Alicia Tiny et al /J. Pharm. Sci. & Res. Vol. 12(8), 2020, 1124-1130

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Biogenic Synthesise of Silver nanoparticle using *Syzygium samarangense* leaf extract and its Antioxidant, Antibacterial and Drug Conjugation studies

Alicia Tiny, Jesteena Johney, Ragunathan R*
Centre for Bioscience and Nanoscience Research,
Amman Nagar, L and T Bye Pass Road, Eachanari, Coimbatore -21, Tamilnadu, India

Abstract

The present research work was done by the aim of to identify the medicinal importance of *syzygium samarangense* leaf extract. Primary study was done by antioxidant study and followed by the sample was synthesized with $AgNO_3$ solution and characterized by UV- Visible study, FTIR, SEM and XRD. The nanoparticle was used for antibacterial activity (Viable count method), conjugation with drug and the efficacy was identified, followed by drug release and DNA damage study was done.

Keywords: Antioxidant activity, Antibacterial activity, Drug Conjugation, Drug release, DNA Damage

INTRODUCTION

Wax apple is a fruit which has a wide diversity among its species. It is a tropical and nonclimacteric fruit. The botanical name of Wax apple is *Syzygiumsamarangense*. The scientific classification of *syzygium samarangense* as follows - it belongs to the kingdom Plantae, Super division - Spermatophyta (seed plants), Class - Dicotlyledons, Family- Myrtaceae, Genus- *Syzygium*, Species – *Syzygium samarangense* (Blume) Merr & Perry. *Syzygium* genus is composed of about 500 species all around the world. *S.samarangense* has many common names based on its location which are wax apple, java apple, wax jambu, rose apple (english). It is called jambusemarang, jambuklampok, in Indonesia. *S.samarangense*is called jambu air mawar in Malay, makopa in Philippines, bellfruit in Taiwan [1].

Syzygium samarangense is known to be originated from Malaysia and other south-eastern countries of Asia. It is widely distributed and cultivated throughout Malaysia, Thailand, India, Indonesia, Laos, Vietnam Taiwan. The wax apple is also grown in the Caribbean [2].

The wax apple is a semi deciduous tree. It need adequate rainfall, humidity and soil for its growth. The tree can thrive in dry seasons. The tree is commonly found along ponds, rivers and streams. It grows well at 12000ft altitude. [3] It grows upto 5-15m in height, and has a trunk of width 3-10m. Its bark is open, radialy growing crown. It is pinkish grey in color. The flowers are white to yellow in color. The morphology of the flower is 3-4cm in diameter 1.5cm long, lobes 3-5mm long, petals four. The flowers are developed in drooping panicles of 3-20 and will fall off within 2-3 days of blooming. Leaving behind the future fruit which will ripen in 2 -3 months [4]. The fruit of wax apple is round and oval in shape. The fruit is a berry, crowned with fleshy calyx with incurved lobes, 3.5-5.5cm x 4.5-5.5cm. The fruit has many color varieties, pink, red, light red , sometimes it is cream or green colored. The flesh of the fruit is crunchy, aromatic, juicy and sweet- sour in taste. Ninety percent of the fruits is edible [5]. The fruit is eaten raw, salted or cooked as sauce. The fruit is good source of flavanoid, phenol and other antioxidant compounds. Wax apple fruit is believed to have many benefits for human health.

The leaf of wax apple is the chosen plant part for this paper. The leaves are asymmetrical, opposite, rounded at the base, 10-25 cm x5-12cm , coriaceous with thin margin, heavily aromatic when it is injured, petiole thick, 3-5 cm long [4]**.** The previous phytochemical analysis on the leaf revealed the presence of phytocompounds like chalcones, antho cyanidins, flavonol glycosides and elllagitannins. The volatile oils of *Syzygium samarangense* leaf extract contain a high percent of terpenoids, tannins and other such compounds [6]. It has been noted that the hexane and ethanolic extracts of *S.samarangense* leaves displayed good immuno-stimulant activity. The ehtanolic extract of the leaves exhibited high antibacterial activity. In traditional medicine the leaves of wax apple is used as an astringent to diagnose fever and stop diarrhea. Powdered leaves is used to treat cracked tongue.

Plants have been used for its medicinal property since time memorial, Wax apple (*Syzygium samarangense)* is one such plant that has many medicinal property. Wax apple has antioxidant property and hence can be used to treat humans having oxidative stress related health issues such as diabetes. Plant extract is reported to show antidiabetic, antihyperglycemic anticancer, immunomodulatory activity [7]. In this paper we have chosen to work with the leaf extract of *Syzygiumsamarangense* to identify its antibacterial antibacterial activity by observing its total viable count method.

Nanotechnology is one of the latest and most rapidly evolving field in interdisciplinary scientific research. It has become the crucial and fascinating aspect in the modern scientific world. The present study was aimed on the synthesis of silver nanoparticle using leaf extract of *Syzygium samarangense* and its characterization by UV-visible spectrophotometer, FTIR, SEM and XRD. To identify the antibacterial property using viable count method, Phytochemical analysis and antioxidant activity of the leaf extract also done to identify the bioactive compounds. This is followed by drug conjugation, drug releasing capacity, DNA damage study also done to

finalize the expediency of the *Syzygium samarangense* leaf extract.

MATERIALS AND METHOD

Collection of plant material

Syzygium samarangense (Wax apple) leaves were collected from Ayyanthole, Thrissur district, Kerala, India. About 20- 30 leave samples were collected. The collected leaf sample was dried under the shade of sun light for 2- 4 days.

Preparation of leaf extract

1 gram of the dried leaves was grounded to paste with the help of mortar and pestle. The powdered leaf was mixed with 20ml of distilled water and was kept in shaker overnight in the condition ofat 40ºC, 60-70 rpm. The extract was then filtered using Whatman no.1 filter paper to remove the macromolecules, and the extract was used for further study.

Phytochemical analysis

The qualitative phytochemicals analysis of the leaf extract of *Syzygium samarangense* were performed to confirm the presence or absence of phytohormones (alkaloids, terpenoids, phenol, sugar, saponins, flavonoids, quinines, protein, steroid) according to the procedure of [8].

Antioxidant activity

Antioxidant activity of the *Syzygium samarangense* was characterized with mainly three methods. ie; total antioxidant activity, SOD and with H_2O_2 activity. The methods used was given below;

Total antioxidant test [9]

The antioxidant activity was tested by phosphomolybdenum method. The phosphomolybdate reaction mixture was prepared by adding 1ml 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. 1ml of plant extract was mixed with 1ml of reaction mixture (given above) in a test tube, and mixed well. The mixture was then incubated at 50ºC for 90min. After incubation the test tube was cooled down to room temperature. The absorbance was measured at 695nm in UV spectrophotometer (Microprocessor Labtronics LT-291). Ascorbic acid was used as positive reference standard and mg/g (20, 40, 60, 80, 100 mg/ml) of total antioxidant was calculated.

Superoxide dismutase (SOD) [10]

The Superoxide dismutase is an enzyme which catalyses the dismutation of superoxide radical to ordinary oxygen. The activity of Superoxide dismutase (SOD) was evaluated by the inhibition of the production of nitrobluetetrazolium by the SOD enzyme. 0.1ml of leaf extract sample was added to 1ml of reaction mixture I (0.075ml of 20mM L- Methionine, 1ml of 50mM PBS, 0.1ml of 50mM EDTA, 0.04ml of 10mM Hydroxyl amine hydochloride). This mixture was properly mixed and incubated at temperature of 30ºC for 5 minutes. To the incubated mixture 50µM of riboflavin was added, the sample was then exposed to 200V fluorescent light. After this treatment 1ml of reaction mixture II (1% sulphanilamide in 5% phosphoric acid) was added. The OD was measured at 543nm using UV- spectrophotometer (Microprocessor Labtronics LT-291). The activity was expressed in U SOD/ mg (10, 20, 30, 40, 50mg/ml) of protein.

Hydrogen peroxide [11, 12]

Hydrogen peroxide activity was performed by the protocol of Ruch et al., and Khan et al with slight modification. 0.1 ml of sample, and 2ml of 20mM hydrogen peroxide was mixed and followed by added ethanol, and make upto 3ml. The test tube was kept for incubation at room temperature for few minutes. The absorbance of the solution was measured at 230nm using a UV- spectrophotometer (Microprocessor Labtronics LT-291). The results were reported in mg/g (20, 40,60,80, 100 mg/ml) by using ascorbic acid as a standard.

Synthesis of leaf extract with $AgNO_3$ [13]

Silver nanoparticle was synthesized in a conical flask with 1mM $AgNO_3$ (prepared by using standard formula, MW of AgNO3 – 169.87) and equal amount of the plant extract, after adding the solution was mixed and incubated in dark room temperature for 24 hrs and followed by the incubated sample was allowed to expose under sunlight for 2-4minutes to get clear color change.

Characterization of silver nanoparticle

The primary detection of silver nanoparticle was done by visual identification of any color change of the reaction sample which was kept after overnight incubation. Followed by **UV-visible** spectroscopy measurement (300nm - 600nm) were recorded on a UV visible labtronics LT 291 spectrophotometer. F**TIR** -To identify the functional groups present in the synthesised silver nanoparticles, Fourier Transform Infrared spectroscopy (FTIR) analysis was carried out.The spectra was recorded between range 400 cm^{-1} and 4000cm^{-1}. **SEM** - The surface structure, size and shape of the sample was identified by Scanning Electron Microscope(SEM), after making a fine powder by centrifugation followed by drying. **XRD** - To understand the crystal structure of the synthesized silver nanoparticle X-ray diffraction (XRD) analysis was conducted. XRD is used to outline the crystallographic structure, preferred orientation in polycrystalline or powdered solid sample, grain size. XRD operated at 35kV and 28mA in flat plane geometry mode with each scan taking about 2 seconds. The corresponding patterns were compiled over a 2θ range of 20º to 90º.

Antibacterial activity using Viable count method

Antibacterial activity of the synthesized sample was identified using viable count method. Primarily nutrient broth was prepared by dissolving 13gm in 1000ml of distilled water and sterilized under autoclave at 121^0C for 15minutes. The sterilized sample was cooled to room temperature and transferred to test tube, marked as control, plant extract, and nanoparticle. Each tubes contain 5ml of nutrient broth and 100 µl of the *E.coli* culture, along with plant extract and nanoparticle separately (100µl each). All the tubes were incubated for overnight at 37^0C. After incubation 100µl of the sample was transferred to petriplate and followed by added 15-20 ml of the sterilized nutrient agar (prepared by dissolving 28gm in 1000ml of distilled water and autoclaving). The plate was mixed clock wise and anticlock wise, followed by incubated at 37^0C for 24hrs, the number of organism was counted.

Drug conjugation and Efficacy study [13]

Drug conjugation was done by preparing a stock solution of the drug amoxicillin. The stock solution was prepared by adding 50mg of drug (amoxicillin) in 2ml of phosphate buffer solution in an eppendorf tube, and was thoroughly shaken. From this 1ml of the drug solution and 1ml of the nanoparticle was mixed and incubated for overnight. Efficacy of the conjugated sample was confirmed by well diffusion method. Mueller Hinton Agar medium was prepared by dissolving 39g in 1000ml of distilled water, and autoclaved. The media was poured to petriplate and after solidification 70µl of *Escherichia coli* culture was swabbed. Wells were made on the agar medium using a sterile stainless corck borer and added the samples (25µl).Then the plate was kept for incubation at 37ºC for 24 hrs. Zone of inhibition was measured with a measuring scale.

Drug releasing analysis

Pretreated dialysis bag was taken and it was activated by rinsing with distilled water, followed by phosphate buffer solution. Drug coated silver nanoparticle was transferred to the bag and tightly tied the both ends. The dialysis bag was suspended in a beaker containing phosphate buffer. Time dependant release study was performed and the result was calculated spectrophotometrically (Microprocessor Labtronics LT291). The reading was taken after 2 hours followed by 24 hours of incubation. Reading was read at 254nm using spectrophotometer and the drug release was calculated by using the formula;

% of drug release = Control OD - Sample OD / Control OD X 100

DNA damage study

DNA damage is principally an alteration in the structure of DNA. DNA damage study was done to identify the ability of synthesized nanoparticle to protect the DNA from damages. Primarily *E.coli* DNA was isolated using phenol chloroform method [14] and the samples was mixed, the steps involved preparing a series of mixtures [15], mixture 1 (5µl DNA and 5µl synthesized nanoparticle), mixture 2 (5µl DNA and 5µl of leaf extract). Sample was kept for incubation at room temperature for 2-3 hours. After incubation this was loaded along with control DNA (without the addition of sample) with 2 µl of bromophenol blue dye on 0.8% agarose gel electrophoresis at 100V for 45minutes. The result was viewed on UV transilluminator to identify the damage.

RESULT AND DISCUSSION

Phytochemical analysis

The phytochemical analysis was done on *Syzygium samarangense* leaf extracts to detect the presence or absence of phytohormones. The test was confirmed by visual observation of color change in the result. The obtained results of the qualitative confirmation of the phytocompounds are given in fig (1). Alkaloids, terpenoids, phenol, saponins, and protein was present and sugar, flavanoids, quinines, and steroids are shown absence in this qualitative test.

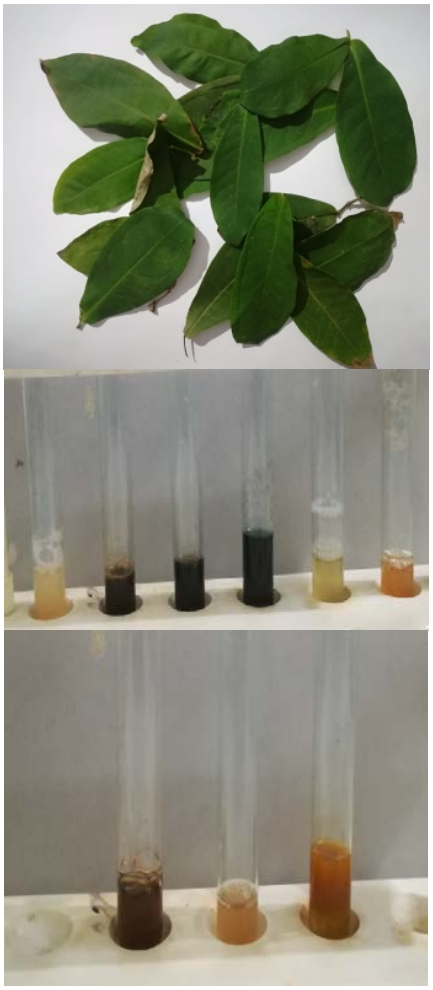

Figure 1; *Syzygium samarangense* leaf and phytochemical analysis result

[16], investigated on the topic of phytochemical and physiochemical analysis of three different types of the apples. The result shows that all the screened apple contain the bioactive compounds of flavonoids, phenols, tannin, saponin and alkaloids content. [17] Reported that these compounds are helping the pharmaceutical activities. Phytochemical like phenol and flavonoids are found to be the most responsible things for free radical scavenging activities of many medicinal plants.

Antioxidant analysis

The total antioxidant study of the leaf extract was carried out by using the phosphomolybdate method. 456 mg /gm of the total antioxidant activity was observed. Super oxide dismutes Activity was finalized by mg/ml of the protein content and was 38mg/ml. The activity of the enzyme superoxide dismutes was evaluated to determine its ability to convert superoxide radical to normal oxygen. Hydrogen peroxide activity was evaluated with the standard graph of ascorbic acid and it shows 56mg/g of the H_2O_2 activity.

In the study of [18] methanolic extract of *S.samarangense* shown 159.99 ±0.33 mg/g of the total antioxidant content. [19] done study with *S.alternifolium* extracts (hexane, ethyl acetate, water, ethanol) and which gave good antioxidant activities that are; DPPH activity, H_2O_2 and Nitric oxide activity. Higher activity was

observed in water extract compare with other solvents. There are very few research on the total antioxidant activity, SOD and also about H_2O_2 activity of the *S.samarangense*. The current research giving quantitative measurement of the *Syzygium samarangense.*[20] Free radicals are chemical constituents and are exist individually with one or more unpaired electrons. The formation of free radicals is able to transport thousands of reactions and hence cause widespread tissue damage. DNA, Lipids and proteins are subject to attack by free radicals, antioxidants may offer resistance not in favor of oxidative stress by scavenging the free radicals.

Synthesis of silver nanoparticle

During the synthesis of silver nanoparticle, the color of the test sample was light yellowish green color. The plant extract and silver nitrate solution (AgNPs) was observed after 24 hrs of incubation its turned to reddish brown. This can be considered a primary confirmation of the synthesis of silver nanoparticle.

UV-visible analysis of synthesized nanoparticle

The UV- visible spectrum, the higher plasmon peak was found at 435nm. It was observed that optimum result was obtained at equal concentration of plant leaf extract and silver nitrate solution. This test primarily gives good confirmation about the synthesis of silver nanoparticle.

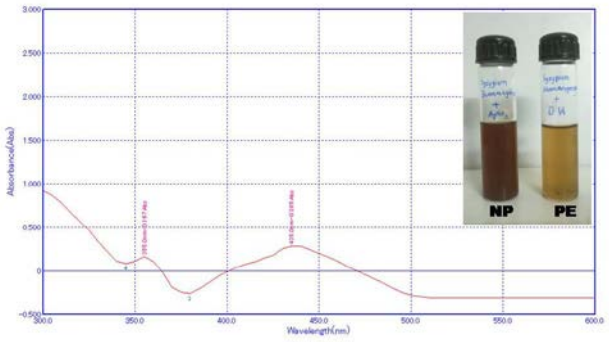

Figure 2; Result of UV-Visible spectroscopy

The figure 2; was denoting the presence of silver nanoparticle, 355nm and 435 nm were observed in this study and this was confirming the presence of nanoparticle. Inside the graph the two tubes were differentiating (based upon the color change) the conversion of nanoparticle (NP) and the plane plant extract (PE). [21] Reported that the absorption spectrum of spherical silver nanoparticles present a maximum between 400 and 450 nm when the particle size diminishes or increases, respectively.

FTIR

Fourier Transform infrared (FTIR) spectroscopy is a chemical analytical technique, determines the presence of nanoparticle, and also infer the functional group present in the nanoparticle. The FT-IR spectrum of the silver nanoparticle from the leaf extract of *Syzygium samarangense* was given in figure 3;

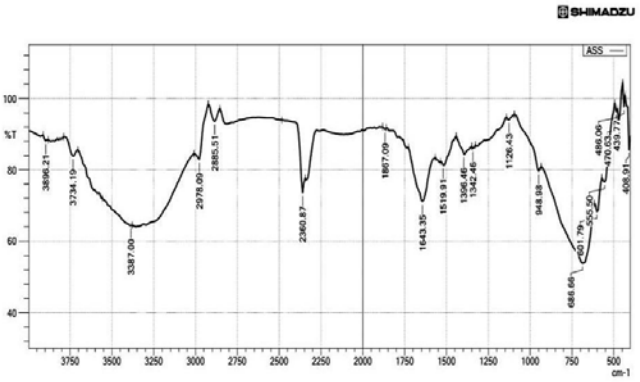

Figure 3; FTIR Analysis result

The synthesized sample showed twenty different peaks from 4000cm-1 to 400cm-1. 408 cm-1 ,439cm-1, 470cm-1,486cm-1 these lower peak are may be the presence of C-l bond,555cm-1 is C-Br group,601cm-1 and686cm-1 is corresponding to strong C-Cl group,1126cm-1 is C-OH strong stretch,1342cm-1is corresponding to NO2 stretch, 1396cm-1 is CH3 bend,1519cm-1 is corresponding to C=O amide,1643cm-1 corresponding to C=C alkene,1867cm-1,2360cm-1,2885cm-1-C-H aldehyde,2978cm-1 is carboxylic acid OH stretch group, 3387cm-1- N-H stretch, 3734cm-1 and 3896cm-1 corresponding to OH stretch water content.

[22] reported that Absorption peaks at 663.52 cm-1 assigned to C-Cl stretching corresponding to halogen compounds, 3361.02 cm-1 assigned to O-H stretching for alcohols and phenols, 1401.31 cm-1 is assigned to C-O stretching for alcohol and phenols, 1642.41 cm-1 assigned to C=O stretching for tertiary amides. IR spectroscopic study of [23], reveal that carbonyl group form proteins and amino acid residues has the stronger capacity to bind metal, could probably form a layer casing the metal nanoparticles to prevent agglomeration and thereby stabilize the extracts.

SEM

The synthesized nanoparticle was done for Scanning electron microscope analysis. The image shown the morphology was circle and cuboidal shape for the nanoparticle and the size of the nanoparticle was 25-46nm.

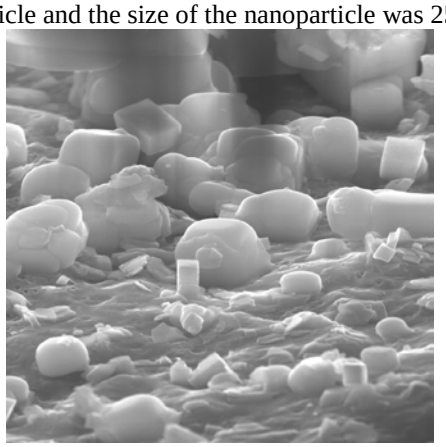

Figure 4; Scanning Electron Microscopy image

This nano particle has smooth surface also. [24], done research on the antibacterial and photo catalytic degradation efficacy of silver nanoparticle using *cordial dichotoma* plant extract, and observed the size and morphology of the sample was uniformly spread throughout the sample and shown 10-20 nm in size.

XRD

The XRD pattern of the synthesized sample was done to determine the crystalline nature of the AgNPs. The resulted high peaks were found at 28.30^0 and 40.62^0. with the height of 29.69 and 18.40.

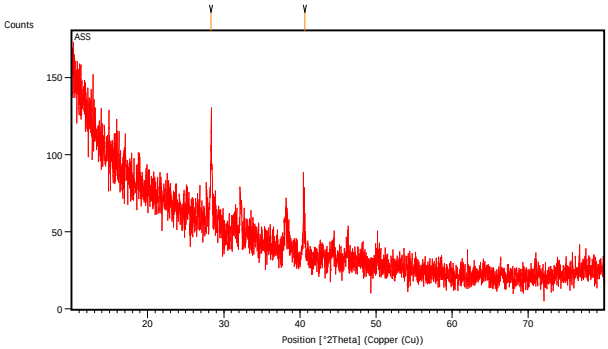

Figure 5; Result of XRD analysis

[25], reported that the XRD pattern of green synthesized AgNPs were corresponds to (111), (200), (220) and (311) of cubic crystalline silver. [26], investigated about the detailing of XRD Diffraction of green synthesised silver nanoparticle and they got Four peaks at 2θ values of 38.04, 44.13, 64.30 and 77.32 degrees corresponding to (111), (200), (220) and (311) planes of silver have been observed.

[27],**)** XRD pattern of silver nanoparticles showed five peaks, corresponding to (111), (101), (220), (202) and (311) planes of standard XRD peak of silver crystals. Two high peaks in 32.44◦ and 46.24◦ matches the standard XRD data of Ag_2CO_3 (PDF No.: 04-0783). This structural characteristic pattern confirms the AgNPs have cubic crystalline structure.

Antibacterial activity using Viable count method

This method was tested against the *E.coli* bacteria. After incubation the nanoparticle added culture plate was observed as absence of bacterial growth, compare with control *E.coli* plate and plant extract added plate. The observed result was given below;

Most of the research article detailing about the antibacterial activity using well diffusion, disk diffusion or with MIC in different method. Here we tried to finalize the viability of the organism using plating method, which is also giving a clear idea about the live and the number of organism with the treated samples. From the result which is clear that the control *E.coli* plate (marked as 2) contain too numerous growth after the incubation. Plant extract added (Marked as 1) shown less growth, in the addition of nanoparticle the *E.coli* has no growth after the incubation which is clear in the above image. So the result suggesting that the nanoparticle has good anti-bacterial activity against the *E.coli* bacteria.

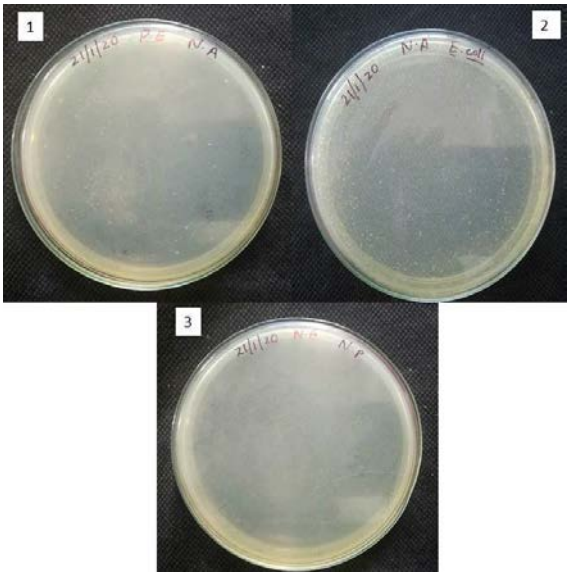

Figure 6; result of antibacterial activity using Viable count method

[28], investigated that the *Syzygium samarangense* leaf essential oil at various concentrations for antibacterial activity against Gram-negative and Gram-positive bacteria strains and the oil shown marked activity against all tested microorganisms. The leaf oil (25%) exposed noticeable activity against, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (20-25mm/50µL inhibition zone). [29], done activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. They conveyed that the extract have good activity against all the used pathogens.

Drug Conjugation and Efficacy study

In our study the conjugation was done with silver nanoparticle and amoxicillin drug. Efficacy study was done on Mueller Hinton Agar plate and which shown zone of inhibition after the incubation period. The conjugated sample shown high zone of inhibition compare with other sample used, the result was given below;

Table 1; Result of efficacy study

Efficacy study for Amoxcilin and Leaf extract	**Zone of inhibition (mm)**
Leaf extract + silver nanoparticle	5mm
Drug + Nanoparticle	8mm
Drug alone	4mm

Recently nano medicine showed a viable alternative to organic drugs particularly in the improvement of new effective antimicrobials. Because of lacking in drug approvals, increasing resistance capacity of microbes, nano conjugation are anticipated as next generation of antimicrobial [30]**.** Nanoparticles have effective drug

delivery agents to increase the bioavailability of drugs for target sites, due to the nano size it can be loaded in high amount of drug molecules this will help to reduce the side effects and toxicity. Compare with other nanoparticle silver nanoparticle has good effectiveness against cancer, nervous system and infectious disease [31].

Nanotechnology has recently emerged as a remarkable tool in the arena of biomedicine, particularly in diagnosis and drug delivery. Numerous nanoparticles conjugated with drugs have been produced and utilized against several infectious diseases caused by resistant microorganism including bacteria, fungi, parasites etc. [32]. The present study revealing the promising result of the nano conjugated sample against pathogens. From the table which is clear that the combination of drug and nanoparticle was giving more antibacterial activity against the pathogens.

Drug release analysis

Drug release of the nanoparticle can be done by two ways first is by sustained release and second by sudden release. Sudden release of drug can help body to fight pathogen immediately while sustained release of the drug can prepare the body for future attacks. The release study of the drug was done using UV- spectrophotometer. The reading after two hours of incubation showed 8.116%, while the reading taken after overnight incubation showed 74.02% release, from the result sample was effective against the pathogens.

DNA damage study

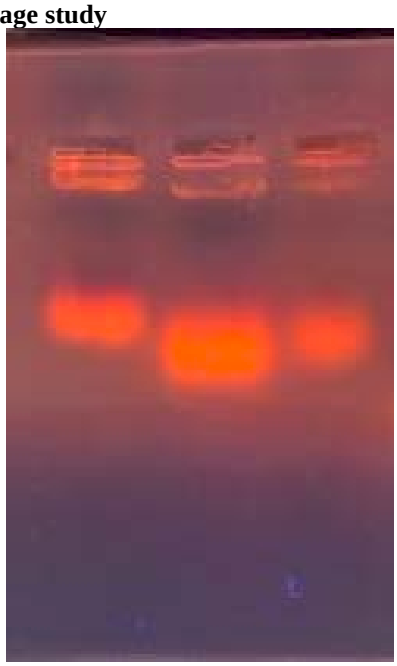

Figure 7; DNA Damage study, Lane 1 : Control DNA ; Lane 2 Plant Extract with DNA ; L3 ; Nanoparticle with DNA

[33], From the study of oxidative DNA damage from nanoparticle exposure and its application to workers health, it is revealed that all the nanoparticles are causing some type of the DNA damage or change to the human being knowingly or unknowingly. It is suggesting that should take the remedy as an extra care in all the field (medical, industry, agri) to avoid the harmful effect of the nanoparticle. On the other side from the result, which giving clear idea to inhibit the growth of the bacteria by treating with nanoparticle. The nanoparticle treated DNA was occurred high level of DNA damage compare with plant extract sample.

CONCLUSION

Syzygium samarangense plant leaf extract was used for the silver synthesizing and further it characterized by using, UV-Visible, FTIR, SEM and XRD study. Before synthesizing, the bioactive compounds were checked by qualitatively and quantitatively for a primary detailing. The synthesized sample was then used for antibacterial activity by viable count method and the nanoparticle was used for drug conjugation, drug release, and DNA damage study. All the study shown significant result and which is a promising sample against the used pathogens and maybe also to other diseases.

Acknowledgement

The authors thank Centre for Bioscience and Nanoscience Research, Eachanari, Coimbatore, Tamilnadu, India, for providing constant guidance and laboratory facilities to carry out this work.

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