Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2018.0405.41**

A STUDY ON ANTIMICROBIAL PROPERTIES OF HERBAL NANOPARTICLES OF SELECTED MANGROVE PLANTS

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ABSTRACT: Homogenous plant powder at nanoscale is the need of the hour for existing and newly emerging biomedical applications, and novel drug delivery with less side effects. Several methods are used for the synthesis of nanoparticles (NPs) such as physical, chemical, enzymatic and biological. Ball milling is one of the physical methods used for synthesis of homogeneous nanoparticles. The herbal nanoparticles were prepared from shade dried selected mangrove plant leaves i.e. *Avicennia marina, Rhizophora apiculata, and Excoecaria agallocha* of Krishna estuary by employing ball milling technique. The XRD analysis revealed that the obtained nanoparticles ranged between 14.38 to 28.70 nm. The nano size of the powdered leaf material was also confirmed by Transverse Electron Microscopy (TEM) and UV-VIS spectrophotometry. The FTIR analysis and EDS confirmed the presence of various functional groups and mineral elements present in the herbal nanoparticles. The nanoparticles with less size formed from *R. apiculata* showed maximum antibacterial and antifungal activity with a zone of inhibition of 26 mm on *Bacillus subtilis*. The present study confirms that smaller nanoparticles are found to exhibit maximum zone of inhibition when compared with larger particles.

KEYWORDS: herbal nanoparticles; mangroves; ball mill; Krishna estuary

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Peer review under responsibility of Life Science Informatics Publications

1.INTRODUCTION

The 21st century revolutionized by the development of nanotechnology and is predicted to be one of the key technologies of this century [1]. Nanoparticles are defined with a particle size between 1 and 100 nm and their size probably occupying major role in all types of industries. Because of their astonishing properties many of these nanomaterials are playing a pivotal role in optics [2, 3], electronics [4] photocatalysis [5], automotive industry [6], water and air treatment [7], fabrics [8, 9], cosmetics [10], and health products [11]. Silver, gold, zinc, copper are generally used as composite metals in preparation of nanoparticles especially in the field of pharma and medicine as drug delivery agents. Of these composite metals silver is widely used metal in a number of biological activities. Silver has been known for its antibacterial effect since ancient times in Greece, Rome, and Macedonia [12]. Nowadays, silver is used for many bactericidal applications, such as wound healing [13], water treatment [14], and flower preservation [15]. Currently the most effective application for silver nanoparticles appears to be their usage as antibacterial/antifungal agent [16, 17]. In spite of silver nanoparticles occupied key role in human health system with wide medicinal uses, several studies have evaluated that Ag-Nps accumulation inside the body may lead to an irrecoverable end to the human life [18]. At present concerns have been raised concerning the environmental impact of nanoparticles and the possible human exposure. Nanomaterial risk assessment is mainly influenced by the mobility of nanoparticles [19] along with nanoparticle size, shape, and surface modification. In addition, due to the large surface area of nanoparticles pollutants can be easily adsorbed to nanoparticles. As nanoparticles such as silver nanoparticles can be absorbed by plants or other living organisms, the particles can reach the food chain [19]. The main nanoparticle uptake possibilities into the human body were via the skin, respiratory tract and gastrointestinal tract [19]. Nanoparticles absorbed via the respiratory tract can reach the lymph stream and the blood circulation [20]. Some studies showed that nanoparticles are able to pass through the blood-brain-barrier [21] and through cell membranes [22, 23] and can thus deposit in organs and interact with biological systems. It has been shown that silver nanoparticles can induce a toxic response of different mammalian cell lines [24-28]. Cytotoxic and genotoxic effect of silver nanoparticles in human cells revealed the dysfunction of mitochondrial as well as induction of reactive oxygen species (ROS) by Ag-nanoparticles results in DNA damage and chromosomal aberrations [29]. Because silver nanoparticles are used in many application fields and previous studies showed the possible hazardous effects of these materials it is important to develop silver devoid non toxic nanoparticles. Nowadays, new discoveries have helped to develop herbal drugs that have no side effects and have high therapeutic activities [30]. Herbs have been an integral part of our therapeutic use since thousands of years, but are still under investigation. Herbal extracts were used initially as crude drugs in the form of powder, tincture, poultice and other formulations [31]. The antimicrobial activity of herbal products has already been investigated in traditional

medicines [32]. The most important properties of herbal materials are their non-hazardous nature and exert less or no side effect when compared with synthetic drugs [33]. These attracted researchers to extract and modify the active constituents from plants and evaluate their biological potential which finally led to drug discovery [34]. The knowledge and a comprehensive study on the herbal plants powders at nanoscale in the present context is essential for existing and its newly emerging different biomedical applications. Apart from most preferred "Bottom-up" process Green bio synthesis of nano particles the "Top-down" approach is employed in preparation of herbal nanoparticles. In Top-down approach, Bulk material is broken down into particles at nano scale with various lithographic techniques e.g. grinding, ball milling etc. Ball milling is one of the physical methods employed for synthesis of homogenous herbal nanoparticles. Mangroves are halophytic plants growing along the tropical and subtropical coastline in the areas where river water mixes with sea water under extreme environmental conditions such as high salinity, temperature and radiation [35, 36]. Mangroves try to adjust to continuous changing environment by synthesizing number of secondary metabolites and survive. Mangroves are known for their medicinal properties since ages and have been used in folklore medicine. Several species of mangroves produce bioactive compounds that have anti microbial activity against pathogenic strains. The secondary metabolites extracted like alkaloids, phenolics, steriods and terpenoids have been characterized from mangroves are unique to these plants and are reported to have antibacterial, antioxidant, and antifungal, anti cancer properties [37]. Green synthesis methods has been largely applied to different mangrove plants for production of nanoparticles [38] and very little is known about production of herbal nanoparticles from mangroves. Therefore the focus of the present study is to synthesize herbal leaf powders of selected mangrove plants at nano scale through ball milling and ascertain the influence of particle size on antimicrobial activity.

2. MATERIALS AND METHODS

2.1 Collection of plant material

The present study is conducted on mangroves growing at Krishna estuary located in the Krishna delta between $15°42^{II}$ – $15°55^{II}$ N and $80^{o}42^{II}$ – $81^{o}01^{II}$ E in Krishna and Guntur districts of Andhra Pradesh, India. Three mangrove plants viz. *Avicennia marina, Rhizophora apiculata, and Excoecaria agallocha* are selected for the present study.

2.1.1 Synthesis of herbal Nanoparticles

Healthy and fresh matured leaves were collected from selected three mangroves i.e. *Avicennia marina, Rhizophora apiculata, and Excoecaria agallocha* of Krishna estuary. The collected leaves were washed with tap water and double distilled water until dust is removed from surface of the leaves. The leaves are shade dried at room temperature. An electric mixer grinder was initially used to grind the shade dried leaves until coarse powder is obtained. Further the coarse powder is grinded with planetary ball mill (Fritzsch Pulverisette P6) with a steel vial of 250 ml volume. In

each vial 40 zirconium balls of 20 mm were taken. Particles were grounded in the planetary ball mill at 300 rpm for about 10hr. These samples were then characterized with SEM and TEM.

2.2 Characterization of herbal nanoparticles

2.2.1 X-Ray diffraction studies

The obtained nanoparticles were comprehensively characterized using X-ray diffraction (XRD). The powder obtained was coated onto XRD grid and analyzed for nanoparticles by using Schimadzu – 6100 X Ray Diffractometer operated at a voltage of 40 kv and current of 30 mA with cu kal radiation. The diffracted intensities were recorded from 10° to 60° of 20 angles. The size of the nanoparticles is estimated by Debye-Scherrer formula (Instrumental broadening) [39] and particle sizes are calculated for all selected plants.

$$D = 0.94 \lambda / \beta \text{ Cos } \theta$$

Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle.

2.2.2 Fourier transform infrared spectroscopy (FTIR)

The spectra of herbal nanoparticles were obtained through Fourier transform infrared spectroscopy (FTIR). FTIR studies were done using Schimatzu IR Affinity – 1S spectrophotometer. The KBr used was of IR grade (SD Fines). About 500 mg of KBr was placed into a mortar and grind it until there is no evidence of crystalinity. The KBr powder was transferred into the drying box at a temperature of 400 °C. A 10 mg of solid sample was placed into the mortar and again grind it until a fine powder is formed. One milligram of solid fine powder of sample (as per requirement of the die) and 200 mg of dry fine powder of KBr were weighed and the quantities were transferred into a mortar and mixed well with the help of a spatula. Bottom and top portion of KBr were assembled at press assembly and one of the 13 mm die with the polished surface up inside the press. The KBr sample mixture was transferred to KBr press assembly. The sample was slowly compressed in KBr press assembly at a pressure of 2000 kg/cm^2 for about 60 sec. The prepared disc was then subjected for scanning between 500-4000^{-1} cm.

2.2.3 Ultraviolet – Visible spectroscopy (UV-VIS)

The herbal nanopowder samples were dispersed in methanol and placed in a 1 cm square quartz cuvette for optical analysis at room temperature. Optical properties of the dispersed herbal nanoparticles were analyzed using UV-Vis spectrophotometer (LM-44; Perkin Elmer, Germany) operated from the UV to NIR (200-900 nm) spectral regions at a step size of 5A.

2.2.4 Scanning and Transmission electron microscopy (SEM and TEM)

Grain size and surface morphology of nanopowders were examined through TEM (CM200; Philips, Eindhoven, the Netherlands) operated at 120 kV. The average particle size was defined from the obtained image. Scanning Electron Microscopy (SEM) was used to determine size and

shape of green nanopowder. SEM analysis was carried out using Carl Zeiss Japan, model machine. Thin film of nanoparticle powder sample was prepared on carbon-coated tape by adhering small amount of dried fine powder of sample on the grid, excess sample was removed with the help of blotting paper. The film on the SEM grid was allowed to dry under a mercury lamp for 5 min. The AMT Camera System was operated at an accelerating voltage of 100 kV at 10000 X magnification.

2.2.5 EDS Observation of Nanoparticles

Energy-dispersive X-ray spectrum (EDS) analysis was carried out using high resolution scanning electron microscope (JEOL JEM 2100) to confirm the presence of elements in the herbal nanoparticles as well as to detect atomic weight of the elements.

2.3 Antimicrobial Activity

The antibacterial activity of the obtained herbal nanoparticles was carried out by agar well diffusion method. Nutrient agar (NA) was used for culturing the test bacteria. NA medium (100 ml) was sterilized at 15 lbs pressure (121°C) for 15 min, cooled and inoculated with 0.1 ml of test bacterial suspension. The inoculated plates were incubated at 30 °C and the diameter of the inhibition zone was measured after 24 h.

3. RESULTS AND DISCUSSION

3.1 Herbal nanoparticles characterization studies

3.1.1 X-ray diffraction

The X-ray diffraction profile (EDAX) for the herbal nanoparticles was analyzed (Figure 1). The EDAX pattern shows clearly that the nanoparticles were crystalline in nature. The present study confirmed the presence of nanoparticles with strong signal energy peaks in the range 2–4 keV. The size of the nanoparticles formed was calculated and *R. apiculata* recorded smaller nanoparticles 14.38 nm in size (Table 1 and Figure 1).

Table 1: XRD Data of studied Herbal nanoparticles

Plant name	Peak Position (2θ)	Avg. Crystallite size (nm)
Rhizophora apiculata	31.38	14.38
Avicennia marina	31.58	26.38
Excooecaria agallocha	31.43	28.70

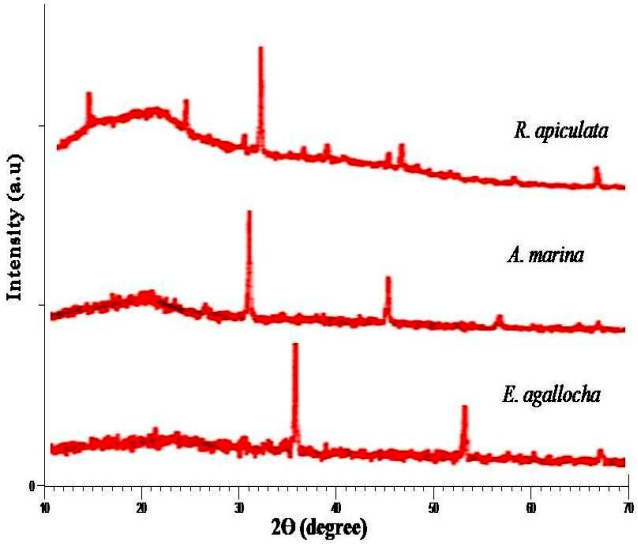


Figure 1: XRD of ball milled leaf powders of selected mangrove plants

3.1.2 FTIR

Size distribution and characterization of the herbal nanoparticles were explored further using FTIR. FTIR spectra in (Figure 2) clearly shows the existence of corresponding functional groups for alkanes, carbonyls, aromatics, alcohols, carboxylic acids, and so on in the dry-leaf nanoparticles. Table 2 shows the functional groups observed under frequencies corresponding to various functional groups. The FTIR recorded for the dry leaf powder of *R. apiculata* showed strong bands at 3287, 2889-2942, 1616, 1500, 1059-1431, 841, 707, 661 cm^{-1}. In case of *Avicennia marina* and *Excoecaria agallocha* FTIR spectra are 3333, 2909-2942, 2300, 1637, 1334, 1239, 1165, 1059, 628 and 3374, 3380, 2916, 2306, 1729, 1357, 1215-1046, 768, 602, 535 respectively.

Table 2: FTIR Spectroscopical data and functional group identification

Wave number (cm^{-1})	Functional Groups
600-800	Alkyl Halide (C-Cl)
1080-1360	Amine group (C-N)
1400-1600	Aromatic group (C=C)
1670-1820	Carbonyl group (C=O)
1800-1830	Anhydride (C=O)

3.1.3 UV-VIS

Ultraviolet-visible spectrometry was used to examine the size and shape of the nanoparticles in aqueous suspension. The UV absorption spectra data of all the herbal nanoparticles from selected mangrove plants showed U.V absorption at 223,267, 281,285, 341, 409, 663, 664 nm in *Avicennia marina*, *Rhizophora apiculata* and *Excoecaria agallocha* respectively (Figure 3).

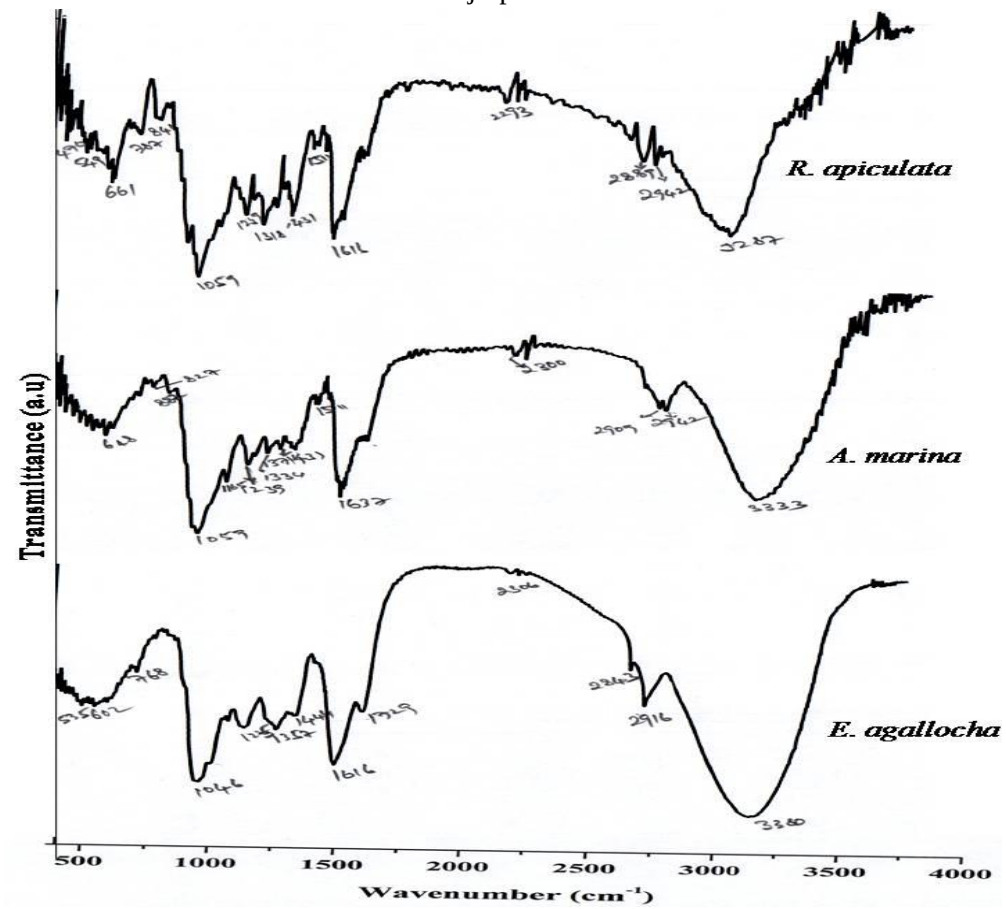


Figure 2: FTIR analysis of Herbal nano powders of selected mangrove plants

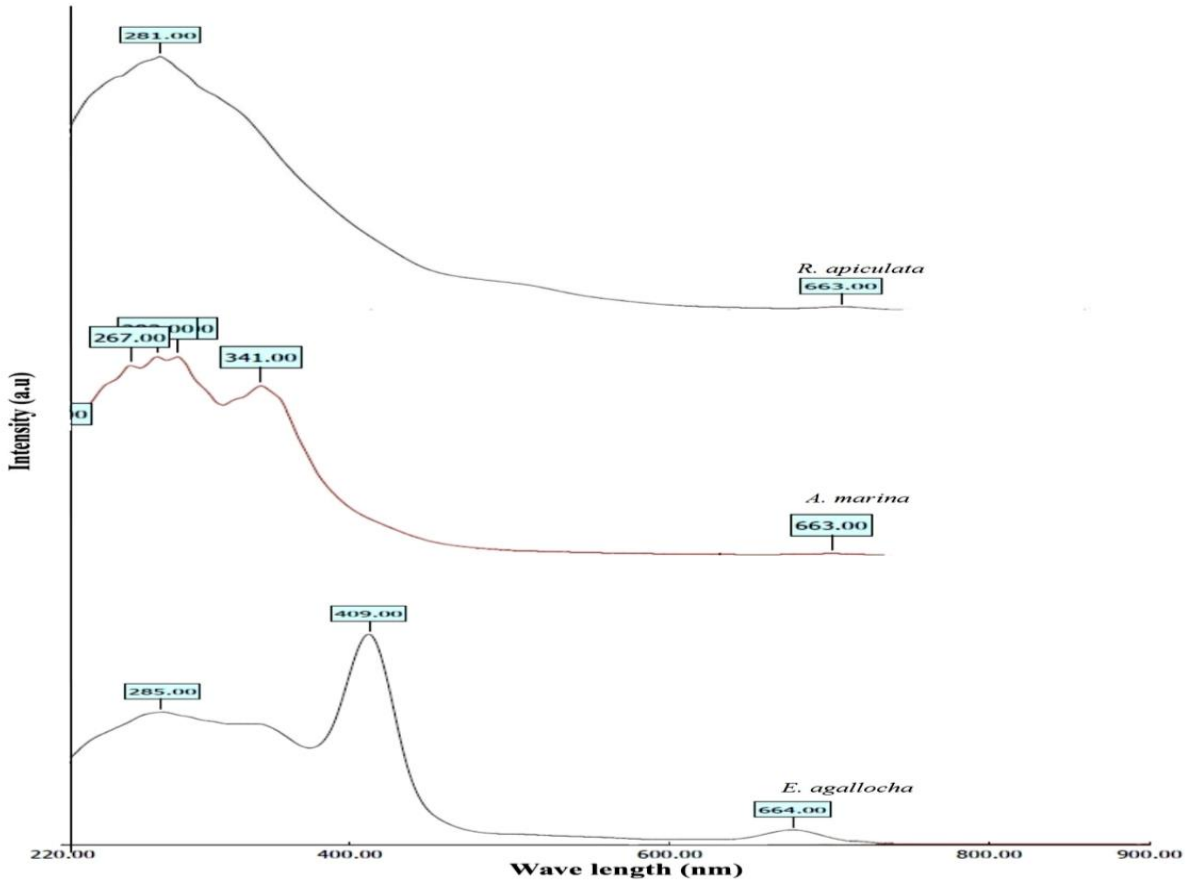


Figure 3: UV-VIS spectra of Herbal nano powders of selected mangrove plants

3.1.4 SEM

The SEM images of the herbal nanoparticles from three selected mangrove plants showed the topographical analysis of herbal nanoparticles in each plant. The nanoparticles obtained in XRD are 14.38, 26.28, and 28.70 nm size in *R. apiculata*, *A. marina*, and *E. agallocha* coordinated with the SEM sizes and the lowest particle size was observed in *R. apiculata* (43.58 nm) followed by *A. marina* (61.20 nm) and *E. agallocha* (82.10 nm). The results are in agree with that of XRD where *R. apiculata* recorded 14.38 nm of particle size.

3.1.5 TEM

The size of the obtained herbal nanoparticles was also confirmed by Transverse Electron Microscopy studies (TEM) at 200 nm range. Analysis of the nanoparticles by TEM confirmed that they were in the nano range, approximately spherical in shape (Figure 4). Most of the nanoparticles were roughly circular in shape with smooth edges. Some nanoparticles had anisotropic structures with irregular contours, as shown in Figure 4. It can be seen that most of the herbal nanoparticles in the TEM images are in close physical contact but separated by a fairly uniform inter rparticle distance.

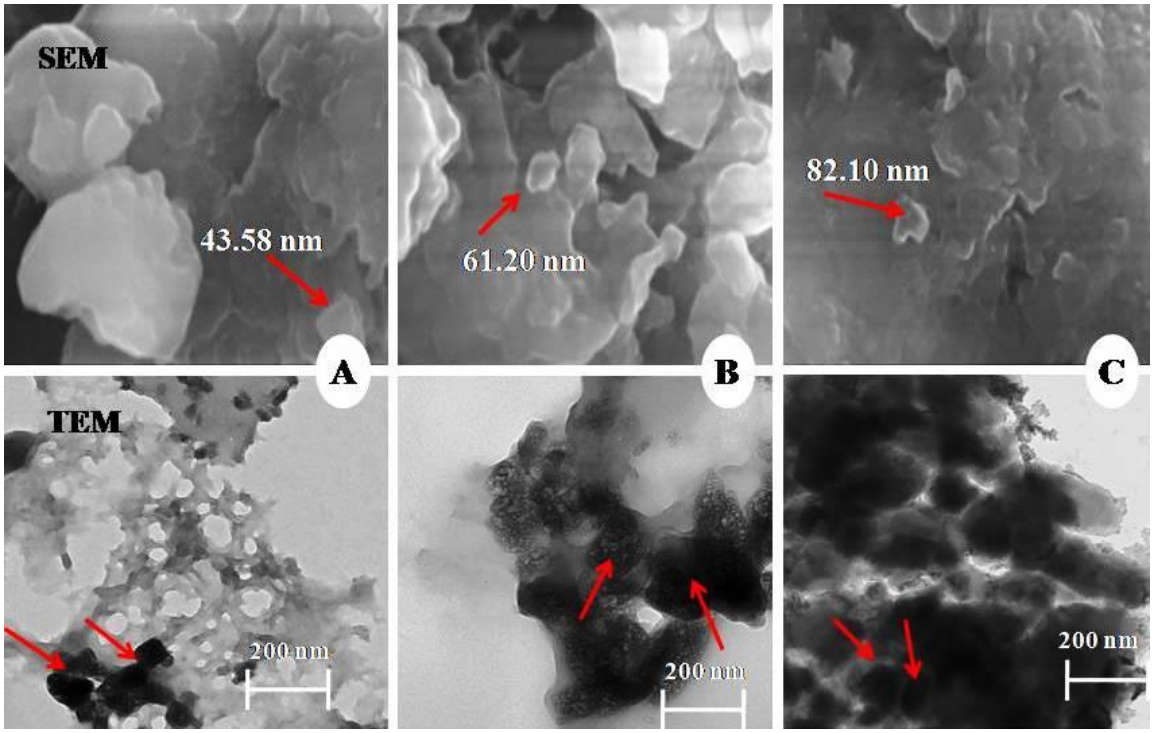
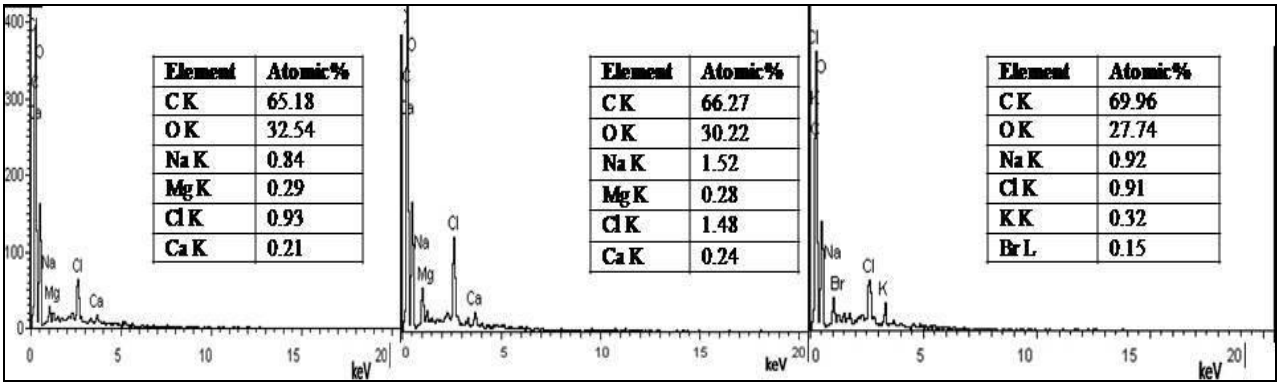


Figure 4: SEM and TEM images of a) *Avicinnea marina* b) *Rhizophora apiculata* c) *Exocoecaria agallocha*

3.1.6 EDS analysis

Analysis through EDS spectrometer confirms the presence of elements in selected mangrove leaf nanoparticles as shown in Figure 4. Identification lines for the major emission energies for carbon, oxygen, magnesium, sodium, chloride and calcium are observed which reveals the abundance of organic materials like cellulose in the prepared leaf nanopowders. Particularly the available

percentage of carbon and oxygen in present EDS confirms the presence of organic molecules (Figure 5).



a) ***Avicinnea marina*** **b)** ***Rhizophora apiculata*** **c)** ***Exocoecaria agallocha***

Figure 5: EDS analysis of herbal nanopowders

3.2 The morpho-physiological studies of plant revealed that the plant powders of R. apiculata showed lower nanoparticles size with a confined shape. These obtained nanopowder were further tested for their antimicrobial potentiality.

The herbal nanoparticles obtained from selected mangrove plants showed significant antimicrobial and antifungal activity with degrees of variation. The antimicrobial assessment corresponds to the size of the herbal nanoparticles. The maximum zone of inhibition was observed in *R. apiculata* which showed the smallest size of the nanoparticles in the selected mangrove plants. The maximum zone of inhibition was observed for *Staphylococcus aureus, Bacillus subtilis, Escheria coli* and *Candida albicans* with a maximum zone of inhibition at 150 µl of *R. apiculata* i.e. 26 mm, 23 mm, 18 mm and 23 mm respectively (Table 3 and Plate 1 and Plate 2).

Table 3: Antimicrobial activity of leaf nanoparticles by well diffusion method

Plant Name	Zone of inhibition (mm/µL)											
	S. aureus			***B. subtilis***			***E. coli***			***C. albicans***		
	50 µl	100 µl	150 µl	50 µl	100 µl	150 µl	50 µl	100 µl	150 µl	50 µl	100 µl	150 µl
R. apiculata	14	19	26	14	19	23	5	13	18	13	18	23
A. marina	14	18	24	13	16	20	5	12	16	13	16	22
E. agallocha	13	12	17	12	16	19	5	9	11	12	14	20

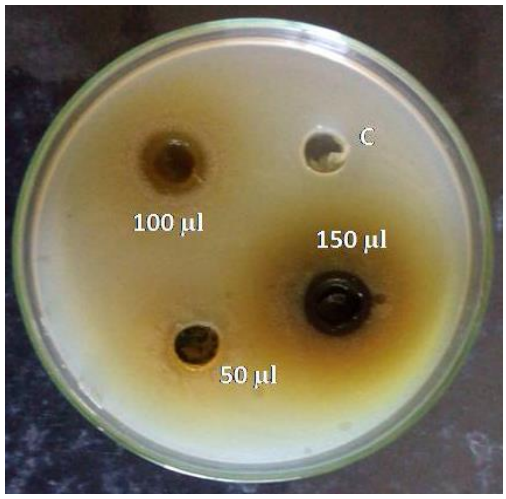


Plate 1: Antimicrobial activity of leaf nanoparticles of *Avicennia marina* against *Staphylococcus aureus*

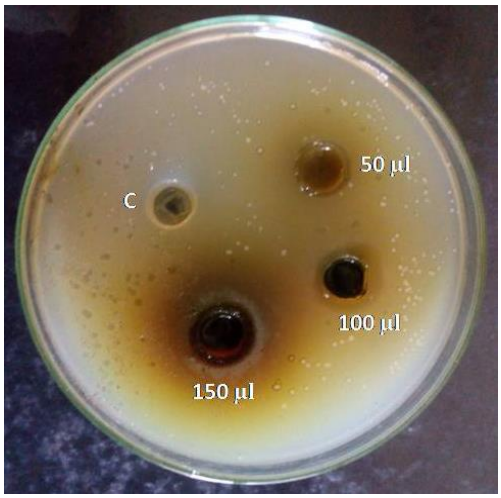


Plate 2: Antifungal activity of Rhizophora apiculata leaf nanoparticles against *Candida albicans*

DISCUSSION

The focus of present study is preparation of herbal nanoparticles through top down method from the selected mangrove leaves and to test the influence of nanoparticle size on antimicrobial potentiality. The XRD patterns in the present study confirm the crystalline nature of the nanoparticles prepared through physical method of ball milling [40]. In present study the XRD spectra results of obtained herbal nanoparticle size is ranged between 14.38-28.70 nm and are tally with the results of different mangrove plant derived metallic nanoparticles synthesized through bioreduction [41]. On the other hand the obtained herbal nanoparticles size is lower than the silver nanoparticles produced through green synthesis from mangrove leaves of *Rhizphora apiculata* (60-95 nm), *Ceriops tagal* (30 nm), *Avicennia marina* (71-110 nm) [42, 43]. The UV absorption spectra data of all the herbal nanoparticles from selected mangrove plants showed U.V absorption at 223,267, 281,285, 341, 409, 663, 664 nm which confirms the existence of

nanoparticles as per other studies reported in case of silver nanoparticles(AgNPs) [44, 45]. UV/Vis absorption spectra of the silver nanoparticles dispersed in *R. mucronata* extracts with absorption peak (SPR) in the visible range at 426 nmv [42]. A peak at 420 nm in leaf extracts of *A. marina* indicating the production of silver nanoparticles [46]. The herbal nanoparticles showed the absorption bands at wave lengths much lower or near the silver nanoparticles reported elsewhere. Similar peaks were observed at 279,437 and 678 on herbal nano particles synthesized by ball milling from *Tridax procumbens* leaf material [45]. The possible phytoconstituents of nanoparticles is revealed by the FTIR studies, which can help in further functionalization with various molecules for various applications [47]. In *Rhizophora apiculata* peaks at 661 $^{cm-1}$,707 $^{cm-1}$,841 $^{cm-1}$,1059-1431 $^{cm-1}$,1500 $^{cm-1}$,2889-2942 $^{cm-1}$ and 3287 $^{cm-1}$ correspond to amines NH_2 bending and vibrating, alchols, alkenes, carboxylic acids with strong O-C bond, arenes C=C strong nitro compound, alkanes and alchol with strong O-H usually broad. In *Avicennia marina* peaks at 628 $^{cm-1}$,1059-1239 $^{cm-1}$,1165 $^{cm-1}$,1334 $^{cm-1}$,1637 $^{cm-1}$,2300 $^{cm-1}$,2909-2942 $^{cm-1}$,and 3333 $^{cm-1}$ correspond to alkynes with strong C-H deformation, carboxylic acids, ester C-O, alchol Alkenes C=C, Si-H silane, alkanes and alkynes with strong sharp C-H stretching. In *Excoecaria agallocha* peaks at 602 $^{cm-1}$,768 $^{cm-1}$, 1046-1215 $^{cm-1}$,1357-1729^{cm-1},2306^{cm-1},2916^{cm-1}and 3380^{cm-1} were obtained. They correspond to functional class alkynes, alchols, carboxylic acids, aldehydes, Si-H silane, alkanes and amines with weak NH bond aliphatic primary amine. The observed peaks are related to major functional groups in different chemical classes such as flavonoids, triterpenoids and polyphenols [48]. The peaks observed between 800-1700 $^{cm-1}$ in the herbal nanoparticles studied indicate the presence of flavanoids and which suggest good antimicrobial activity [49]. The results of the present study also confirm that the smaller particle size showed greater antimicrobial activity. Smaller size of the nanoparticles restricts the DNA replication easily when compared to the large sized nanoparticles as evident in other studies. Higher zone of inhibition was observed in R. *apiculata, A. marina* relative to their smaller nano particle sizes (Table 3). Further smaller nanoparticles with large surface area facilitate easy penetration and thus denaturation of bacterial cell wall [50]. On the other side the phytochemical composition of the herbal nanoparticles can influence and enhance the antimicrobial assessment.

4. CONCLUSION

The herbal nanoparticles prepared through ball milling technique proved to be having excellent antimicrobial activity. The nanoparticles obtained were confirmed through peaks in UV-Vis spectrometry and confirms the crystalline nature as per obtained peaks in the XRD spectra. The smaller particle size plays an important role in the formation of homogeneous nanoparticles, which enhances the antimicrobial activity. This study confirms that smaller nanoparticles with high surface area showed the maximum zone of inhibition than higher particle size with smaller surface area. In addition, size dependent antimicrobial analysis of nanoparticles gives the promising

results in this study. Hence, preparation of herbal nano particles through ball milling will be more helpful in obtaining homogeneous herbal nanoparticles. In the field of biomedical applications this method will lead to a newer concept of "Herbonanoceuticals". Moreover the green synthesis of AgNp's is confined to laboratory scale and production in bulk amounts is very costly whereas herbal nanoparticles can be produced on large-scale with less cost.

ACKNOWLEDGEMENT

Authors are greateful to University Grants Commission (UGC) and Department of Science and Technology (DST), Govt. of India for providing financial assistance to complete the present work.

CONFLICT OF INTEREST

Authors don't have any conflict of interest.

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