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PHYTO-MEDIATED SYNTHESIS OF SILVER NANOPARTICLES FROM *ANNONA MURICATA* FRUIT EXTRACT, ASSESSMENT OF THEIR BIOMEDICAL AND PHOTOCATALYTIC POTENTIAL

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Keywords:

Biosynthesis, Silver nanoparticles, Annona muricata, FTIR, Antioxidant potential, Photocatalytic activity

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ABSTRACT: Plant extract-mediated biosynthesis of nanoparticles has proven to be a novel, rapid, cost-effective, non-toxic and eco-friendly method. For the first time, biological material in the form of aqueous extract of *Annona muricata* fruit (AMF) was successfully utilized for the synthesis of silver nanoparticles (AgNPs). The various parameters such as metal ion concentration, volume of the extract, temperature, reaction time and pH influencing nanoparticle synthesis have also been optimized. The structural and elemental components of the synthesized AgNPs were characterized. The UV-vis spectra gave surface plasmon resonance peak at 421nm. Fourier transform infrared spectroscopy (FTIR) revealed capping of nanoparticles with plant constituents. X-ray diffraction (XRD) demonstrated a face-centered crystalline nature with an average size of 19 nm. Field Emission scanning electron microscopy (FESEM) showed their spherical shape and the presence of elemental silver were determined by Energy Dispersive X-ray spectroscopy (EDX). Further, assessment of the antioxidant activity, total phenolic content and photocatalytic property of biogenic silver nanoparticles attest to their utility for several biomedical and industrial applications.

INTRODUCTION: Nanotechnology has attracted profound scientific interest worldwide during recent times due to its wider scope for applications in biology and medicine. Nanoparticles are clusters of atoms in the size range of 1-100 nm. Metal nanoparticles such as Ag, Au, Pt and Pd are being intensely studied due to their unique optical, electrical, catalytic properties amenable to biological tagging and pharmaceutical applications [1-2]. Among these, silver nanoparticles (AgNPs) have found extensive use in textiles, cosmetics and food industries [3].

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More importantly, in the medical field, AgNPs are being increasingly employed in wound dressings, surgical instruments, prosthetic- therapeutic devices and for anticancer studies including targeted drug delivery [4, 5].

A variety of techniques of AgNPs synthesis rely on chemical reduction using sodium borohydride, citrate, ascorbate and hydrazine as reducing agents; other chemical methods include ion sputtering, sol-gel synthesis, electrolysis and spray-pyrolysis [6]. Physical methods of synthesis involve vapour condensation and arc-discharge [7-9].

The AgNPs synthesized by any or all of the above mentioned techniques, however, have attracted criticism as these involve multistep reactions requiring high energy consumption but with poor material conversions. Overall, the hazardous nature of the lingering reactants and the products very

often make purification of the nanoparticles cumbersome, non-ecofriendly and expensive. Biological systems offer an attractive option which is relatively simple, rapid, cheap and more importantly, environment-friendly and thereby overcomes most of the aforementioned issues associated with chemical and physical synthesis of nanoparticles [10].

Over the past decade a variety of microorganisms such as bacteria [11-13], fungi [14-16] and yeast [17] have been used to synthesize AgNPs. Among the various biological methods of AgNPs synthesis, microbe-mediated synthesis is not feasible industrially as it requires maintenance of sterile conditions. The use of plant extracts for silver nanosynthesis in comparison to microbial systems is gaining momemtum as it eliminates elaborate culture maintenance rituals followed by multiple purification steps [18]. A great majority of studies have been carried out employing various plant parts such as (i) leaf extracts of *Vinca rosea* [19], *Plukenetia volubilis* [20], *Mukia scabrella* [21], *Ocimum sanctum* [22], *Mangifera indica* [23], *Hibiscus cannabinus* [24], *Acalypha indica* [25], (ii) seed extracts of *Macrotyloma uniflorum* [26], *Jatropha curca* [27], *Elettaria cardamomum* [28], (iii) fruit extracts of Oak [29], *Dillenia indica* [30], *Carica papaya* [31] and (iv) bark extract of *Zizyphus xylopyrus* [32].

Annona muricata (Graviola), belonging to family *Annonaceae*, is widely distributed in most of tropical countries. It is a small upright, evergreen tree attaining heights upto 4 m. It produces heart-shaped edible fruits of about 5-20 cm in diameter, green in color with a white flesh inside called sour soup. The phytochemicals present in *Annona muricata* include carbohydrates, alkaloids, flavonoids, cardiac glycosides, saponins, tannins, phytosterol, terpenoids and proteins [33]. This fruit with its custard flavor, widely used for making various products such as juice blends, syrups, jams, jellies and ice-creams, possesses much potential in the international market.

In traditional medicine *Annona muricata* plant has been used for the treatment of cancers especially of liver and breast. It also shows antiviral, antimicrobial, wound healing and antioxidant activities [34]. The present investigation deals with green synthesis of silver nanoparticles using *A.muricata* fruit extract. The study focused on the effects of reaction parameters such as volume of the extract, metal ion concentration, influence of temperature, reaction time and pH on the overall synthesis of silver nanoparticles. Furthermore, the antioxidant property and photocatalytic activity of the biogenic AgNPs *per se* have also been evaluated by us.

MATERIALS AND METHODS:

Chemicals: Silver nitrate and other chemicals used for the experiments were purchased from Sisco Research Laboratories (SRL), Mumbai, India.

Plant material and preparation of the extract: Fruits of *A.muricata* were collected from Thrissur and Malappuram districts of Kerala state. Fresh mature fruits were cut into pieces, shade-dried and powdered finely. For extract preparation, 10g of fruit powder was weighed and mixed with 200 mL milli-Q water and boiled for 15 min. After cooling, the extract was filtered through layers of muslin cloth and whatman No.1 filter paper and stored at 4°C in the refrigerator [35].

Synthesis of silver nanoparticles: For synthesis of AgNPs, different parameters such as volume of fruit extract (2.0, 4.0, 6.0, 8.0 and 10.0 mL), concentration of metal ion (0.5, 1.0, 2.0, 3.0 and 5.0 mM), temperature (37, 60, 70, 80, 90, 95, and 100°C), pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and time (0, 30, 60, 90, 120, 150 min) were optimized. The reaction mixture was periodically monitored in the range of 200-700 nm using a UV-Vis spectrophotometer (Perkin Elmer - Lambda25) to detect the formation of silver nanoparticles as indicated by the appearance of brown color.

Characterization of Silver nanoparticles: *Annona muricata* fruit extract-mediated synthesis of silver nanoparticles (AMFAgNPs) was initially characterized spectrophotometrically using a small aliquot of the sample diluted with distilled water. For further characterization, synthesized AgNPs were purified by repeated centrifugations at 12000 rpm for 20 min. The resultant pellet was re-suspended in milli-Q water and lyophilized (Scanvac, coolsafe). To characterize the bioactive constituents present in the extract, the freeze dried powder was pelletized with potassium bromide (KBr) powder and subjected to FTIR analysis. The

spectra were recorded using a Jasco 4100 FTIR spectrophotometer in the wavelengths ranging from 4000 – 400 cm^{-1}. X-ray diffraction was performed to determine the crystal structure of nanoparticles. For this, the AMFAgNPs were coated on a glass substrate and XRD measurements were carried out using a Rigaku miniflex X-ray diffractometer instrument with Cu Kα radiation (40 kV, 15 mA) in 2θ configuration ranging from 20-70°. Debye-Scherrer's formula was applied to determine the particle size using full-width at half maximum. For morphological characterization, purified AMFAgNPs were subjected to FESEM analysis using Hitachi SEM instrument- S46600 connected to Horiba EDX system.

***In vitro* Antioxidant assays:**

Determination of total antioxidant capacity: The antioxidant capacity of AMFAgNPs was evaluated by phosphomolybdenum method according to the procedure of Prieto et al. (1999) [36]. Separate aliquots of the fruit extract and nanoparticles, at different concentrations (200,400,800,1000,1200 µg/mL) in a volume of 0.1mL were combined with 1.0 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After cooling, the absorbance of each solution was measured at 695nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity was expressed as equivalents of ascorbic acid.

Determination of the reducing power: The reducing power of AMFAgNPs was assessed essentially as described by Chen et al. (2008) [37] with slight modifications. In brief, different concentrations (200, 400, 600, 800 and 1000µg/mL) of nanoparticles and AMF extract were mixed individually with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5mL potassium hexacyanoferrate (1%,w/v). The mixture was incubated at 50°C in a water bath for 20 min and then cooled immediately. To this, 0.5 mL of trichloroacetic acid (10%, w/v) was added and centrifuged at 3000 rpm for 10 min. From the supernatant, 1.0 mL was taken and mixed with an equal volume of distilled water. Finally 0.1 mL of freshly prepared 0.1% ferric chloride was added and the absorbance was recorded at 700nm. Ascorbic acid was used as standard. An increase in the absorbance of reaction mixture indicated stronger reducing power in a dose-dependent manner.

DPPH free radical scavenging assay: DPPH free radical scavenging activity was assayed based on the method of Chen et al. (2008) [37]. One milliliter of 100 mM DPPH (in methanol) was mixed with an equal volume of AMF extract and AMFAgNPs at different concentrations (200, 400, 600, 800, 1000 µg/mL). The mixture was shaken and incubated in dark for 30 min at 25°C. Absorbance was evaluated at 517 nm. Taking ascorbic acid as the standard. Free radical scavenging activity was calculated using the formula:

DPPH scavenging activity (%) = [(Absorbance of control - Absorbance of sample)/Absorbance of control] ×100 (1)

Determination of total phenolic content (TPC): The total phenolic content was determined by Folin-Ciocalteau method [38] using gallic acid as standard. Briefly, separate aliquots of the AMF extract and AMFAgNPs (2mL) mixed with 200 µL Folin-Ciocalteau's reagent were kept for 10 min at room temperature; 300 µL of 15% Na_2CO_3 was added to the mixture. It was then mixed thoroughly and allowed to stand at 25°C for 2 h. Absorbance was measured at 765 nm using UV-visible spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE).

Nitric oxide scavenging assay: Nitric oxide (NO) radicals generated from sodium nitroprusside in aqueous interacts with oxygen to produce nitrite ions which were measured by Griess reaction reagent [39] with slight modifications. Briefly, separate aliquots (500µl) of AMFAgNPs and AMF extract at varying concentrations (200 - 1200 µg/mL) were mixed with 2mL of 10mM sodium nitroprusside followed by 0.5 mL of phosphate buffered saline. It was then incubated at 25°C for 150 minutes. From this reaction mixture, 0.5mL was added to 1.0 mL of sulfanilic acid followed by addition of 1.0 mL of Naphthyl-ethylene-diamine dichloride (0.1%w/v) and incubation at 25°C for 30 minutes. Absorbance was measured at 546nm. Nitric oxide radical scavenging assay was evaluated by Equation (1).

Photocatalytic activity by methylene blue degradation assay: The photocatalytic activity of AMFAgNPs was evaluated by monitoring degradation of methylene blue in the presence of sunlight. Initially, 5 mg of methylene blue was mixed with 500 mL double distilled water to be used as stock solution. To100 mL of the stock solution, 10 mg of AMFAgNPs was added and mixed well using a magnetic stirrer. A control set up was also maintained without nanoparticles. It was then exposed to sunlight and monitored from morning to sunset. At specific time intervals, 1-2 mL of the solution was withdrawn to evaluate degradation of the dye. Concentration of dye during degradation was calculated by the absorbance value at 665 nm.

Percentage of dye degradation was obtained by the following formula:

$$\% \text{ Decolorization} = \frac{(C_0 - C) \times 100}{C_0} \qquad (2)$$

Where C_0 is the initial concentration of the dye and C is the concentration of dye solution after photocatalytic degradation [40].

RESULTS AND DISCUSSION:

Biosynthesis of nanoparticles: The factors involved in the reduction of $AgNO_3$ to AgNPs in the presence of AMF extract were studied. The reducing environment provided by the constituents present in the extract triggered the biosynthetic process within a few minutes. This was discernible by the change in the color of the reaction mixture from colorless to yellowish brown indicating the presence of silver nanoparticles (**Fig. 1**). The characteristic absorption peak observed at 421 nm in the UV-vis spectra confirmed the excitation of surface plasmon resonance (SPR) effect and reduction of $AgNO_3$ (**Fig. 2**).

Fruit extract volume was also optimized with good absorbance obtained at 8 mL extract per 92 mL of silver nitrate solution (**Fig. 3**). Interestingly, terpenoids have been reported to be surface active molecules for nanoparticle sysnthesis in *Azadirachta indica* leaf extract [41]. It was observed that biosynthesis was influenced by both temperature and time of incubation. Effective synthesis was observed at 90°C compared to that at lower temperatures [42, 43] within about 30 minutes of incubation (**Fig. 4** and **Fig. 5).** Likewise, the minimum concentration observed for effective synthesis was found to be 1.0 mM. Conditions beyond these limits resulted in aggregation of nanoparticles as evidenced by the absorbance profiles (**Fig. 6**).

The effect of pH on biosynthesis of AgNPs was also evaluated. The absorbance value was found to increase gradually with increase in pH from 4.0 to 9.0. However, effective synthesis was found to occur at pH 7.0 beyond which aggregation was observed (**Fig. 7**). Taken together, the reaction conditions for optimized synthesis of AMFAgNPs in a 100 mL reaction mixture was found to be 8ml of AMF extract in 1mM AgNO3, pH 7, at 90°C for 30 min.

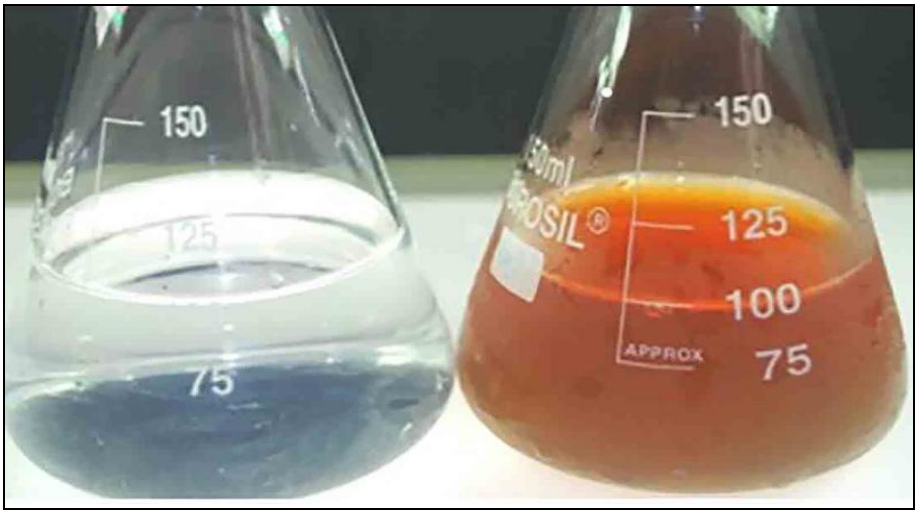


FIG. 1: AQUEOUS SOLUTION OF 1MM $AGNO_3$ (A) BEFORE ADDITION OF THE AMF EXTRACT (B) COLOR CHANGE AFTER ADDITION OF AMF EXTRACT AT 30 MIN

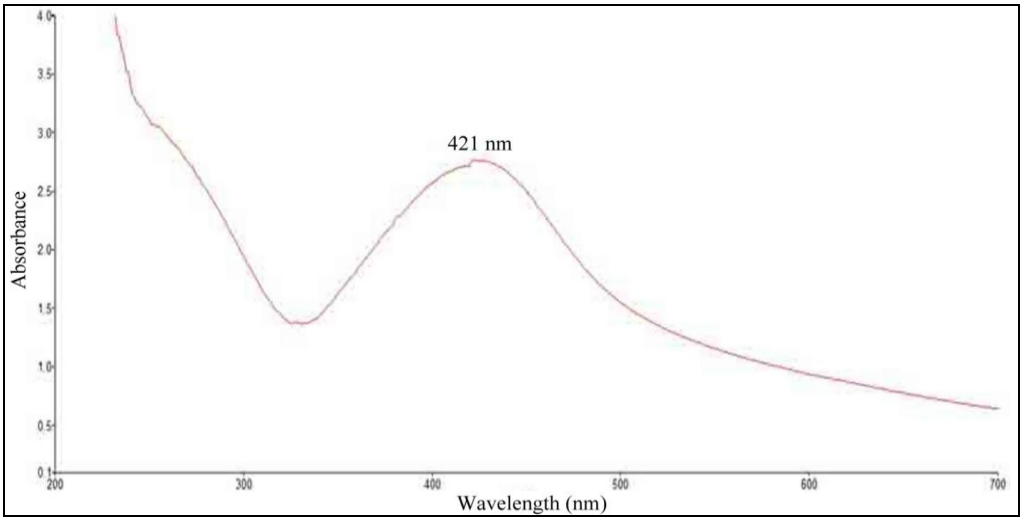


FIG. 2: UV-VISIBLE SPECTRA OF AMF EXTRACT - REDUCED AGNPS.

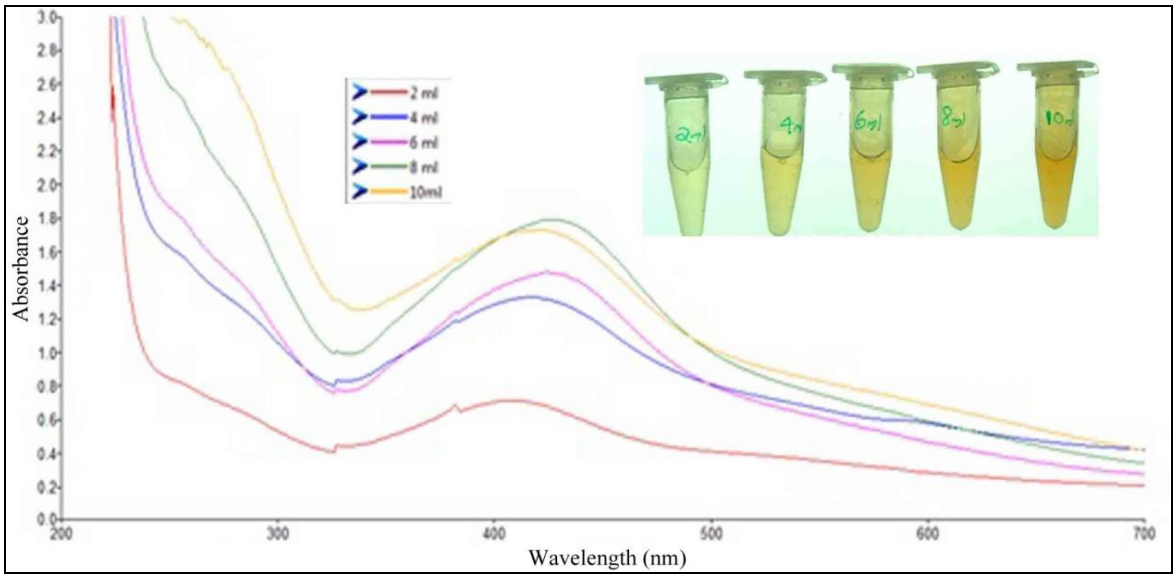


FIG. 3: UV-VIS SPECTRA OF AGNPS SYNTHESIZED IN THE PRESENCE OF VARIABLE VOLUMES (2 – 10 ML) OF AMF EXTRACT

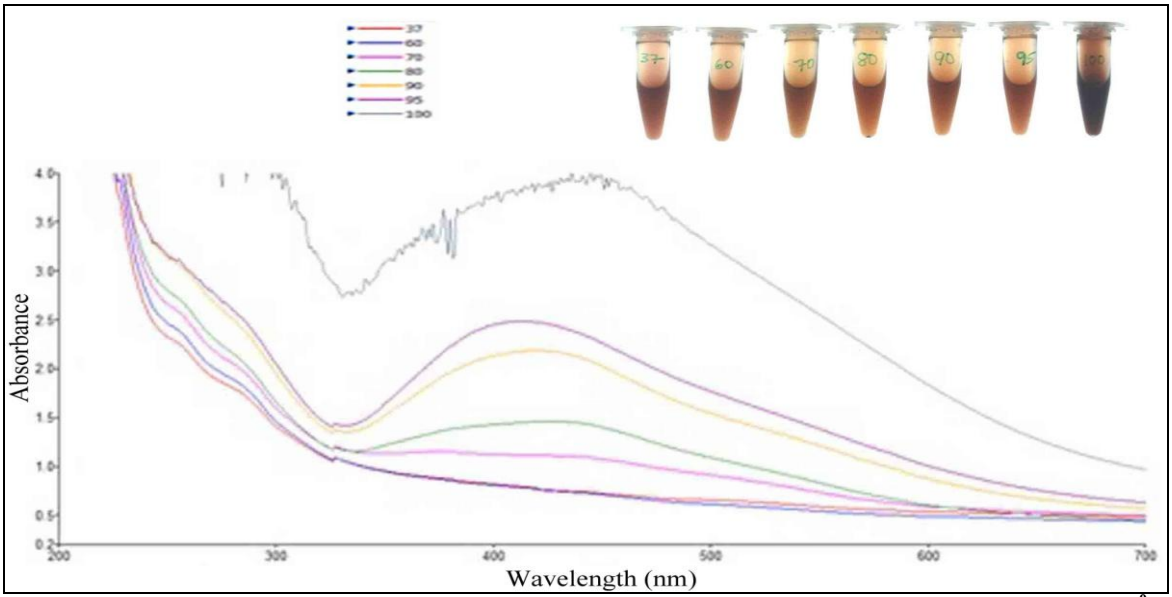


FIG. 4: UV- VIS SPECTRAL PEAK OF AMFAGNPS SYNTHESIZED AT DIFFERENT TEMPERATURES (37 – 100 ^{0}C).

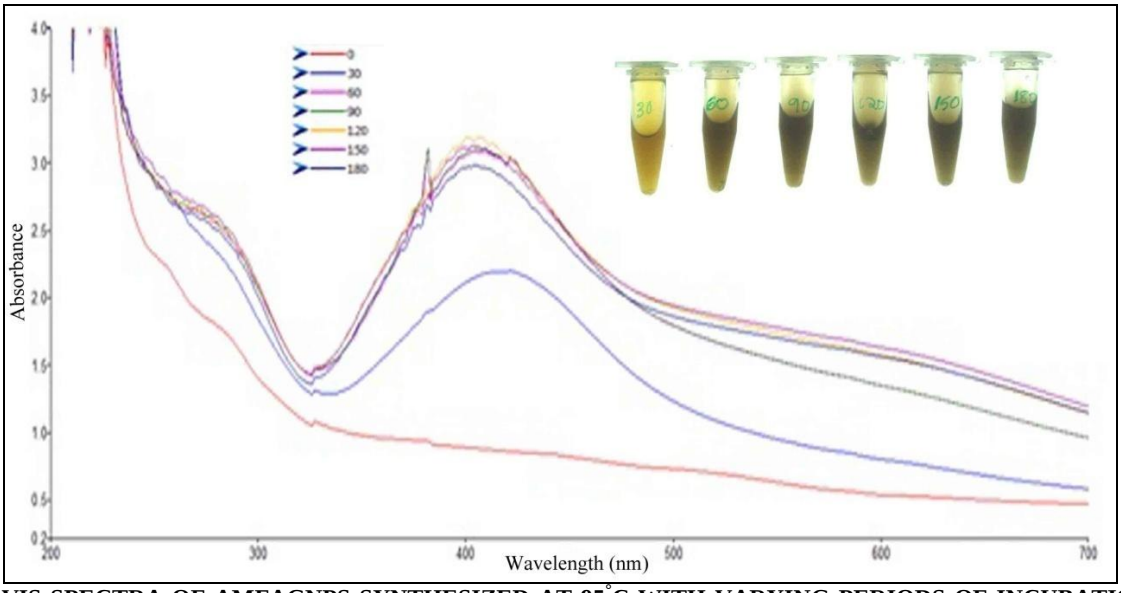


FIG. 5: UV-VIS SPECTRA OF AMFAGNPS SYNTHESIZED AT 95°C WITH VARYING PERIODS OF INCUBATION (0 – 180 MIN.)

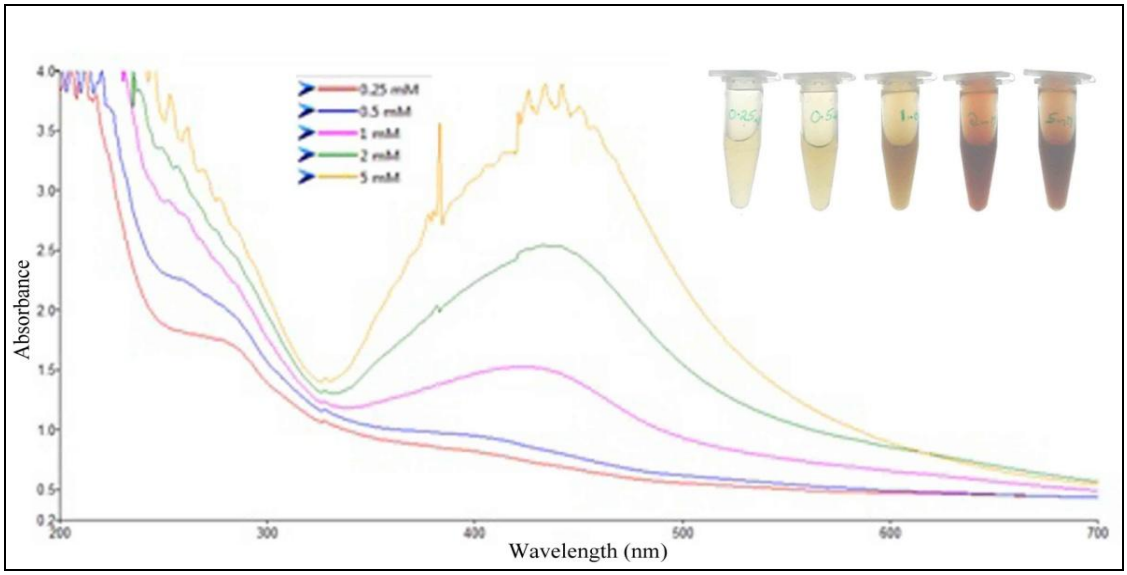


FIG. 6: UV-VISIBLE ABSORPTION SPECTRA OF AMFAGNPS AT VARYING CONCENTRATIONS OF $AGNO_3$

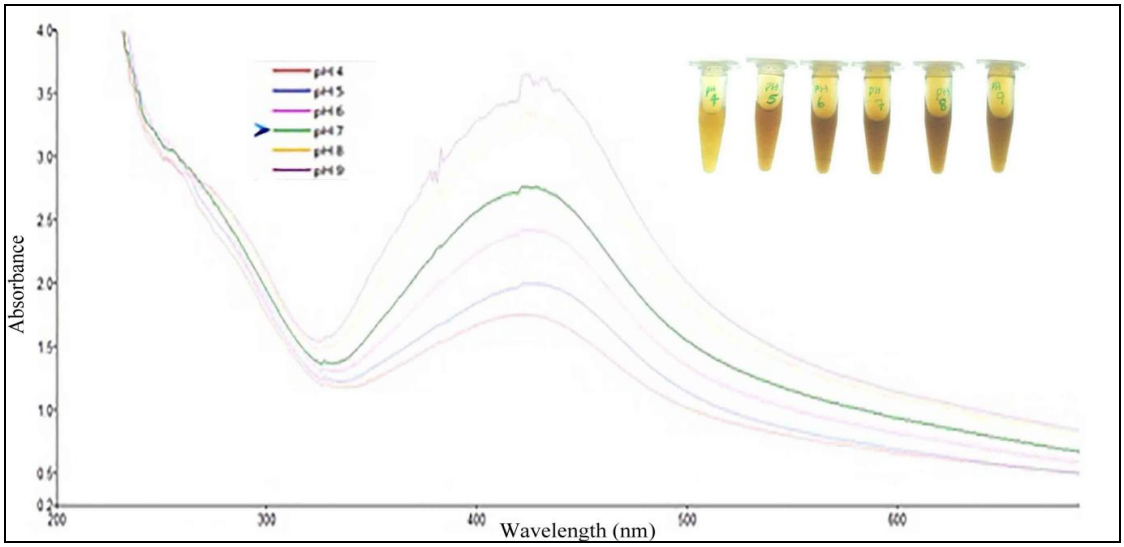


FIG. 7: UV-VISIBLE SPECTRAL PEAK OF AMFAGNPS SYNTHESIZED AT VARYING pH CONDITIONS

Characterization of Silver nanoparticles: FTIR spectroscopic analysis was carried out to examine possible bioreducing and capping agents present in the fruit extract. FTIR results of AMFAgNPs showed an intensive peak at 3650cm^{-1} corresponding to O-H stretching of alcohols and phenols. Peaks at 3422cm^{-1} indicated N-H groups. Peaks at 2923cm^{-1}, 2853cm^{-1}, 1457cm^{-1} and 672cm^{-1} represented C-H functional groups. Further, peaks at 1747cm^{-1} represented carbonyl groups (C=O), peaks at 1023cm^{-1} indicated C-O bond. Results obtained depicted that the presence of these functional groups influence the synthesis and stabilization of nanoparticles (**Fig. 8**). The crystalline structure of silver nanoparticles was confirmed using X-ray diffraction analysis (**Fig. 9**). Four intense peaks identified at 2θ values of 27.6^0, 38^o, 46^o and 63.6^o corresponded to (110), (111), (200), (220) planes of face-centered cubic phase obtained from JCPDS card 89-3722. The average crystal size was found to be 19 nm calculated by Sherrer's formula. The morphology and size of the synthesized AMFAgNPs were obtained by FESEM images (**Fig. 10).** The SEM image showed spherical and relatively uniform shape of nanoparticles with diameter varying from 30-100nm. The energy dispersive X-ray analysis (EDX) revealed the presence of elemental silver signal of the silver nanoparticles (**Fig.11).**

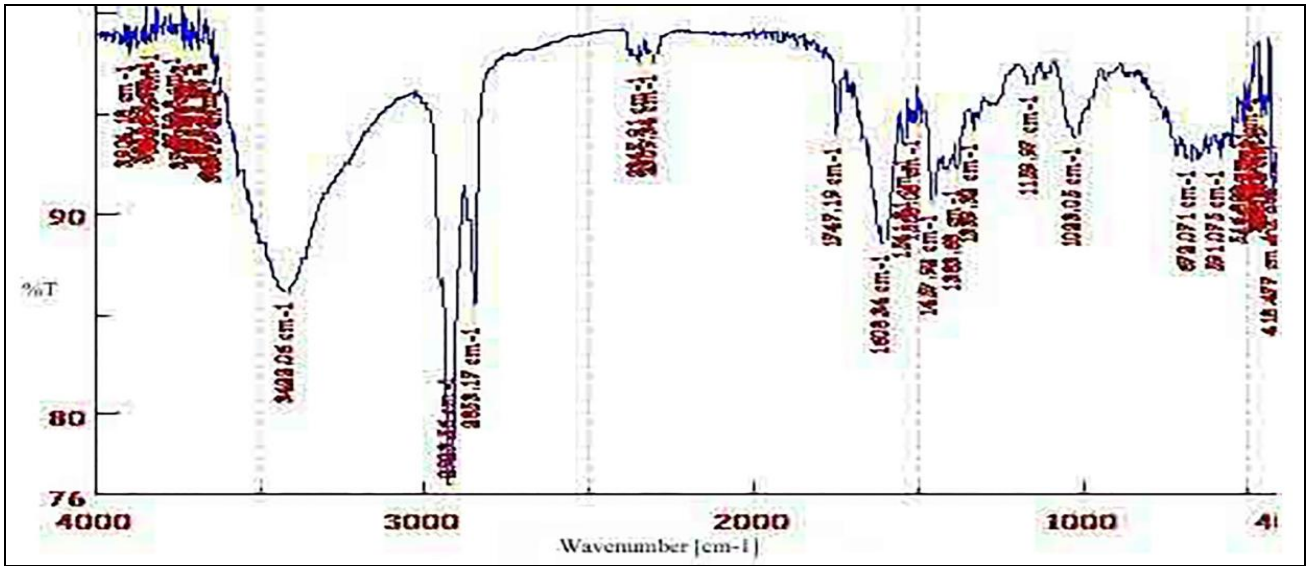


FIG. 8: THE FTIR SPECRUM OF AMFAGNPS

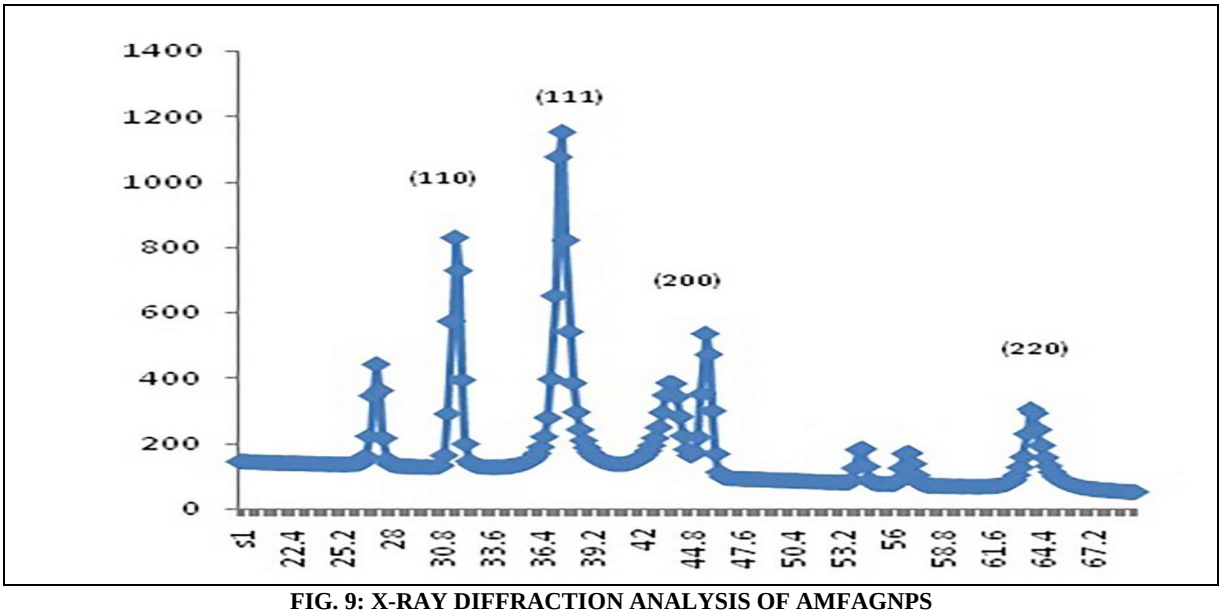


FIG. 9: X-RAY DIFFRACTION ANALYSIS OF AMFAGNPS

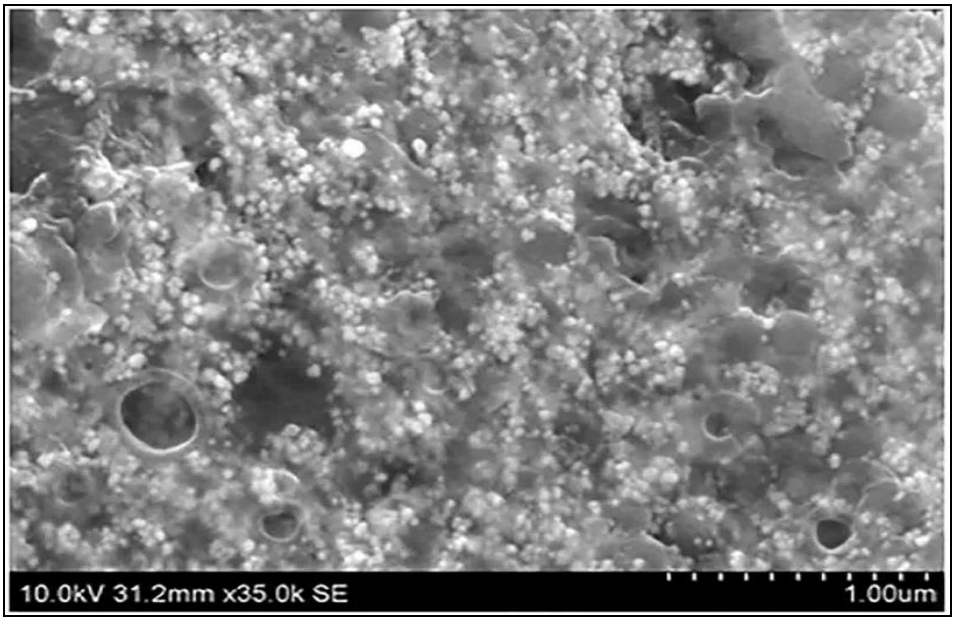


FIG.10: FESEM IMAGE OF AMFAGNPS

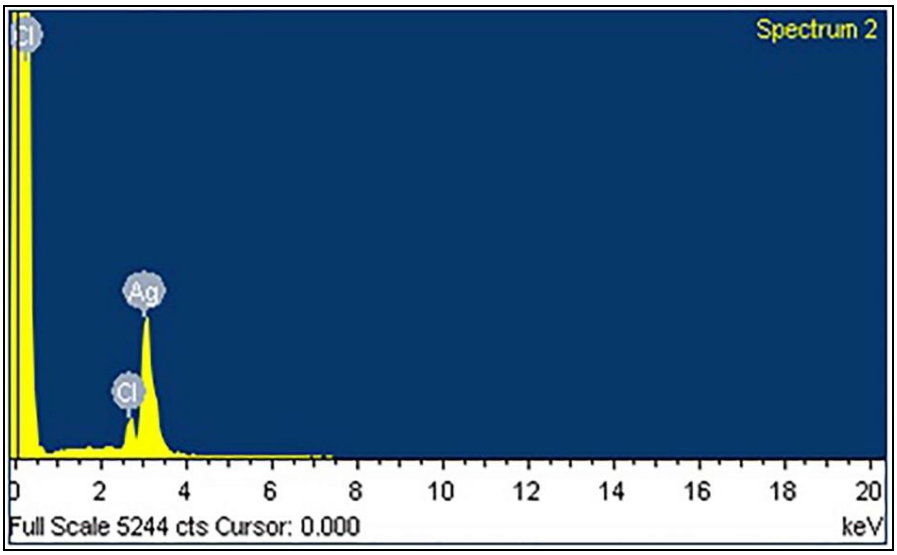


FIG. 11: EDX ANALYSIS OF AMFAGNPS

***In vitro* antioxidant assays:** The antioxidant activity of AMFAgNPs was determined by phosphomolybdenum method **(Fig. 12**). Although, AMFAgNPs showed higher levels of antioxidant activity compared to that of AMF extract alone, the values obtained with the former were observed to be far below compared to that of ascorbic acid. Interestingly, fruit extract-induced silver nanoparticles exhibited enhanced antioxidant activity attributable to their nanoscale size.

The reducing powers of the AMFAgNPs and AMFextract were also measured individually. In this assay, yellow color of the solution changed to green and blue based on the reducing power of the solution. Reducing powers of the samples were found to increase in a dose-dependent manner (**Fig. 13**). Again, AMFAgNPs showed higher reducing power compared to that observed with AMF extract alone.

The DPPH scavenging assay exhibited effective inhibitory activity of both AMFAgNPs and AMF extract compared with ascorbic acid taken as standard (**Fig. 14**). The DPPH activity was found to increase in a dose-dependent manner with AMFAgNPs displaying more than 50% inhibition at higher doses in comparison to the standard.

Likewise, the phenolic content of the nanoparticles and that of the extract as measured by Folin-Ciocalteau method revealed the presence of

significantly higher amounts (upto 95%) of phenolics attached to AMFAgNPs compared to that found in AMF extract alone (**Table 1**). Deepankar et al (2012) [44] have reported much lower amounts of adsorbed phenolics (< 10%) in *Iresine* leaf extract - induced synthesis of silver nanoparticles. Nitric oxide plays an important role in nervous, immune and cardiovascular systems as a bioregulatory molecule [45]. AMFAgNPs were found to possess good NO scavenging activity at higher concentrations - as high as 80.02% - close to the values of the standard ascorbic acid (**Fig.15**).

The antioxidant potential of plant extracts depends much on the total phenolic content, reducing power and other free radical scavenging constituents present therein. [46-48]. It is well established that natural antioxidants reduce the risk of chronic diseases including cancer and heart disease. The present studies reveal that the fruit extract-mediated silver nanoparticles exhibit antioxidant activity which qualifies them to be considered as an alternative to natural antioxidants [49].

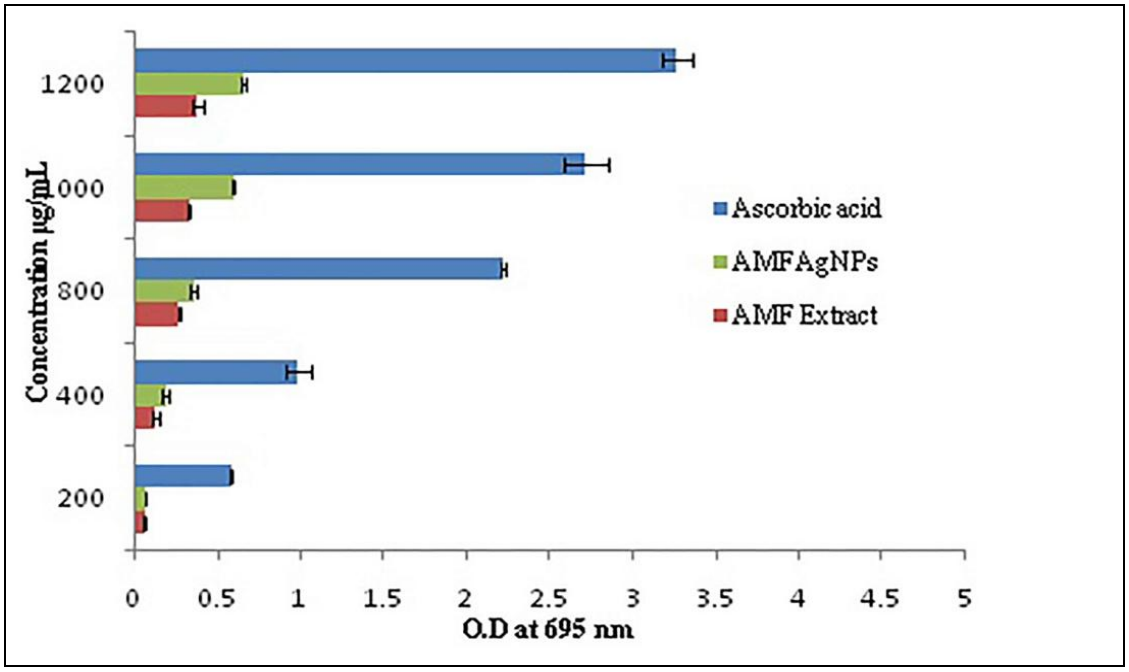


FIG. 12: TOTAL ANTIOXIDANT ASSAY OF AMFAGNPS, AMF EXTRACT AND ASCORBIC ACID

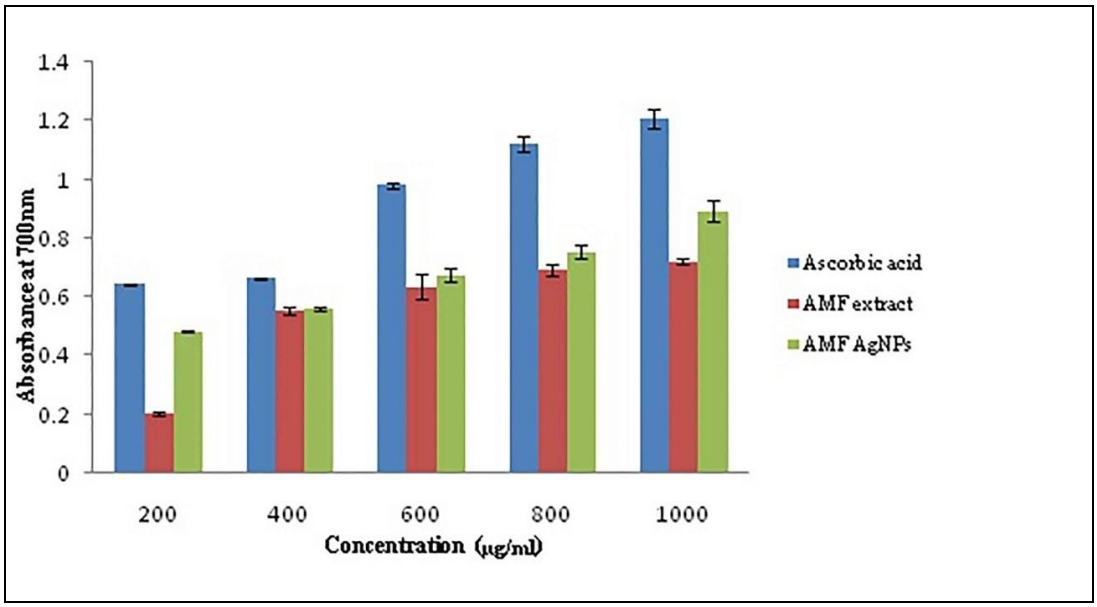


FIG. 13: REDUCING POWER ASSAY OF AMFAGNPS, AMF EXTRACT AND ASCORBIC ACID

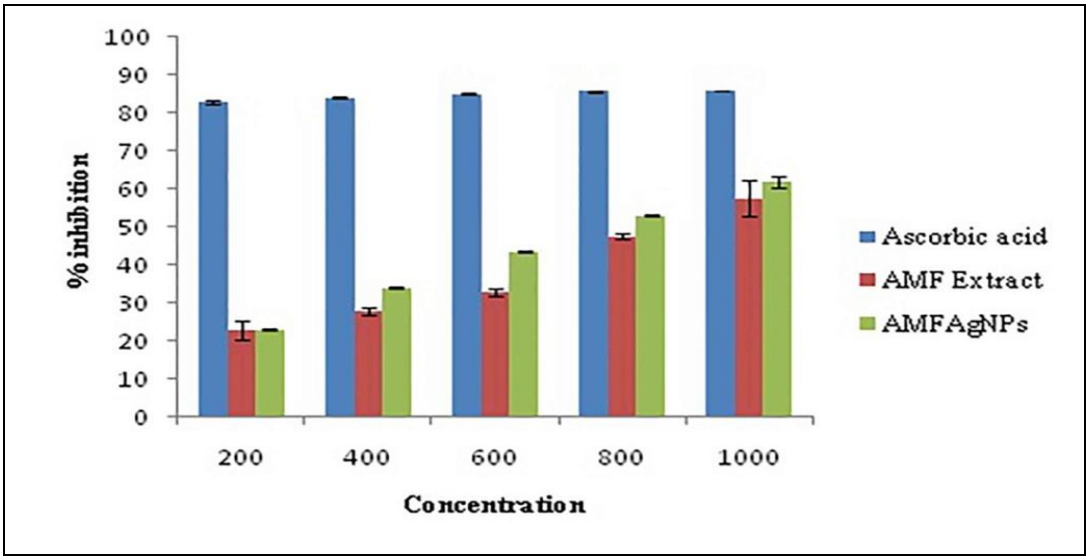


FIG.14: DPPH ASSAY OF AMFAGNPS, AMF EXTRACT AND ASCORBIC ACID

Table 1 the phenolic compounds present in the AMF extract and AMFAGNPS.

TABLE 1: THE PHENOLIC COMPOUNDS PRESENT IN THE PLANT EXTRACT AND NANOPARTICLE

Sample	Phenol content (mgGA/g sample)
AMF extract	51.00 ± 1.00
AMFAgNPs	48.5 ± 0.5

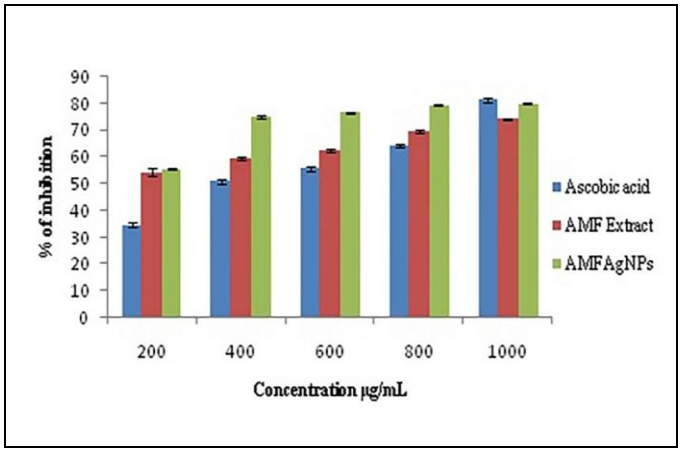
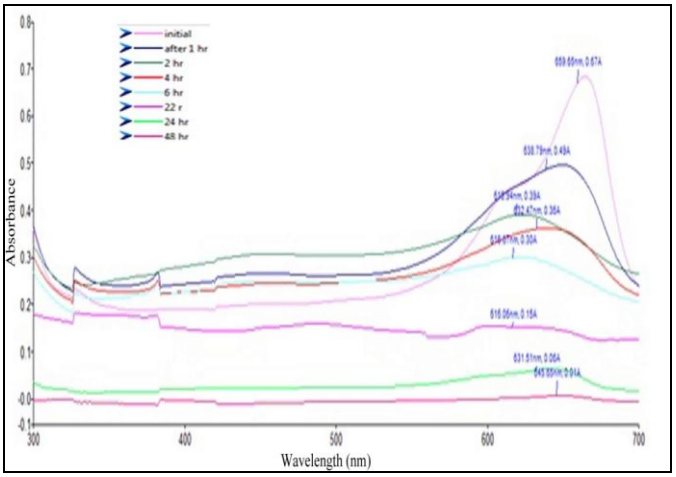


FIG.15: NITRIC OXIDE SCAVENGING OF AMFAGNPS, AMF EXTRACT AND ASCORBIC ACID

Photocatalytic degradation of dye: Dye degradation was initially identified by color change. The deep blue color of methylene blue changed to light blue in the presence of AMFAgNPs after 1 h incubation. Subsequently, on continued incubation the solution became colorless by about 48 h. The characteristic absorption peak of methylene blue at 660 nm decreased gradually with an increase in exposure time indicating almost complete photocatalytic degradation of the dye [**Fig. 16 (a)].** The degradation efficiency of AMFAgNPs was calculated as 98% at 48 hour [**Fig. 16 (b)].** This property of these biogenic nanoparticles has potential applications in textile industry and water purification plants [50].



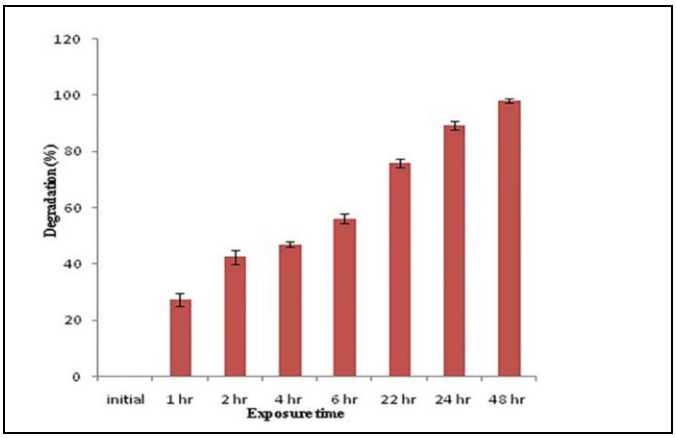


FIG.16: PHOTOCATALYTIC ACTIVITY OF AMFAGNPS BY METHYLENE BLUE DEGRADATION ASSAYS (A) ABSORPTION SPECTRA OF DYE AND (B) PERCENTAGE OF DYE DEGRADATION

CONCLUSIONS: Green synthesis of silver nanoparticles has proven to be an attractive option due to its simplicity and ecofriendliness. It is cost-effective, rapid and an energetically efficient process. Biogenic silver nanoparticles were synthesized employing *Annona muricata* fruit extract as the reducing and capping agent(s). UV-vis spectrophotometry of these nanoparticles showed a characteristic absorption peak at 430 nm. SEM and EDX techniques showed that these were spherical in shape with sizes ranging from 30- 100 nm. Phytoconstituents in the nanoparticles were confirmed by FTIR analysis and their crystalline structure validated by XRD. The significant antioxidant and photocatalytic properties of biosynthesized AMFAgNPs attest to their potential exploitability for medical and industrial purposes.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interests.

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