suspension of test fungus was prepared by scraping the spores from 7-day-old PDA slant culture. 10µl spore suspension was picked up from slant through micropipette, checked the CFU and poured into each fresh Potato dextrose agar plate. 10 µl of the test samples from each concentration were loaded into the 5 mm diameter well in five test fungus plates and incubated at 28°C for 48 hrs. The MIC end-point criterion was defined as the lowest AgNPs concentration showing no visible growth after 48 hrs incubation. MIC values were calculated by comparing the germination of spores in PDA plates containing different concentrations of AgNPs. The MFC was determined from the concentration of the compound in which no fungal growth was found. To determine MFC, 2, 4, 8, and 10 times higher concentrations of MIC were taken and the colony-forming units (CFU) were counted after 24 hrs of incubation at 37°C to observe complete growth inhibition of the fungal organisms.

2.10. In Vitro antibacterial activity of green synthesized AgNPs

2.10.1. Antibacterial activity by the agar well diffusion method

Assessment of antibacterial activity of AgNPs sample against two Gram-positive bacteria (*Bacillus subtilis*, *Streptococcus mutans*) and two Gram-negative bacteria (*E. coli* and *Salmonella typhimurium*) was measured by the agar well diffusion method [30]. 8 mm wells were cut in each fresh inoculated bacterial plate and 10 µl of different concentration of the test sample was loaded into the 5 mm diameter well seeded with test bacteria and incubated for 24 hrs at 37 °C. The potency was compared by measuring zone diameter of growth inhibition with standard antibiotics, Streptomycin (10 µg/ml).

2.11. *Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

The MIC values of synthesized AgNPs against four Bacterial pathogens were determined using the standard protocol [33]. The stock AgNPs sample were serially diluted up to 5 µg/ml and MIC values were measured using NA media. 10 µl of the test sample of different concentrations was loaded into the well of pre-inoculated nutrient agar plate of target bacteria, incubated for 24 hrs at 37 °C and observed for zone of growth inhibition. The MBC was determined by checking the viability of the bacterial cells after treating with 2 × concentration of MIC of AgNPs and dilution plating on a nutrient agar plate. In brief, the actively growing bacterial strains (log phase growth) were treated with test samples (different AgNPs samples) at higher concentration of MIC and incubated for 1 hr. The treated culture was then plated on a nutrient agar plate at a dilution of 10^{-2} to 10^{-4} in triplicates and incubated in similar conditions for observation for any viable colony formation. MBC is noted as the concentration where no viable cells were noticed [34].

2.12. Synergistic activity with antibiotics

To performed this experiment, 6 mm diameter sterile Whatman No.-1 filter paper discs were soaked with the AgNPs sample at MIC values (5.5 µg/ ml) and filter sterilized antibiotic solutions at MIC values (i.e. Streptomycin, 0.5 µg/ml and Ciprofloxacin, 0.5 µg/ ml) and placed at the centre of the each culture plate seeded with target bacterium and incubated at 37 °C for 24 hrs and were observed growth inhibition. The synergistic potential was determined by comparing the magnitude of antibacterial activity of AgNPs and antibiotics with the antibiotics alone using the following formula: FI-Fold Increase (FI) = [(b − a)/a] × 100; where, 'b' stands for

'inhibition zone diameter (mm) for antibiotics + AgNPs; 'a' stands for 'inhibition zone diameter (mm) for antibiotics alone [35].

3. STATISTICAL ANALYSIS[a5]

All the data of phytochemical regime and antimicrobial activity were analyzed using one way ANOVA, Tukey HSD and Pearson correlation[36]. All the statistical analysis was performed using the statistical program SPSS v. 13.0 (SPSS, 2004).

4. RESULTS AND DISCUSSION

4.1. Phytochemical Regime

The biochemical analyses of fruit epicarp extract represent variation in secondary (Phenols, flavonoids, tannin, saponins, alkaloids, phytate and oxalate) metabolites which are very much similar to the Roy and Barik, 2010 experiment[19]. The phytochemical regime of the plant is presented in Fig.1. All Secondary chemicals were higher than other plant parts.

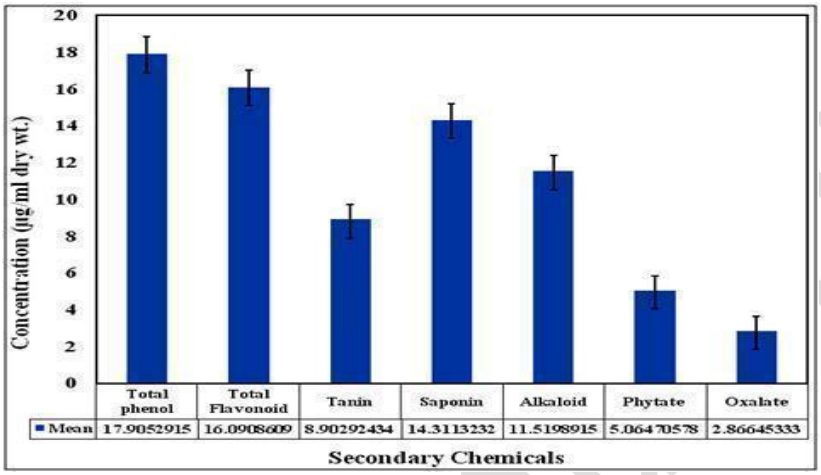


Fig. 1[a6]:-Phytochemical variations of *Glycosmis pentaphylla* fruit epicarp extract (Mean ±5 observations).

4.2. UV-VIS spectroscopy characteristics of AgNPs

During the green synthesis of AgNPs through the fruit epicarp extract changes colour from green to brown as previously reported by researchers (Fig. 2) Parvekar et al, 2020 [37]. The brown colour due to the reduction of Ag^+ confirms the formation of AgNPs and was characterized by UV-Vis Spectroscopy as in Roy and Barik, 2010. The reduction of pure silver ions (Ag^+) was estimated by UV-Vis spectral analysis in the frequency range of 270 to 500 nm at room temperature and which was represented the peak at around 420-430 nm for a long time interval (10-300 minutes) specific for the synthesis of AgNPs with longer stability (Fig. 3). The band at 420 - 430 nm can be attributed to the property surface plasmon resonance (SPR) due to the oscillation of electrons (Mie scattering) for the strong interaction of light with the AgNPs. In both cases EtOH act as blank. The λ_{max} of AgNPs was observed at around 430 nm whereas in EtOH extract it was at around 380 nm, respectively within the time span of 10-300 minutes. The UV-visible spectra showed absorption bands in the 350 to 550 nm region which confirms the formation of AgNPs [38, 39].

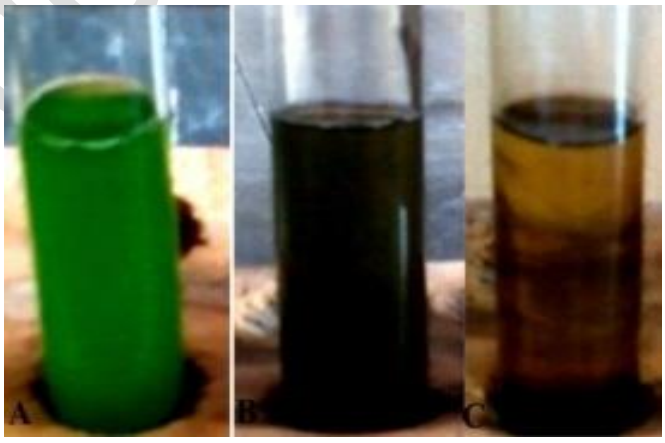


Fig. 2: The colour changes from green (A) to brown (B and C) during the reaction of Ag^+ into AgNPs due to the photochemical present in fruit epicarp extract of *Glycosmis pentaphylla*.

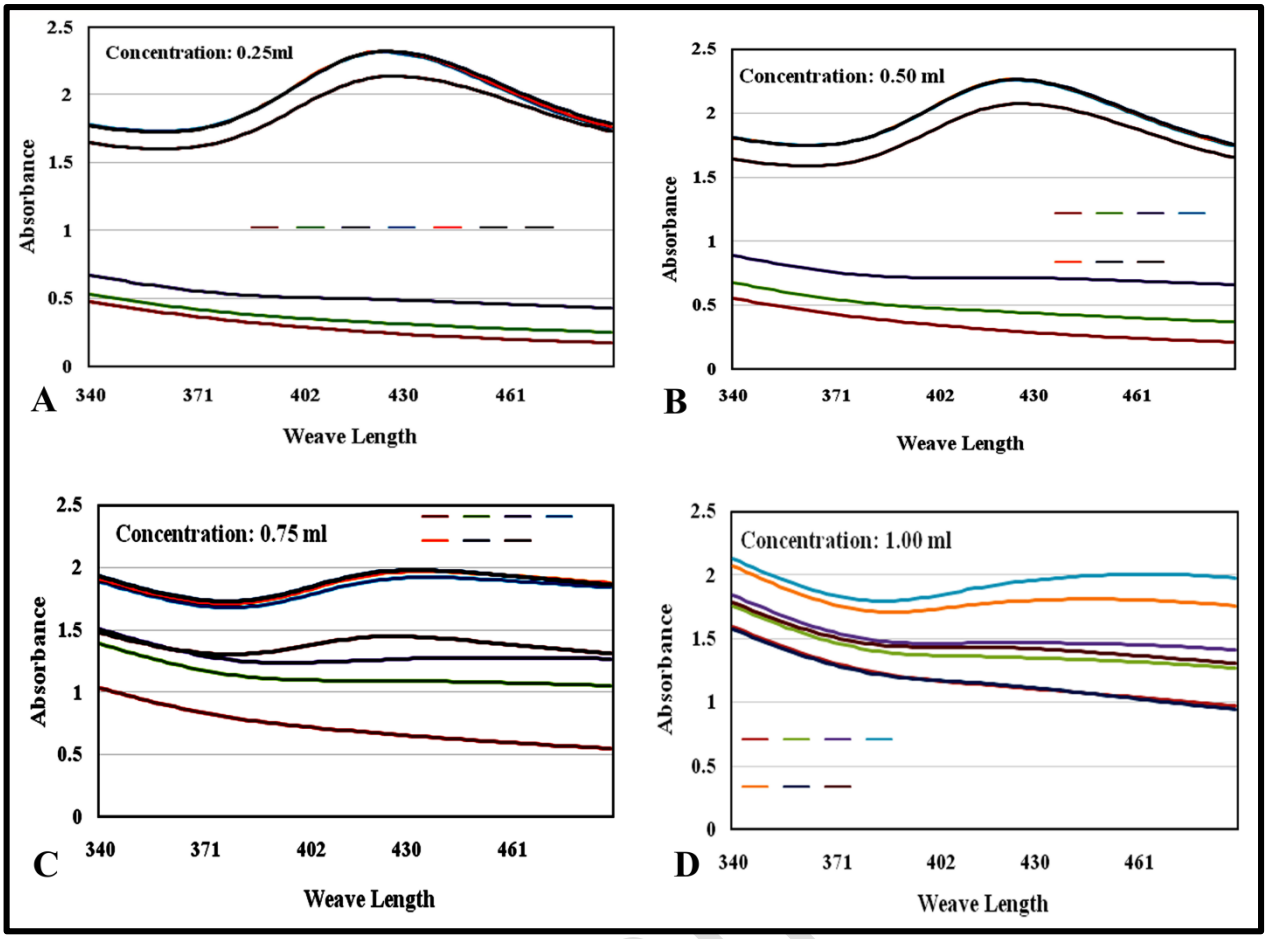


Fig. 3:- UV-Vis absorption spectra recorded at different time intervals (10 min, 30 min, 1h, 24h, 48h, 72h) of AgNPs synthesized from fruit epicarp extract *Glycosmis pentaphylla* (Mean of 3 observations).

4.3. SEM and TGA characteristics of AgNPs

Microscopic surface features including morphology and particle size of synthesized AgNPs was assessed by SEM analysis. The SEM image provided roughly spherical topography of AgNPs was about 75±5 nm in size (Fig. 3). The SEM image also confirms that the synthesized nanoparticles are well separated with no aggregation (Fig. 4). TGA data of the synthesized AgNPs showed steady weight loss due to desorption of its bioorganic compounds with an increasing temperature range of 160–550°C [19]. Previously reported SEM images of AgNPs from different extracts showed spherical particles, aggregated spherical particles, irregularly shaped particles, and cubic particles [40, 41]. The moderate particles sizes observed were [42, 41, 40, 43, 44], and 50 nm, for AgNPs synthesized by different biological extracts using water as a solvent. This observation aligns well with the previously reported particle sizes [40, 41, 45, 46, 47]. In an article comparing the advantages and drawbacks of these methods and the applications of nanoparticles in various domains, a synthesis of chemical, physical, and biological methods for obtaining AgNPs of different shapes and dimensions (from 2 to 300 nm) was described [48].

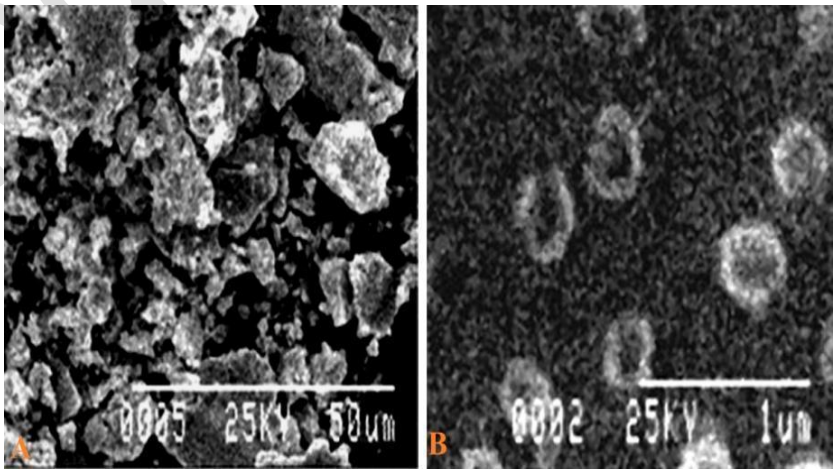


Fig. 4:- The SEM images of AgNPs synthesized from fruit epicarp extract *Glycosmis pentaphylla* at 25.0 kV × 1 k.

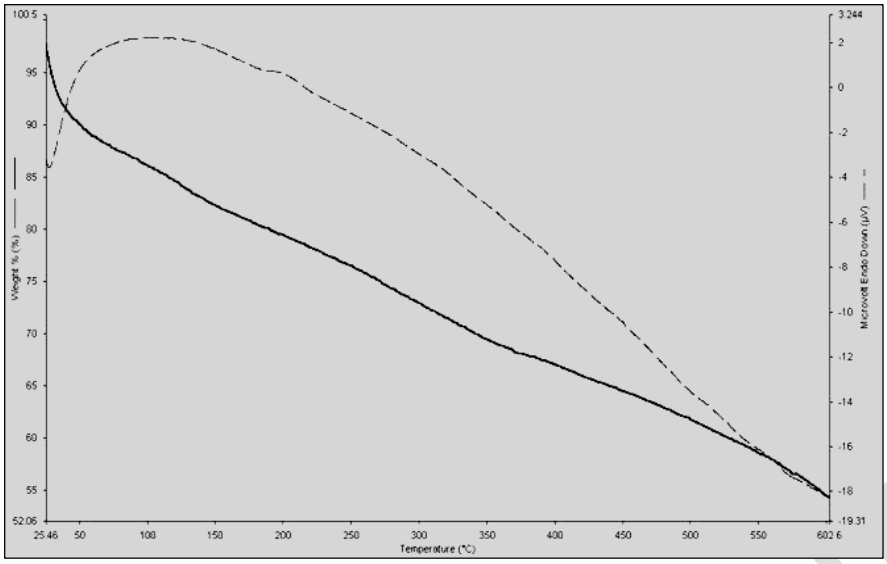


Fig. 5:- TGA of the synthesized AgNPs showed steady weight loss within the temperature range of 160 –550˚C.

4.4. Invitro antifungal potentiality of AgNPs

The antimicrobial activities of AgNPs against various crop pathogenic funguses were investigated as shown in Fig. 6. The biosynthesized AgNPs inhibits the growth of *Fusarium oxysporum, Fusarium moliniliforme, Alternaria alternata, Colletotrichum lindemuthianum* and *Colletotrichum gloeosporioides.* Thus, AgNPs could be considered as excellent broad-spectrum antifungal agents for sustainable crop production and also could potentially be used widely in clinical applications against human pathogenic fungi. The various research reports of AgNPs synthesized from plants and microbes had broad range antifungal activity against *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium tricinctum*, and *Alternaria* sp. by agar well diffusion method [49-51]. The antifungal potentiality in other reports of AgNPs showed against crop pathogens such as *Aspergillus niger, Rhizoctonia solani, Curvularia lunata, Colletotrichum* sp. and *Fusarium* sp.[52, 53].

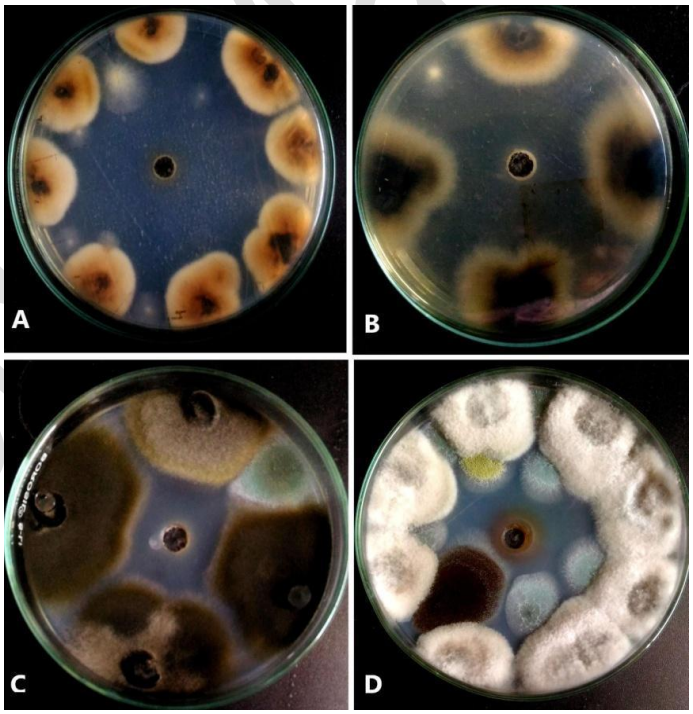


Fig.6:- Antifungal activity of AgNPs in PDA media by agar well diffusion method; (A) *Colletitrichum lindemuthianum;* (B) *Alternaria alternata; (C) Colletotrichum gloeosporioides; (D)Fusarium moniliforme*

4.5. Determination of MIC and MFC of AgNPs

The minimum inhibitory concentration and minimum fungicidal concentration of AgNPs forfive different fungal strains as shown in Table – 1. [a7]The results suggest that the plant synthesized AgNPs are capable of inhibiting crop fungi like *Fusarium oxysporum, Fusarium moniliforme ,Alternaria alternata, Colletotrichum lindemuthianum* and *Colletotrichum gloeosporioides.* The highest MIC values shown in *Alternaria alternata, Fusarium oxysporum, Fusarium moniliforme* was 6.2 µg/ml and the highest MFC values was 7.14 µg/ml. Green synthesized silver nanoparticles had antimicrobial effects against *A. flavus, F.oxysporum,* and *P. digitatum* on PDA in vitro. Inhibition (97.3%) was obtained against *A. flavus* treated with a 10 µg/ml concentration of silver nanoparticles and the minimal level of inhibition was found

against *P. digitatum* and *F. oxysporum* with 2 µg/ml concentrations of AgNPs [54]. This could be possible to adhere AgNPs to fungal hyphae and deactivate plant pathogenic fungi. DNA loses its ability to replicate upon treatment with Ag+ resulting in inactivated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to ATP production [55, 56].

Table - 1:MIC and MFC values of synthesized AgNPs against different fungal pathogens

Name of the crop pathogens	Concentration of AgNPs (µg/ml)									
	50 µg/ml	25 µg/ml	16.66 µg/ml	12.5 µg/ml	10 µg/ml	8.33 µg/ml	7.14 µg/ml	6.2 µg/ml	5.55 µg/ml	5.26 µg/ml
Alternaria alternata	+	+	+	+	+	+	MFC	MIC	–	–
Colletotrichum gloeosporioides	+	+	+	+	+	+	+	MFC	MIC	–
Colletotrichum lindemuthianum	+	+	+	+	+	+	+	MFC	MIC	–
Fusarium moniliforme	+	+	+	+	+	+	MFC	MIC	–	–
Fusarium oxysporum	+	+	+	+	+	+	MFC	MIC	–	–

Here '+' indicated the positive inhibition zone and '–' indicated the absence of inhibition zone.[a8]

4.6. *Antimicrobial Activity of AgNPs*

The application of AgNPs against human pathogenic bacteria showed significant growth as shown in Fig. 7. The antibacterial activity was measured through the zone of inhibition against *B. subtilis* (18 mm), *S. typhimurium* (17.33 mm), *S. mutans* (17 mm) and *E. coli* (17 mm) which was shown in Fig. 8. It was observed that the AgNPs was a more potent antibacterial compound than other. The comparison of a single application of AgNPs , Streptomycin, Gentamicin and Ciprofloxacin against the Gram-positive and Gram-negative bacterial strains which was shown in Fig. 9. The results showed that antibiotics and AgNPs have more or less parallel potency by means of formation of inhibition zone (mm) by the application of the same volume (10 µl) and same concentration (6 µg/ml).The antimicrobial activity of silver nanoparticles was previously reported to penetrate the cell wall of bacteria and kill them[57]. Due to the presence of a thin peptidoglycan layer in cells of Gram, negative bacteria show potent higher than Gram positive [58]. Anyhow, our study, carryout the new features of antibacterial.

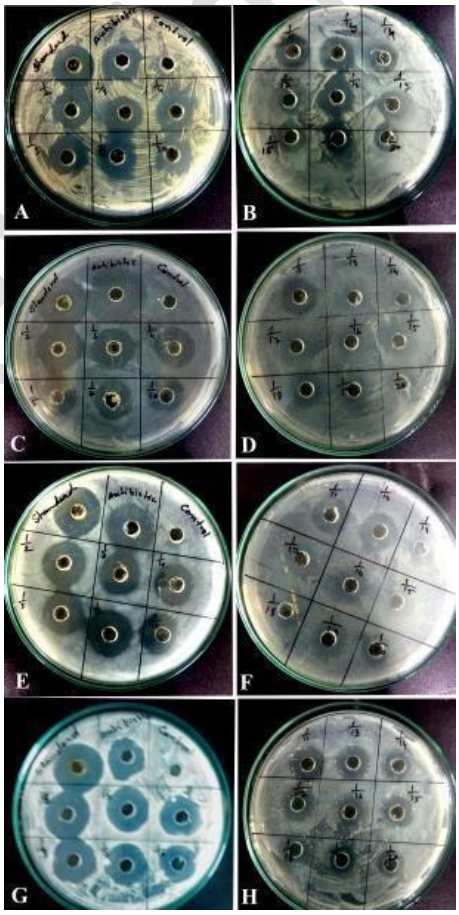
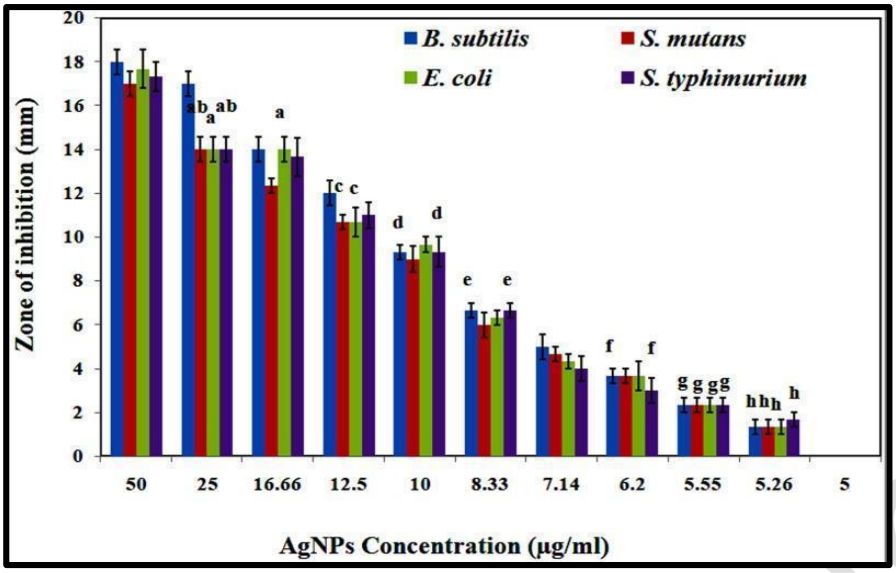


Figure 7: Agar well diffusion assay of the AgNPs .(A-B) against *B. subtilis*; (C-D) against *S. mutans*; (E-F) against *E. coli*; (G-H) against *S. typhimurium*.



The data are displayed as mean ± standard error. Bar with the same letters indicate no significant differences according to Tukey (HSD) test (P < 0.05). Here alphabets a, b, c, d, e, f, g, h indicates bar with standard error.

Fig-8: Antibacterial assessment of the AgNPs. Here, the data were the average diameter of inhibition zone of triplicate trials

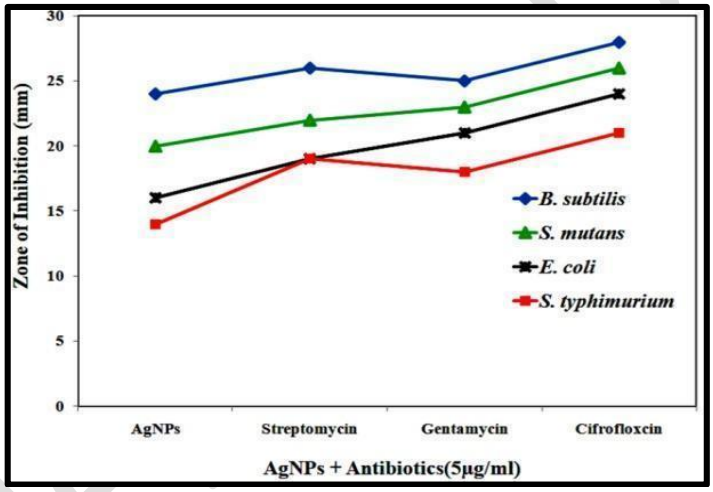


Fig 9: Potency of antimicrobial activity of AgNPs, Streptomycin, Gentamycin and Ciprofloxacin against the Gram-positive and Gram-negative bacterial strains.

4.7. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The results of Table 3 showed that the MIC values varied from 6~5 µg/ml. The MIC values for *B subtilis* and *E. coli* was 5 µg/ml and 5.26 µg/ml MIC values for *S. mutans* and *S. typhimurium* respectively. Similarly, the MBC value for *B. subtilis* and *E. coli* were 5.26 µg/ml and for *S. mutans* and *S. typhimurium*it was 5.55 µg/ml. From the above observations, it is very much clear that AgNPs have the highest antibacterial effect which causes the highest interaction with the bacterial cell wall. Similar observation on antibacterial activity was also observed by the previous studies [59]. We found no particular trend of antibacterial effect for four pathogenic bacteria. Antibacterial activity of AgNPs was observed highest in Gram-negative bacteria than Gram-positive due to the presence of a thick peptidoglycan layer [60]. Phytofabricated AgNPs with antimicrobial properties have also been investigated against different microbes which actually depend on size, shape, environmental conditions (pH, ionic strength) and capping agent [61]. Recently, efficient antimicrobial activity of green AgNPs was observed against multi drug resistant (MDR) and highly pathogenic bacteria (*P. aeruginosa*, *S. aureus*, *S. typhi*, *S. epidermidis* and *E. coli*) [62] and fungi (*C. gloeosporioides*) [63]. Our study shows that *S. mutans* (Gram positive) and *S. typhimurium* (Gram negative) were most sensitive which indicates the mode of action was not only affected by cell wall thickness of the bacteria. Ag+ release from AgNP is another reason for antibacterial activity. As the smaller AgNPs have the higher surface areas associated with the faster release of Ag+ 64 and exert higher toxicity. The high affinity of Ag+ towards protein thiol groups of respiratory enzymes inactivated enzymes even died out [65]. AgNPs also plays an important role in biocompatibility as it controls the interaction of AgNP with a living organism.

Table -2: MIC and MBC values of synthesized AgNPs against different Bacterial pathogens.

Sl. No.	Strains	AgNPs (100 µg/ml)									
		50 µg/ml	25 µg/ml	16.66 µg/ml	12.5 µg/ml	10 µg/ml	8.33 µg/ml	7.14 µg/ml	6.25 µg/ml	5.55 µg/ml	5.26 µg/ml	5 µg/ml
Gram-positive	*B. subtilis*	+	+	+	+	+	+	+	+	+	MBC	MIC
	S. mutans	+	+	+	+	+	+	+	+	MBC	MIC	–
Gram negative	*E. coli*	+	+	+	+	+	+	+	+	+	MBC	MIC
	S. typhimurium	+	+	+	+	+	+	+	+	MBC	MIC	–

Here '+' indicated the positive inhibition zone and '–' indicated the absence of inhibition zone.

4.8. Synergistic effect of AgNPs

The combined effect of antibiotics and AgNPs against different human pathogenic bacteria showed in Table -3. The highest inhibition zone was observed in dual (AgNPs + antibiotic) application than single (AgNPs). The combined effect against bacteria increased the diameter of the inhibition zone that may be possible due to bonding between antibiotics and AgNPs as the antibiotics generally contain active groups like hydroxyl or amino, which bind AgNPs by chelation [67]. The application of Streptomycin with AgNPs showed the highest increasing fold 38.24% against *E. coli* followed by 31.89%, 25%, 20% against *B. subtilis, S. mutans* and *S. typhimurium*. Similarly, the combined application of Ciprofloxacin showed the highest fold increase 30% against *B. subtilis* followed by 24.64%, 15.95% and 5.67 against *S. mutans* and *S. typhimurium*. When bacteria acquire antibacterial resistance, synergistic effect plays an important role as AgNPs and antibiotics kill bacteria in different mechanisms [67]. Our study reveals that, synthesized AgNPs is able to decrease the concentration of Streptomycin and Cifrofloxcin against *S. mutans* & *S. typhimurium* with lowering the side effects and cost effectiveness of antibiotics.

Table -3: Synergistic effect of two antibiotics with AgNPs against *B. subtilis, S. mutans* and *E. coli, S. typhimurium*; FI-fold increase FI% = [(b − a)/a) × 100]

Sample	Name of the Pathogens	Inhibition zone diameter (mm) for AgNPs	Inhibition zone diameter (mm) for Streptomycin (AB)			Inhibition zone diameter (mm) for Ciprofloxacin (AB)		
			Only AB (a)	AB+ AgNPs (b)	FI %	Only AB (a)	AB+ AgNPs (b)	FI %
AgNPs	*B. subtilis*	21.000 ± 0.577	24.000 ± 0.577	30.000^{af} ± 0.577	25	23.333^{g} ± 0.333	30.333^{a} ± 0.333	30
	S. mutans	20.333^{d} ± 0.333	23.000^{be} ± 0.577	30.333^{f} ± 0.333	31.89	23.000^{bg} ± 0.577	28.667^{h} ± 0.882	24.64
	E. coli	20.000^{d} ± 0.577	22.667 ± 0.333	31.333 ± 0.667	38.24	23.000^{g} ± 0.577	26.667 ± 0.882	15.95
	S. typhimurium	19.000 ± 0.577	23.333^{e} ± 0.333	28.000^{c} ± 0.577	20	22.667 ± 0.333	28.333^{ch} ± 1.202	5.67

The data are displayed as mean ± standard error according to Tukey (HSD) test (P <0.05)

5. CONCLUSION

The biosynthesis of silver nanoparticles from different parts of plant is low cost, safe, environmentally friendly, less time consuming, and it provides effective satisfactory results without any hazardous chemicals involved. In the present study, AgNPs were successfully synthesized through the green technique at normal room temperature. The SEM studies confirmed that the concentration of the fruit epicarp extract is highly efficient in controlling the shape and size of AgNPs structures. TGA was detecting the steady weight loss due to desorption of its bioorganic compounds with increasing temperature. The synthesized AgNPs are lysis the cell wall integrity against pathogenic bacteria and few crop fungi. The applications of AgNPs and antibiotics together improved its efficiency to reduce the dose and also its cost. These results not only provide a new approach for integrative control of plant pathogens but also reduce or avoid the use of various drugs. From the application point of view, these AgNPs could be used as biofungicide for sustainable agriculture and biomedical use against human pathogenic bacteria in future studies.

6. FUNDING ACKNOWLEDGMENT [a9]

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7. AUTHORS CONTRIBUTION STATEMENT [a10]

Swapan Kumar Chowdhury designed the whole study including sample collection, antibacterial assay, antifungal assay, synergistic effect at Department of Botany, Sreegopal Banerjee College and prepared the manuscript. Nayan Roy conducted chemical analysis, synthesis of AgNPs, Characterization and prepared contribution part of manuscript. Indrani Mukherjee prepared the part of the manuscript. All the authors read and approved the final version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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OTHER COMMENTS

1. CONTENT IN THE SUB HEADING

- No subheadings should have very less content. At least ensure that all the subheadings should at least have 60 words.

2. FOR PLANT OR PHYTO CHEMICAL STUDY OR ANY RELEVANT STUDY

- For plant or phyto chemical study or any relevant study, plant material should be authenticated by suitable botanist or Pharmacognosist or phyto chemistry
- Authentication done by ?whom ?? (For Plant)
- Example

> Street Market, Coimbatore, Tamil Nadu. The selected plant *L. acidissima*. L and fruits were authenticated by Dr. V. Sampath Kumar, Scientist 'D'-in-charge, Botanical Survey of India, Southern Regional Centre, Coimbatore

3. For studies on specific animals

- For studies on specific animals such as fishes, nematodes, insects etc, kindly provide authentication by zoologist for their zoological name.

4. Ethical Committee Approval for Animals:

- Kindly ensure that you include ethical committee approval for your animal study with registration or reference number. See the following examples,
- **Examples:**

> The animal experiment was carried as per the instructions approved by the Ethics Committee of the Institute (CPCSEA Reg No.203/2017).

> All animal experimental procedures of this study were approved by the Human and Animal Research Ethics Committee of Shahid Beheshti University of Medical Sciences (ethical code: IR.SBMU.MSP.REC.1397.515). This study was accomplished with respect to the guidelines of the Specific National Ethics for Biochemical Research issued by the Research and Technology Deputy of the Ministry of Health and Medical Education (MOHME) of Iran (issued 2005).

5. Ethical committee approval for the Patient/human testing

- If your paper is related to patients or human testing , kindly include the Institutional permission statement and / or Human Ethical approval committee reference number for your study in the materials and methods. Mention which protocol was followed (Helinsky declaration or any other) for conduction of the study .Also ensure and include appropriate sentence for getting a written patients consent for this study. See some of the following examples,

- **Examples**

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Naresuan University Institute Review Board (IRB#566/59 and COA No.573/2016). Written consent was taken from the patients/individuals for participating in the study

Informed consent from the patients and ethical clearances from the committee was taken with the IRB No. 2016/P/PROS/76. All procedures performed in the study were conducted in accordance with the ethical standards given in 1964 Declaration of Helsinki, as revised in 2013.

The pilot study was reviewed and approved by the Human Research Ethics Committee of La Trobe University (approval number HEC 17-073), and permission to run the pilot study at Warringal Private Hospital was provided by the Director of Clinical Services. All participants involved provided informed written consent to participate. Finally, the study was registered to the Australian New Zealand Clinical Trials Registry (ACTRN12620000353998).

The study protocol was approved by the Ethics Committee of the University Medical Center Freiburg and the data security official. Patients gave written consent to use their routinely collected data for scientific purposes. Regulations of the European Data Protection Directive [Ref ?] were followed. The study was conducted according to the Declaration of Helsinki [Ref ?]

This study was conducted in accordance with the Declaration of Helsinki of the International Conference on Harmonization, and the laws and regulations of UK. The protocol was approved by local ethics committees, with ref number UK 7787656/DF 002/ dtd 22aug 2018

6. INCLUSION AND EXCLUSION CRITERIA

- For patients or treatments using humans you need to provide INCLUSION CRITERIA and EXCLUSION CRITERIA
- The following link will give some idea about inclusion and exclusion criteria,
  - https://en.wikipedia.org/wiki/Inclusion_and_exclusion_criteria
  - http://researcharticles.com/index.php/inclusion-and-exclusion-criteria-in-research/
  - https://libguides.city.ac.uk/postgraduate_research/criteria
  - https://media.tghn.org/articles/trialprotocoltool/SOURCE/Checklist/StudyPop/Inclusion%20and%20Exclusion.html
- **Example**

Inclusion criteria	Exclusion criteria
Age 18 years and older African American (qualitative only)	Unable to breastfeed due to illness or delivery complications Taking breastfeeding-contraindicated medications or substances Diagnosis of human immunodeficiency virus Department of Social Services involvement Non-English speaking
38 weeks' gestation and older	Admitted to the neonatal intensive care unit Congenital abnormalities that prevented breastfeeding Died

Inclusion criteria	Exclusion criteria
Patients willing to participate in the study	Patients allergic to NSAIDs or opioids
Patients of both genders above 18 years	Pregnant women
Patients who have undergone orthopedic surgery	Patients with known alcohol or drug addiction or abuse
Patients weighing 50 to 80 kg	Patients receiving any other NSAIDs (except for the study medication)
Patients with pain intensity at rest of at least 6 cm on a horizontal 10 cm visual analogue scale (VAS)	Patients receiving CNS depressants or warfarin

INCLUSION CRITERIA

- Those admitted for treatment of hypertension an associated complications as an inpatient.
- Freshly diagnosed as being hypertensive
- Patients of either sex and age above 18 years

EXCLUSION CRITERIA

- Hypertensive patients less than 18 years of age.
- Pregnant and lactating women.
- Patients having a mental illness.
- Patients who are not willing to give informed consent.

Patient inclusion and exclusion criteria

Inclusion Criteria	Exclusion criteria
Age 25 to 65	psychological disorders
Clinical diagnosis of androgenetic alopecia and grading with Hamilton scoring	dermatitis or any dermatosis of the scalp
Good general health without any other pathology of the scalp	chronic metabolic disorders, immunodeficiencies, allergies
Patients willing to return for follow up	patients not willing to return for follow up, or with reduced therapeutic compliance
Informed consent	jobs where hygiene could not be guaranteed and maintained

A total of 2,145 individuals who had experienced hospitalization within the last year were selected from the data. Those who had no caregiving records ($n = 30$) or hospital admission cost records ($n = 286$) were excluded. Of those who had fully answered the survey items, inpatient service users younger than 65 years old ($n = 1,008$) and those who had been admitted to the hospital for cosmetic surgery ($n = 2$) were also excluded. Therefore, a total of 819 elderly inpatients aged more than 65 years were included in the analysis

7. Placement of Tables/Figures/graphs at appropriate places

- All figures should be clear (not less than 300 dpi)
- Place all your tables/figures/graphs at or nearby the places were you are explaining or mentioning them .

8. Number of References

- There should be minimum 25 references and atleast 5 references should be of recent references

9. Discussion:

- Each and every sentences mentioning any earlier studies for discussing for your results should have respective reference number citation. Results should be discussed in support of citing references . Try to cite many references in support of your result interpretations from your result. Ensure that you have atleast 15 references cited in the discussion . See the following example