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RESEARCH ARTICLE

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *Citrus reticulata* FRUIT PEEL AQUEOUS EXTRACT AND THEIR ANTIBACTERIAL ACTIVITY.

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Abstract

Environmental friendly approach or green chemistry synthesis of metallic nanoparticles has become new growing branch in nanobiotechnology. In this present work a simple and environmental friendly silver nanoparticles (AgNPs) were prepared using *Citrus reticulata* fruit peels aqueous extract as the reducing agent guided by the principles of green chemistry. The fruit peels aqueous extract was challenged with silver nitrate solution for the production of AgNPs in room temperature. The crystalline phase and morphology of AgNPs were determined from UV-Vis spectroscopy, Fourier transform infrared (FTIR) spectra, X-ray diffraction (XRD), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX). The UV-Vis spectrum indicated that the surface plasmon broad peak was observed at 466 nm after 6hr of incubation. XRD spectrum revealed that the average size of *Citrus reticulata* fruit peels aqueous extract mediated AgNPs obtained approximately 24 nm by using the Debye-Scherrer equation. SEM image showed uniformly distributed AgNPs on the surface of the cell with high agglomeration. EDX analysis revealed that the presence of silver which was confirmed by the Ag peak at 3.28 keV. In addition, the *Citrus reticulata* fruit peels aqueous extract mediated AgNPs loaded discs were tested for antibacterial properties against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis* *Escherichia coli*, *Salmonella paratyphi* and *Klebsiella pneumoniae* and found that the obtained metallic AgNPs have a good antibacterial activity and can be used for biological applications.

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Introduction:-

Nanoparticles are aromatic or molecular aggregates with at least one dimension between 1 and 100nm that can drastically modify their physic-chemical properties compared to the bulk material (Karnani and Chowdhary, 2013). They are the fundamental building blocks of nanotechnology. The most important and unique property of nanoparticles is that they exhibit larger surface to volume ratio. The most effectively studied nanoparticles today are those made from noble metals, in particular Ag, Pt, Au and Pd (Gurunathan *et al.*, 2009; Jain *et al.*, 2009). The synthesis of metal and semiconductor nanoparticles has attracted considerable attention from physicists, chemists, materials scientists and engineers owing to their potential applications in catalysis, biosensing, recording media and

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electronics (Sathyavathi *et al.*, 2010). Among the above four noble metal, silver nanoparticles play a significant role in the field of biological systems, living organisms and medicine (Parashar *et al.*, 2009). It is observed that silver nanoparticles do not affect living cells so they can be used as an effective anti-microbial agent as they get attached to the cell wall and disturb cell wall permeability and cellular respiration (Singh *et al.*, 2008). Due to this property silver containing particles are used in textile fabrics, as food additives and in packaging to eliminate microorganisms (David *et al.*, 2010). Therefore design and development of simple, one-step, non toxic and eco-friendly method for the production of multifunctional silver nanoparticles is of greatest interest to excel their applications.

Numerous methodologies are formulated in the past to synthesize silver nanoparticles of particular size and shape depending on their specific requirements. The usage of toxic chemicals and solvents in the synthesis of silver nanoparticles limits their application in the clinical fields. So the biopreparation of nanoparticles as an emerging field of the intersection of Nanotechnology and Biotechnology has received increased attention due to a growing need to develop environmentally benign technologies in silver nanoparticles synthesis (Madhumitha and Selvaraj, 2013).

The process for making silver nanoparticles using plant extracts is readily available and less expensive. The plant extracts act as reducing and stabilizing agents in the production of nanoparticles. This is because different plant extracts contain different concentrations of organic reducing agents (Mukunthan and Balaji, 2012). Though, plant mediated synthesis of silver nanoparticles are considered as safe, eco-friendly and cost effective, they also have some drawbacks in using the sustainable plant resources (Haverkamp and Marshall, 2009). For this reason, in the present study we used fruit peel extract of *Citrus reticulata* as alternate source.

The synthesized nanoparticles were confirmed by colour changes and characterized by UV-Visible spectroscopy. Fourier transform infrared (FTIR) spectral measurements were carried out to identify the potential biomolecules. The morphology of the nanoparticles was observed by SEM (Scanning Electron Microscope). The crystalline nature of Ag nanoparticles was confirmed by X-ray diffraction analysis. Further its efficacy to inhibit different pathogenic bacterial growth were evaluated against human pathogens by disc diffusion method.

Materials and Methods:-

Preparation of *Citrus reticulata* fruit peels extract:-

Citrus reticulata fruits were collected from the local market nearby our college. The fresh and healthy *Citrus reticulata* fruits peel were collected, rinsed thoroughly first with tap water for 5-10 min followed by distilled water for 10-20 min to remove all the dust and unwanted visible particles, fruit peels were cut into small pieces and dried at room temperature. To prepare the aqueous extract of *Citrus reticulata* fruits peel, 20g of thoroughly washed and finely chopped fruit peels were added to 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask and then boiled for 10min in heating mantle. The solution was then removed from the heat source and left at room temperature. Following this step the extract was filtered through Whatman filter paper No.1 and used as such for silver nanoparticles synthesis.

Synthesis of silver nanoparticles using *Citrus reticulata* fruit peel extract:-

The aqueous solution of 1mM concentration silver nitrate (AgNO_3) was prepared to synthesize silver nanoparticles from *Citrus reticulata* fruit peel extract. 5ml of *Citrus reticulata* fruit peel aqueous extract was slowly added to 100ml of aqueous solution of 1mM AgNO_3 while stirring, for reduction into Ag ions. The colour change of the reaction mixture from faint yellow to yellowish brown to reddish brown to colloidal brown was monitored after 6hr of incubation at room temperature.

Characterization of Silver Nanoparticles:-

The bio reduction of Ag^+ ion in aqueous solution was monitored with the help of UV-visible spectroscopic analysis. UV-Visible spectroscopy analysis of silver nanoparticles synthesized using *Citrus reticulata* fruit peels were carried out as a function of time needed for bioreduction at room temperature on UV-2600 series Shimadzu spectrophotometer between wavelengths of 300 to 800 nm at a resolution of 1nm. Fourier transform infrared (FTIR) spectra for green synthesised silver nanoparticles was recorded on a Shimadzu FTIR spectrometer 8000 series, with KBr in the wavenumber region of $4,000 - 400 \text{ cm}^{-1}$. Crystalline nature of the nanoparticles was analyzed by XRD at 2θ ranges from 20 to 80°C using X'Pert Pro X-ray diffractometer (PAN analytical BV, The Netherlands) equipped with $\text{Cu/K}\alpha$ radiation source using Ni as filter at a setting of 30kV/30mA. The morphology and elemental

composition of the synthesised silver nanoparticles was identified by Scanning Electron Microscopy (SEM, JEOL JSM-6390) along with Energy Dispersive X-ray Spectroscopy (EDS, Model No. 9582, Oxford Instruments) operating at an accelerating voltage of 20 kV.

Antibacterial Activity:-

The biosynthesized silver nanoparticles using *Citrus reticulata* fruit peels aqueous extract were tested for antibacterial activity by disc diffusion methods against human pathogenic organisms such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella paratyphi* and *Klebsiella pneumoniae*. Pure cultures of bacterial test organisms were grown in nutrient broth at 37°C for 24 h. About 200 μ L of aliquot of each strain (1×10^6 cfu/mL) was spread uniformly onto the individual pre-sterilized Mueller Hinton agar plates using sterile cotton swabs and allowed to dry for 10 to 15 min. On other side, Whatman No. 1 filter paper discs (3 mm in diameter) were prepared and coated with 50 μ L of silver nanoparticles. The silver nanoparticles coated filter paper discs were placed on the surface of each cultured plate, Chloramphenicol disc was used as positive control and *Citrus reticulata* fruit peels aqueous extract was used as negative control. Then, the petridishes were incubated at 37°C for 24 h. After incubation, the inhibition zones were measured in millimeters.

Figure 1:- Biosynthesis of AgNPs using *Citrus reticulata* fruit peels aqueous extract a) *Citrus reticulata* fruit peels aqueous extract b) 1mM AgNO₃ c) Reaction mixture after 6hr incubation

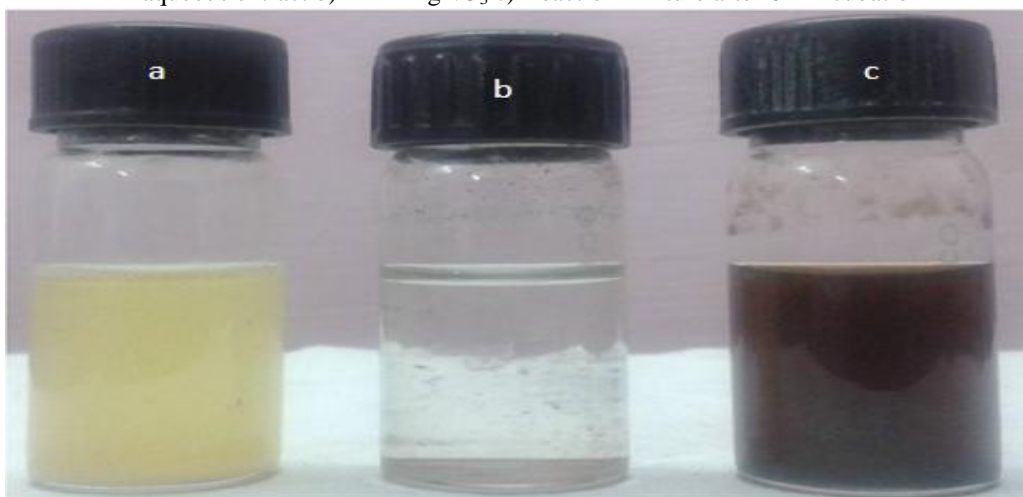


Figure 2:- UV Spectrum of biosynthesized Silver nanoparticles.

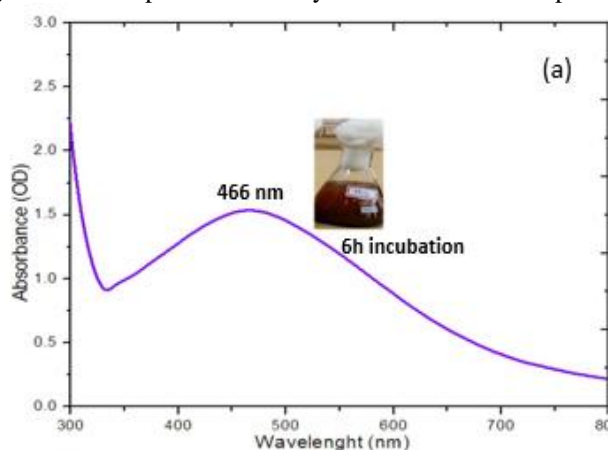


Figure 3:- FTIR Spectrum of Silver nanoparticles.

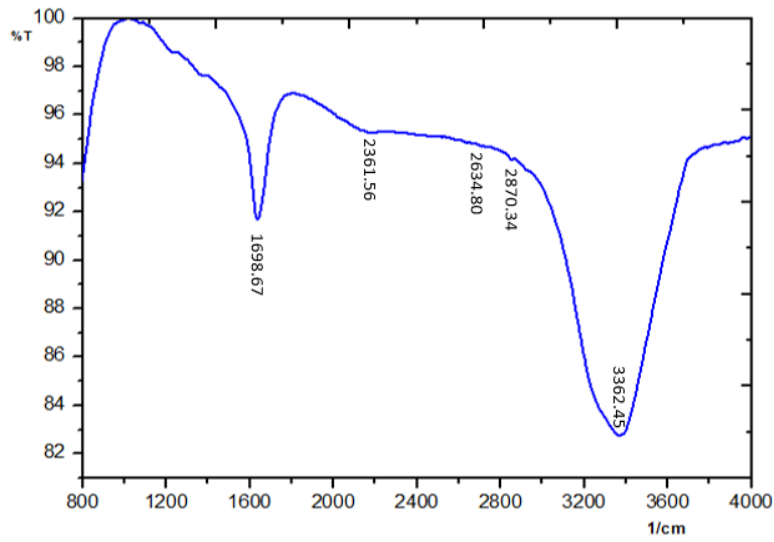


Figure 4:- XRD analysis of Silver nanoparticles

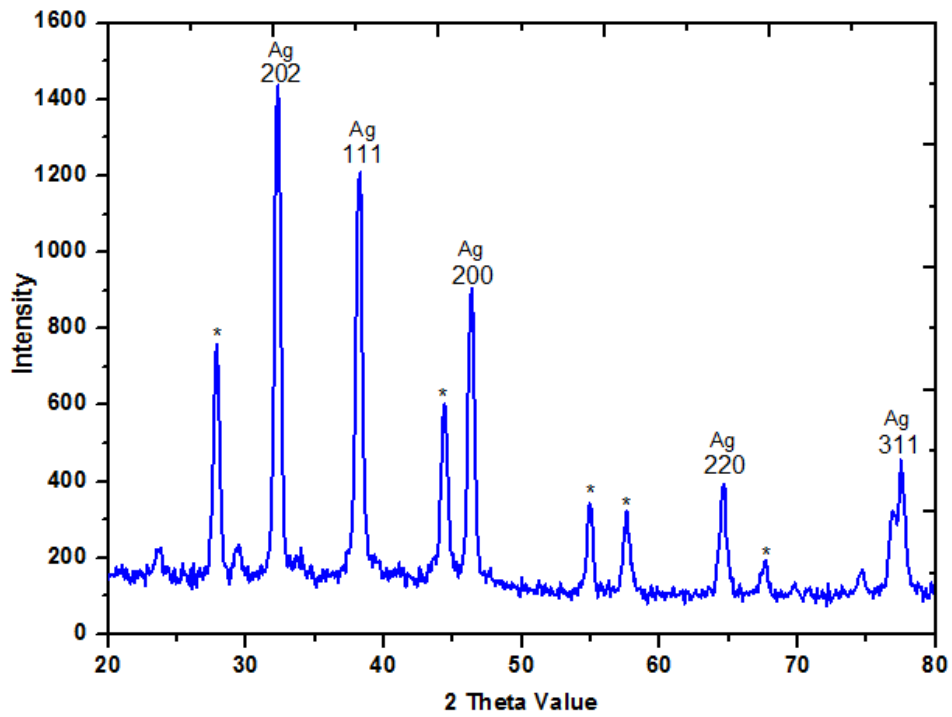


Figure 5:- SEM image of biosynthesised Silver nanoparticles

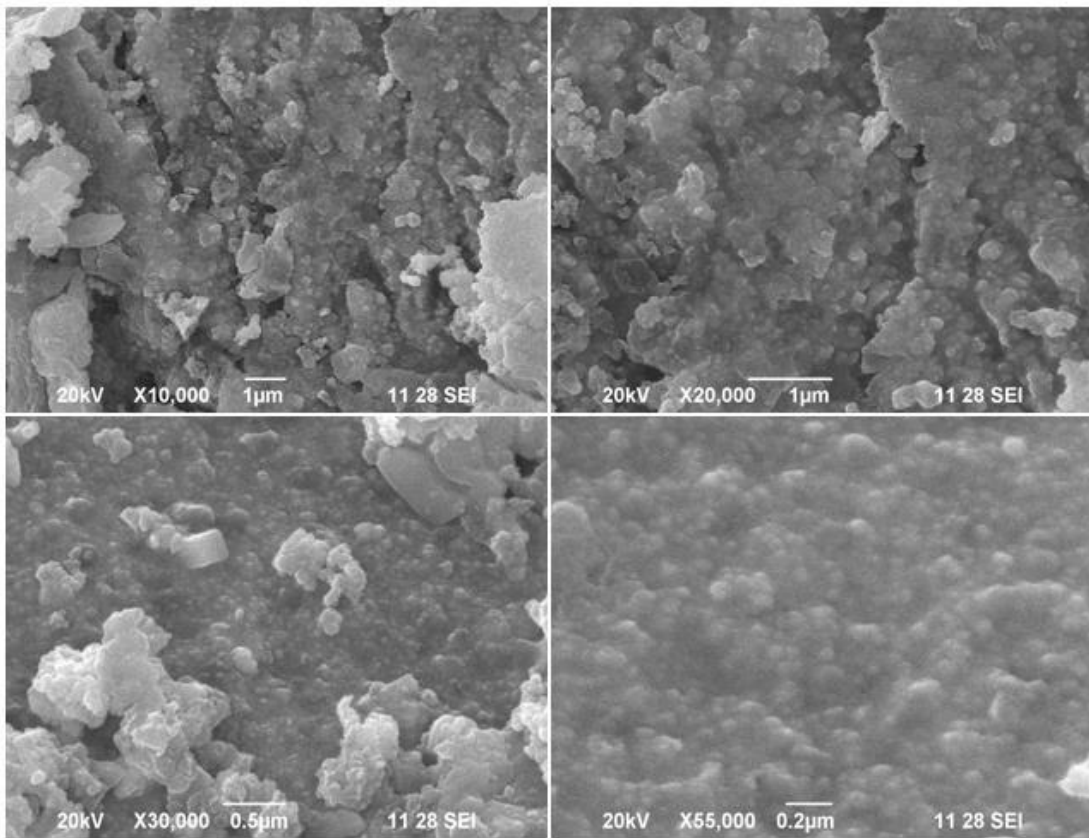


Figure 6:- EDX spectrum of Silver nanoparticles

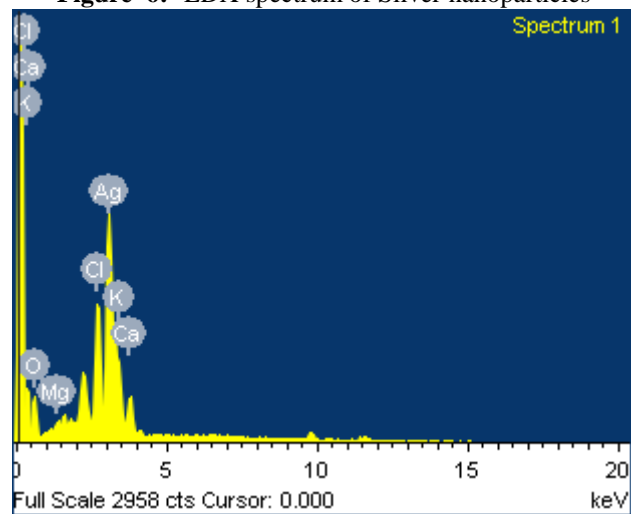
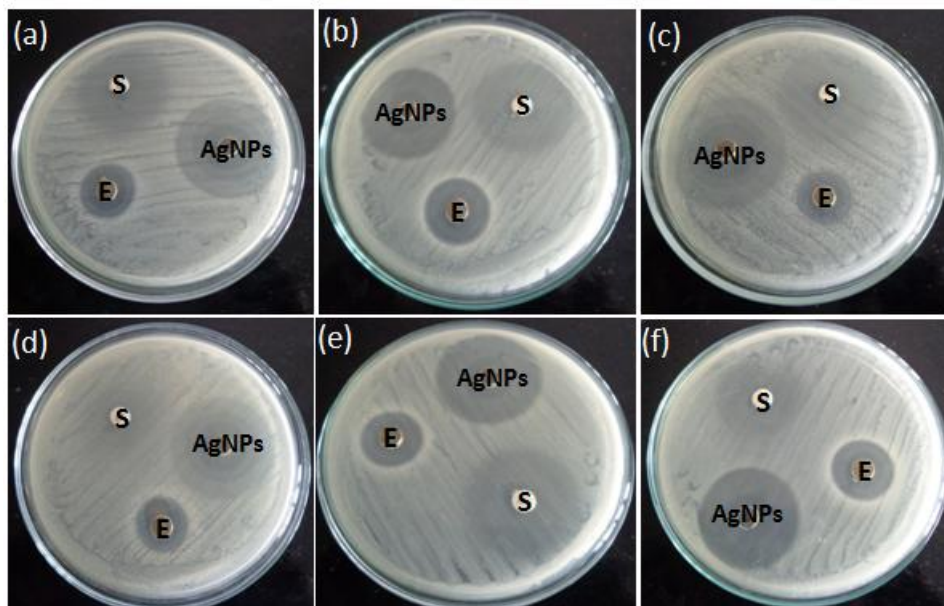


Figure 7:- Antibacterial Activity of *Citrus reticulata* peel mediated silver nanoparticles a) *B. subtilis* b) *E. Coli* c) *K. pneumoniae* d) *S. paratyphi* e) *S. aureus* and f) *S. pyogenes*



Results and Discussion:-

Eco-friendly and reliable methods have been developed to raise significance in the synthesis and application of nanoparticles that are helpful for mankind (Bhattacharya and Gupta, 2009). The major advantage of using plant extracts for silver nanoparticle synthesis is that they are easily available, safe, nontoxic in most cases and have a broad variety of metabolites that can aid in the reduction of silver ions and are quicker than microbes in the synthesis. Reduction of silver ion into silver nanoparticles for the period of exposure to the plant extracts could be followed by colour change. Silver nanoparticles exhibit yellowish-brown colour in aqueous solution due to excitation of surface plasmon vibrations (Elumalai *et al.*, 2010). In the present study, *Citrus reticulata* fruit peels aqueous extract was used as reducing agent for the synthesis of AgNPs using 1mM AgNO₃. The crude extract of *Citrus reticulata* fruit peel was light yellow colour however after addition of AgNO₃ the colour of the reaction mixture turned dark brown colour which indicated the formation of AgNPs after 6h incubation period (Fig.1). This colour change indicated that the nanometric range silver ions in reaction medium have been converted to elemental silver. Metal nanoparticles such as silver have free electrons, which give rise to SPR absorption band (Veerasamy *et al.*, 2011).

UV-Vis Spectrum Analysis:-

The green route synthesized AgNPs using *Citrus reticulata* fruit peel aqueous extract was confirmed by the UV-Vis spectrum analysis at different nm scale ranging from 300 nm to 800 nm. The colour changed from light yellowish to dark brown was due to excitation of Surface Plasmon Vibration which indicated the formation of AgNPs. The Surface Plasmon broad peaks at 466 nm (Fig. 2) throughout the reaction clearly revealed that the green synthesized AgNPs were dispersed in the aqueous solution. It was also reported that the green synthesised silver nanoparticles using aqueous extract of *Citrus reticulata* peel showed the localized surface Plasmon bands at the similar regions (Elias *et al.*, 2015).

FTIR Spectrum Analysis:-

FTIR spectrum analysis had helped to understand the nature of biomolecules present in the *Citrus reticulata* fruit peel extract that involved in the formation of silver nanoparticles. The FT-IR spectrum of *Citrus reticulata* fruit peel extract mediated green synthesized AgNPs showed sharp peak located at 3362.45, 1698.67 and light peak located at 2870.34, 2634.80, and 2361.56 cm⁻¹. The sharp peak at 3362.45 may be assigned to the O-H stretch and 1698.67 may be assigned to the C-N stretch bonding function of secondary alcohol groups. Whereas light peak at 2870.34, 2634.80, and 2361.56 cm⁻¹ may be assigned to the C-O, N-H and O-H stretching that indicated the presence of phenol, amine and carboxylic acid functional group (Fig.3). The FTIR spectrum revealed that the different functional groups present at different position and functional biomolecules like phenols, alcohols and carboxylic

acid are involved in the reduction of silver ions. Hence, the existence of these functional groups is responsible for the stabilization of synthesized silver nanoparticles and also acts as reducing and capping agent (Haytham, 2015).

XRD Analysis:-

X-ray diffraction spectrum of *Citrus reticulata* fruit peel mediated silver nanoparticles showed the Bragg reflections in the XRD pattern at $2\theta = 32.27, 38.25, 46.60, 64.78$ and 78.69 . These Bragg reflections clearly indicated the presence of (202), (111), (200), (220) and (311) sets of lattice planes and further on the basis that they can be indexed as face-centered-cubic (FCC) structure of silver (JCPDS file nos. 04-0783). A comparison of obtained XRD spectrum with the standard, confirmed that the silver particles formed in present experiments were in the form of nanocrystals. X-ray diffraction results clearly showed that the silver nanoparticles formed by the reduction of Ag^+ ions by *Citrus reticulata* fruit peel extract are crystalline in nature (Fig.4). The average crystallite size of green route synthesized silver nanoparticles was estimated from the full width half maximum (FWHM) of (111) reflection by using Scherrer formula, $D = 0.9\lambda / \beta \cos\theta$. Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength source (0.1541 nm), β is the full width at half maximum and θ is the diffraction angle. It was observed that the average diameter of the silver nanoparticles crystal was about 24 nm. Some unassigned intense diffraction peaks (*), might be related to the crystallization of bioorganic phases that attached on the surface of the nanoparticles. The average particle size of silver nanoparticles synthesized by the present green method can be calculated using the Debye-Scherrer equation (Nabikhan *et al.*, 2010).

SEM-EDX Analysis:-

Scanning electron microscopy images showed the shape of the green synthesized AgNPs using *Citrus reticulata* fruit peels aqueous extracts (Fig. 5). The surface morphology of the AgNPs was observed at different magnification and it revealed that orange peels extract mediated AgNPs were uniformly distributed on the surface of the cell with high agglomeration. The observation of larger silver nanoparticles may be due to the high aggregation of the smaller ones (Ranjithkumar *et al.*, 2013). This may be due to availability of different quantity and nature of bioorganic compounds present in the aqueous extract. The SEM equipped with Energy Dispersive X-ray Spectroscopy (EDX) analysis indicated the presence of silver which was confirmed from the Ag peaks (Fig. 6). The strong signals in the silver region around 3.28keV confirmed the formation of AgNPs. Throughout the scanning range, some additional peaks belonging to other organic compounds present in the extract of *Citrus reticulata* fruit peel were also recorded and it was suggested that they were mixed precipitates of fruit peel extracts (Usha and Gladys, 2014).

Antibacterial Activity:-

Silver have long been known to have strong inhibitory and bactericidal effects, as well as a broad spectrum of antimicrobial activities which have been used for centuries to prevent and treat various diseases most notably infections. Silver nanoparticles are reported to possess anti-bacterial and anti-fungal properties (Caroling *et al.*, 2013). In this study, antibacterial activity of *Citrus reticulata* fruit peels extract mediated synthesized biogenic silver nanoparticles was evaluated by using standard Zone of Inhibition (ZOI) microbiology assay against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella paratyphi* and *Klebsiella pneumoniae* and found that *Citrus reticulata* fruit peels extract mediated AgNPs have significant antibacterial activity against tested pathogens (Fig. 7). The maximum ZOI of *Citrus reticulata* fruit peel extract mediated AgNPs loaded disc was found to be 13 mm against *Klebsiella pneumoniae* and *Bacillus subtilis*, whereas other bacterial strains like *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella paratyphi* showed 11 mm and 10 mm zone of inhibition respectively. Previous study for the silver nanoparticles synthesized using orange peel extract showed the maximum of 6 mm zone of inhibition against tested *E. coli*, *Pseudomonas sp.* and *Salmonella sp.* (Awad *et al.*, 2014). The analysis of antibacterial activity of silver nanoparticles synthesized from *Citrus sinensis* showed utmost zone against *E.coli* (17 mm) at 2mM AgNO_3 and *S.aureus* (16.5 mm) at 2mM AgNO_3 (Chandrasekar *et al.*, 2015). Likewise, our present results suggested that the plant mediated metallic silver nanoparticles have been a good antibacterial activity which can be used as effective material for biological applications.

Conclusion:-

Green chemistry nanoparticles are gaining importance due to the free of toxic chemicals and provide effective synthesis of expected products in an economic manner. In this present work, we developed an environmental friendly and convenient green chemistry method for the synthesis of silver nanoparticles from fruit waste as reducing agent and *Citrus reticulata* fruit peels aqueous extract is found to be suitable for the production of AgNPs at room temperature by green approach. Production of AgNPs after incubation was identified by the colour change

that occurs due to Surface Plasmon Resonance during the reaction with the organic compounds present in the *Citrus reticulata* fruit peels aqueous extract. The formation of AgNPs was confirmed by UV-Vis spectrum and Surface Plasmon broad peak observed nearby 466 nm. The FTIR spectrum indicated the different functional biomolecules present at different position such as phenols, alcohols and carboxylic acid which are involved in the reduction of silver ions. XRD and SEM-EDX indicated that the *Citrus reticulata* fruit peels aqueous extract mediated AgNPs were uniformly distributed on the surface of the cell with high agglomeration. In addition, *Citrus reticulata* fruit peels aqueous extract mediated green synthesized silver nanoparticles showed excellent antibacterial properties against human pathogens at low concentration level.

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