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SYNERGISTIC ANTIBACTERIAL EVALUATION OF *TAGETES ERECTA* SILVER NANOPARTICLES WITH COMMERCIAL ANTIBIOTICS

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ABSTRACT

In the present study silver nanoparticles were synthesized using aqueous extract of fresh flowers of Tagetes erecta. The aqueous silver ions when exposed to fresh flower extract were reduced and resulted in green synthesis of silver nanoparticles. The synthesized silver nanoparticles were characterized by UV-visible spectroscopy, determination of zeta potential, FTIR, X-ray diffraction and SEM analysis. The UV spectrum of synthesized silver nanoparticles showed maximum absorbance at 430nm i.e. at a conc. of 1.5 ml of extract/10 ml silver nitrate solution. Different functional groups were identified by FTIR image, which contributed to antimicrobial activity. From SEM analysis crystalline nature of synthesized nanoparticles was evident. XRD peaks indicated the structure of nanoparticles and proved that nanoparticles are crystalline in nature. Zeta potential of synthesized nanoparticles was found to be -31.9mv which showed that the green synthesized nanoparticles are stable. Synergistic antimicrobial activity of selected concentration of the plant extract along with the antibiotics A(Penicillin) B(cefixime) showed more antibacterial activity against the selected gram positive and gram negative bacteria (Staphylococcus aureus, Klebsiella, Pneumonia, Escherichia coli, Bacillus subtilis.).Among different combinations the synergistic effect of penicillin is more with the synthesized silver nanoparticles against Klebsiella pneumoniae showing a zone of inhibition of 4.5 cm. The synergistic effect of cefixime is more with the synthesized silver nanoparticles against Bacillus subtilis showing a zone of inhibition of 3.5 cm.

KEYWORDS: *nanoparticles, characterization, Synergy, antimicrobial activity*

INTRODUCTION

Plants are very diverse in nature. Nanoparticles (1 nm–100 nm) confer a very large surface area and this gives high reactivity and use in different dosage forms and routes. Synthesis of nanoparticles using plant extracts has been studied using variety of plants like Azadirachta indica (1) , C. papaya fruit (2) and they are exploited for antimicrobial applications (3).The green synthesis of nanoparticles results in controlled size of nanoparticles and the process employed is easy for scale up (4).Extracts from different plant parts contain various phytoconstituents like Flavonoids, terpenoids ,proteins that act as reducing and capping agents.(5).The various phytoconstituents in marigold flower exhibit antimicrobial activity[16].The other advantages of green synthesis include well-defined and controlled size of the nanoparticles. They are devoid of contaminants and the process is easy to scale-up (4).The literature survey reveals use of noble metal silver for its antimicrobial properties(6).Silver nanoparticles have been used for their wide applications like in the treatment of ulcers (7) , brucellosis (8), mosquito larvicidal properties (9), etc. Recently development of multi drug resistant species of microorganisms is on the rise. Green synthesized silver nanoparticles showed antibacterial activity against multidrug resistant microorganisms (10). silver nanoparticles can be synthesized using different synthetic methods like physical, chemical, and biological methods (11).The silver nanoparticles have diverse *in vitro* and *in vivo* applications (12).Nanoparticles are synthesized from aqueous extract of Terminalia arjuna leaves and the synthesized nanoparticles are assessed for their antimicrobial activity [13]. Characterization of silver nanoparticles and their antibacterial activity was evaluated using *Pedalium murex* leaf extract [14]. *Tagetes erecta* commonly known as marigold has been widely used in homoeopathic medicine for the treatment of many diseases. [17]. The flowers are bright yellow, brownish-yellow or orange. Antimicrobial activity of gold nanoparticles of flower extract was reported [25]. The florets and

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leaves of marigold are also used as emmenagogue, diuretic and vermifuge. [18]. The flowers show presence of lutein esters of dipalmitate, dimyristate and monomyristate quercetagetin (3, 3', 4, 5, 6, 7-hexahydroxyflavone) and quercetagetin (quercetagetin-7-glucoside) isolated from the Indian varieties(19).In the present work, an attempt has been made to synthesize silver nanoparticles using aqueous flower extract of *T.erecta* (Fig. 1). The characterization was done using
Infrared spectral and microscopic analysis. The synthesized silver nanoparticles were evaluated for their synergistic antimicrobial activity with two commercial antibiotics.

MATERIALS AND METHODS

Collection of Plant Material

Orange colored marigold flowers are bought from local garden Dundigal, Hyderabad. The plant material collected was authenticated by the Dept. of Pharmacognosy, MLRIP; Dundigal. The chemicals used for the experimental work were purchased from SDFCL, Hyderabad. The pure antibiotic samples to study synergistic antibacterial activity were obtained as gift samples from Aurobindo pharmaceuticals, Hyderabad. Ultra-purified water was used for the experiment.

Fig 1: *Tagetes erecta* flower

Preparation of the marigold flower extract

Fresh, orange and fully blown flowers were collected and thoroughly washed with distilled water. The flowers were finely cut into small pieces. The flower extract was prepared by taking 5 g of washed and cut flowers in a 250 ml Erlenmeyer flask with 100 ml of sterile ultra-pure water and then the mixture was boiled the for 10 min. The flower extract was filtered through Whatman No. 1 filter paper in a separate conical flask and stored at 4^{o}C until further use. The flower extract prepared was used as reducing and capping agent for synthesis of silver nanoparticles.

Phytochemical screening

The flower extract obtained was screened for various phytoconstituents using standard methods (22)

Synthesis of silver nanoparticles

Aqueous solution (1 mM) of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles.0.5 ml, 1.0 ml 1.5 ml 2 ml of extract was added separately to 10 ml of 1 mM $AgNO_3$ solution for the reduction of Ag^+ ions to Ag^0. The synthesis of silver nanoparticles was carried out at room temperature (25 °C ± 2 °C) for 24 h in dark. Upon start of the reaction the solution was colorless and gradually it changed from transparent yellow to orange red and finally dark brown colour appeared indicating the formation of silver nanoparticles [20]. The silver nanoparticle solution thus obtained was purified by repeated centrifugation at 8,000 rpm for 20 minutes. The obtained pellet was redispersed into acetone. Purified silver nanoparticles were obtained after air drying. The green synthesized silver nanoparticles were stored at 4 °C until further experimental work.

Characterization of synthesized Nano particles

The simplest and important technique to confirm the formation of silver nanoparticles was by measuring Plasmon resonance of silver nanoparticles in response to electromagnetic waves. The UV–Vis spectra of synthesized silver nanoparticles were monitored as a function of time using UV-VIS Spectrophotometer (UV Lab India 19-1950-21-0002) in 200–800 nm range operated at a resolution of 10 nm. The XRD patterns were analyzed using X-Ray diffraction. The formation of Ag nanoparticles was determined by an X-ray diffractometer (Bruker AXS) operated at a voltage of 40 kV and a current of 30 mA with Cu-Kα radiation (λ=0.15418nm). The scanning was done in the region of 2θ from 10° to 80°. The size of the nanoparticles was calculated through the Scherrer's equation. SEM (Scanning electron microscopy) analysis was done to visualize texture (external morphology), crystalline structure, and orientation of the synthesized nanoparticles in the sample using SEM- HITACHI S-3700 at an accelerated voltage of 15 KV. Various functional groups present on the surface of synthesized AgNPs were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) (Bruker FTIR) in the scan range of 400–4000 cm-1 at a resolution of 4 cm^{-1}. The zeta potential of the prepared AgNPs was determined using Zetasizer Nano ZS 90.

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Antimicrobial Activity

The antibacterial activity of the formed nanoparticles was carried out by adopting the method of modified Kirby Bauer (21).The antibacterial activity of flower extract alone , the synthesized nanoparticles and the synergistic antibacterial activity of the synthesized nanoparticles with two commercial antibiotics [Penicillin(A) and Cefixime(B)] was determined by testing against two gram positive (*Staphylococcus aureus* MTCC 3160, *Bacillus subtilis* MTCC 5981) and two gram negative bacteria (*Klebsiella Pneumonia* MTCC 10309, *Escherichia coli* MTCC 1698) by agar well diffusion method. In the synergistic study the minimum inhibitory concentration (MIC) of the commercial antibiotics was used (23, 24). Two different concentrations of standard antibiotics were used: S_1 is the MIC and S_2 is slightly higher concentration than MIC. Sterilized medium was poured in to sterile Petri plates to about 10 mm depth. About 100 μl of the test organism was spread uniformly using sterile L-Shaped glass rod on solidified agar medium. Cylinders or cups of about 5 mm diameter were bored using sterile borer. 20 μl *of* each sample to be tested is aseptically poured into each cylinder. For evaluation of synergistic activity 10 μl of commercial antibiotic and 10 μl of silver nanoparticles were added to each cylinder. Each concentration is tested for its zone of inhibition in triplicate. The loaded Petri plates were incubated at 37 ^{0}C for 18-24 hrs. The zones of inhibition were measured, and the results were tabulated.

RESULTS AND DISCUSSION

phytochemical screening

Different phytoconstituents like terpenoids Flavonoids, Alkaloids, Carbohydrates Tannins Phenols were identified qualitatively (table 1).

S.NO	Phytochemical Test	Aqueous extract
1.	Terpenoids	+
2.	Proteins	+
3.	Flavonoids	+
4.	Alkaloids	+
5.	Carbohydrates	+
6.	Saponins	+
7.	Glycosides	-
8.	Tannins	+
9.	Phenols	+
10.	Steroids	-

Table 1: Phytoconstituents in *Tagetes erecta* flower extract

Visual examination

Green synthesis of silver nanoparticles was carried using aqueous flower extract of *Tagetes erecta*. Upon addition of colorless silver nitrate solution to light yellow colored *T.erecta* flower extract change in colour to dark brown to black occurred due to surface Plasmon resonance (fig 2). The number of silver nanoparticles formed was directly proportional to the intensity of the colour developed. Within 4 hrs. the solution turned black.

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Fig 2: Silver Nanoparticles of *Tagetes erecta* flower extract

Characterization

UV Spectral analysis

UV Spectral analysis revealed that maximum absorbance was found at 430 nm. Among different quantities of the extract analyzed 1.5 ml showed maximum absorbance of 0.974 (fig 3). Hence further characterization was carried out using this quantity of silver nanoparticles. The synthesized nanoparticles exhibited negative charge suggested by the Zeta potential value. The stability of the synthesized nanoparticles is indicated by zeta potential. Zeta potential value of > ± 30 mv indicates stable suspension. The synthesized silver nanoparticles exhibited a Zeta potential value of -39.1 mv (fig 4).

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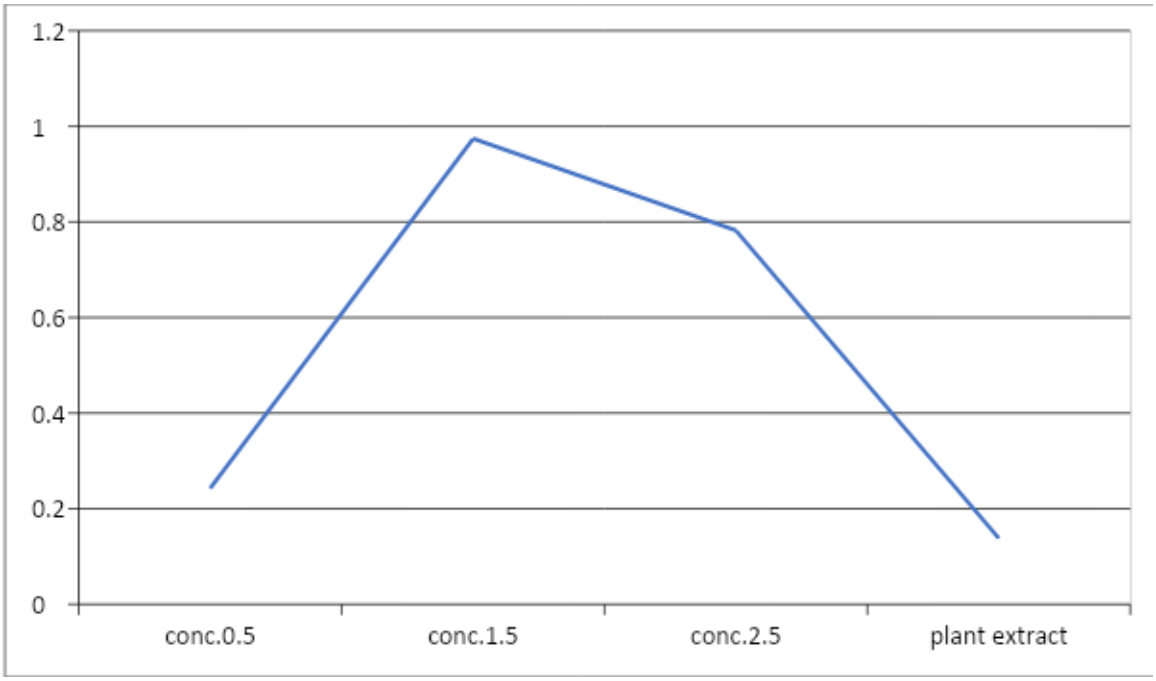

Fig.3 UV absorbance of plant extracts of different concentrations

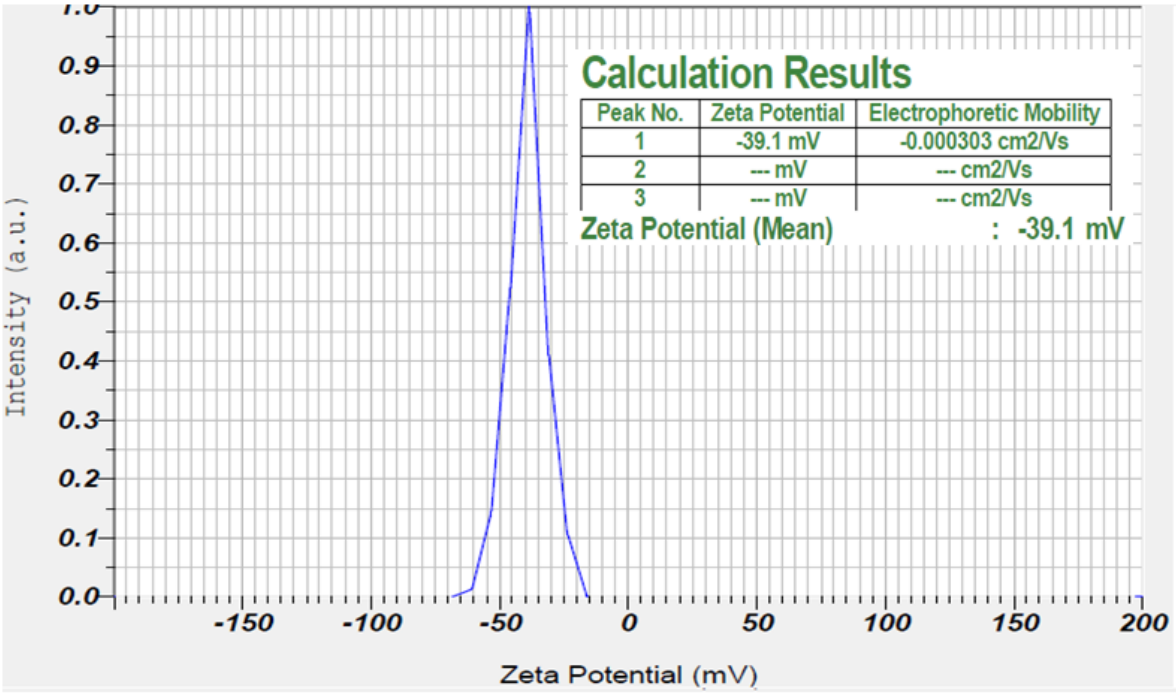

Fig 4: Zeta Potential of the synthesized Nanoparticles

FTIR Spectral analysis

Different biomolecules which contributed for the reduction of silver ions are analyzed by FTIR spectra. The FTIR spectrum of the green synthesized nanoparticles is shown (fig 5) in the figure. The capping of silver ions in the silver nanoparticles is due to the functional groups which are identified from the absorption peaks. The absorption peak around 3318 cm^{-1} is due to amino and hydroxyl

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groups which indicate presence of amino acids and phenols. Thus proteins and phenols acted as effective reducing agents. The peaks at 3450 cm^{-1} indicate N-H stretching 1620 cm^{-1} indicate C=O functional groups. So Flavonoids in the green synthesized nanoparticles are effective in reducing the silver ions. The peaks observed at 1554 cm^{-1} indicate C=C stretching. The absorption peaks at 611 cm^{-1}, 626 cm^{-1} , 565 cm^{-1} correspond to halogens C-Cl and C-Br stretching respectively. Thus Flavonoids and phenols might have contributed more in the reduction and capping of silver as they are water soluble. Similar type of results were observed in synthesis of silver nanoparticles using *Calliandra haematocephala* leaf extract [26].The possible mechanism of bio reduction needs further investigation.

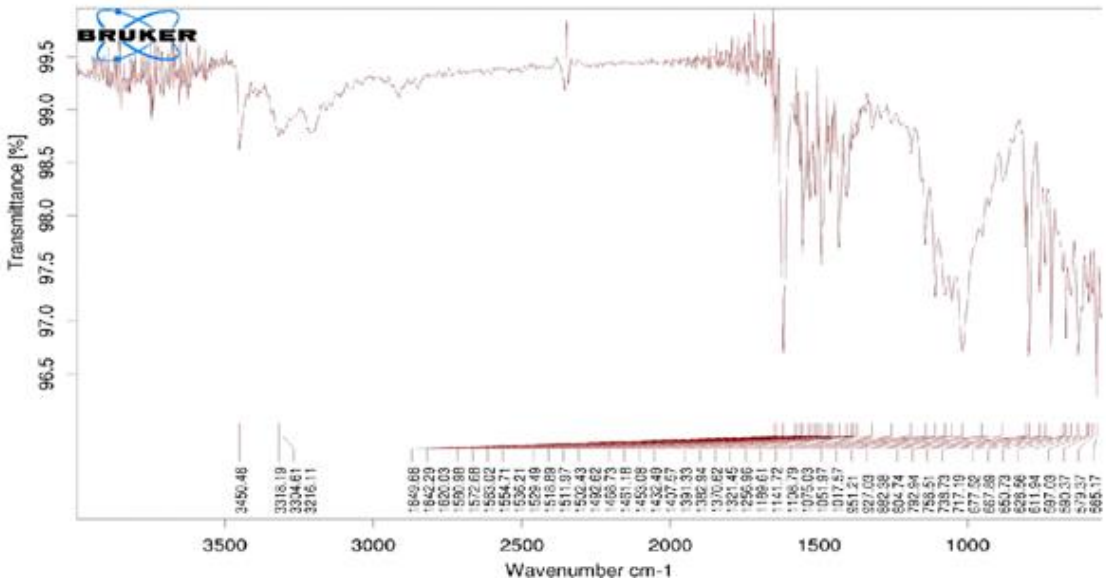

Fig 5: FTIR Spectrum of Synthesized Nanoparticles

SEM Analysis

The SEM image (fig 6) at a magnification of 2000 times shows crystalline nature of the synthesized silver nanoparticles. Most of the synthesized silver nanoparticles are spherical in shape that shows very small particle size of 20-30 nm. Some of the nanoparticles are polygonal of about 200 nm but most of the particles were observed as agglomerates which might be formed due to long standing.

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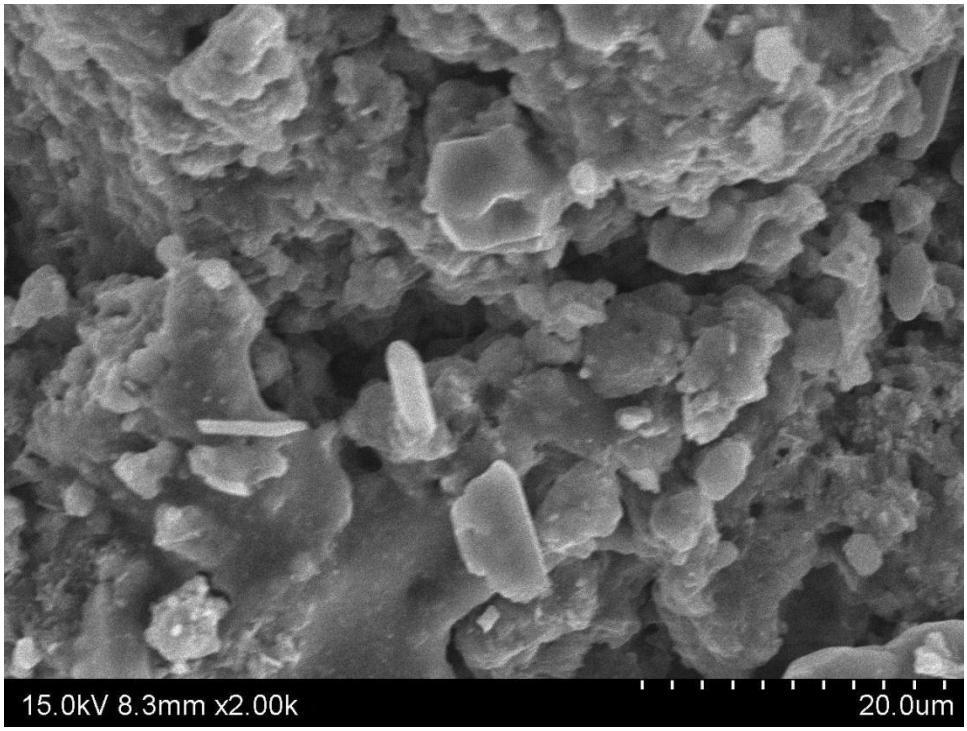

Fig 6: SEM Image of the synthesized Nanoparticles

XRD

The synthesis of silver nanoparticles was further confirmed by X ray diffraction studies (fig 7). Number of peaks observed at 2Θ values of 17.79^0, 19.47^0, 20.65^0, 24.54^0, 27.16^0 27.8^0 45.44^0 that correspond to different diffraction faces of the nanoparticles. The XRD results indicate crystalline nature of synthesized silver nano particles. The size of the crystals was calculated using Scherrer equation ($D = (0.94 \times \lambda) / (\beta \times \cos \theta)$ where D=Average Crystallite size, β = full width at half maximum (FWHM) in radians, θ = Bragg angle, λ = X-Ray wavelength. the mean particle size of the green synthesized nanoparticles was 8.25 nm.

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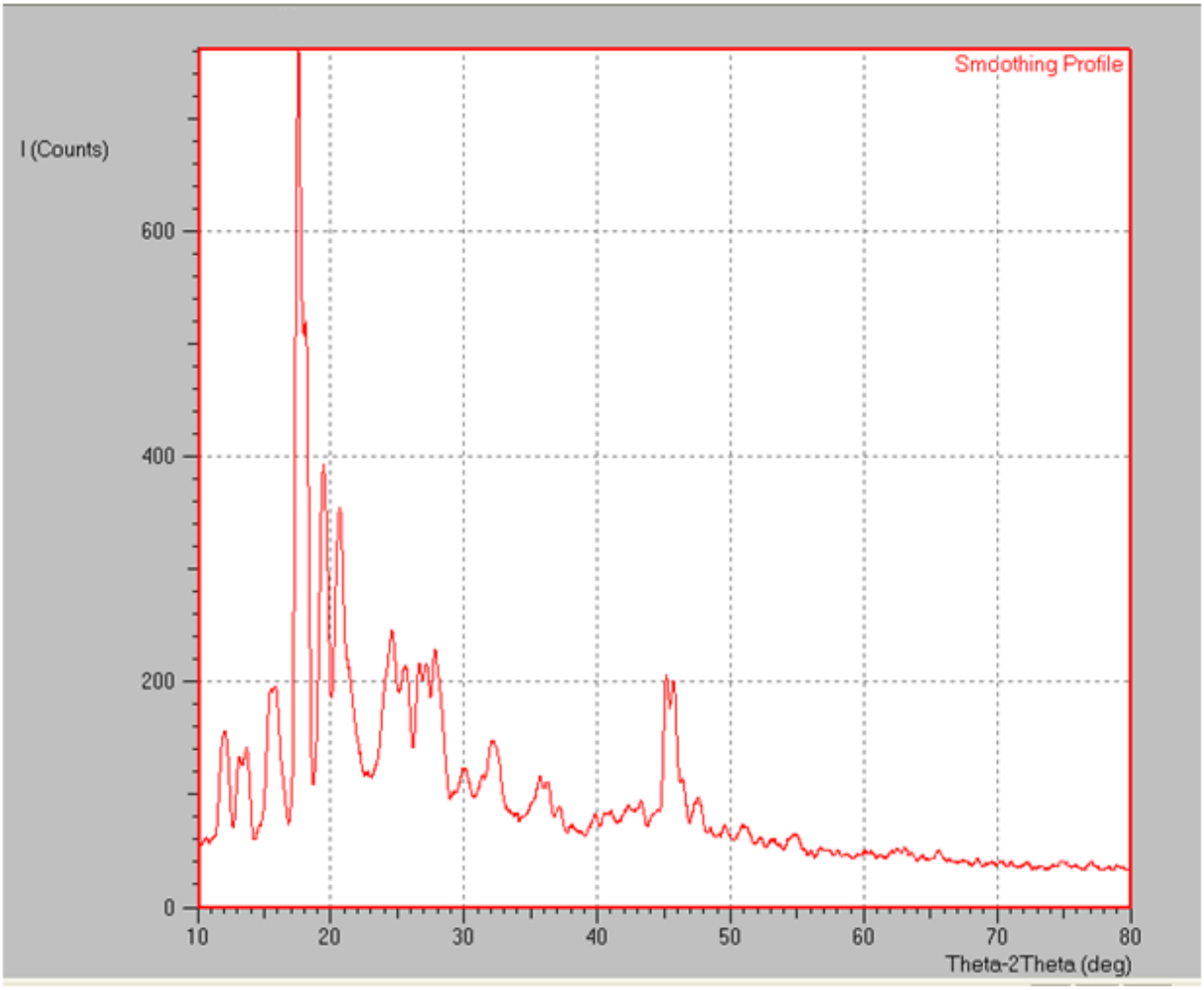

Fig 7: XRD Spectrum of the synthesized Nanoparticles

Antibacterial activity

The antibacterial evaluation suggested that the combination of antibiotic and silver nanoparticles exhibited more antibacterial activity than plant extract alone or standard antibiotics (penicillin & cefixime) alone. As it is revealed in fig 8 gram negative and gram-positive bacteria are more susceptible to silver nitrate than plant extract. The silver nanoparticles exhibited more or less similar antibacterial activity with that of plant extract. The zone of inhibition of silver nanoparticles against *S. aureus*, *E.coli*, *B. subtilis* and *Klebsiella pneumoniae* was 10, 15, 11, 13 mm respectively (table 2). The two commercial antibiotic samples showed much considerable antibacterial activity compared to plant extract and synthesized nanoparticles. The zone of inhibition for *S. aureus* is 22 mm with penicillin and 25 mm for combination of penicillin and silver nanoparticles. The zone of inhibition for *E.coli* is 29 mm with penicillin alone and 34 mm for combination of penicillin and silver nanoparticles. The zone of inhibition for *B. subtilis* is 20 mm and 30 mm for penicillin and combination of penicillin and silver nanoparticles respectively. The zone of inhibition for *Klebsiella pneumoniae* is 25 mm and 45 mm for penicillin and combination of penicillin and silver nanoparticles, respectively. The zone of inhibition for *S. aureus* is similar (30 mm) for cefixime and combination of cefixime and silver nanoparticles. The zone of inhibition for *E.coli* is 45 mm and 29 mm with cefixime and combination of cefixime and silver nanoparticles respectively. The zone of inhibition for *B. subtilis* is 30 mm and 35 mm for cefixime and combination of cefixime and silver nanoparticles respectively. The zone of inhibition for *Klebsiella pneumoniae* is same (35 mm) for cefixime and combination of cefixime and silver nanoparticles. The synergistic antibacterial activity of Cefixime and cefixime plus nanoparticles was similar against *S. aureus* and *Klebsiella pneumoniae.* Thus, the silver nanoparticles exhibited synergistic activity against all the four pathogenic strains with penicillin. Cefixime showed synergistic activity against *E.coli* and B. *subtilis*.

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S.no		Zones of Inhibition(cm)			
	Concentrations	*Staphylococcus aureus*	*E. coli*	*Bacillus subtilis*	*Klebsiella pneumoniae*
1	Plant extract	1±0.20	1	1.3±0.04	1.5±0.04
2	AgNo3	2.8±0.31	1.7±0.04	1.1±0.04	1±0.04
3	AgNPs	1±0.2	1.5±0.08	1.1±0.04	1.3±0.04
4	AS1	2.2±0.08	2.9±0.04	2±0.04	2.5±0.06
6	BS1	3±0.04	4.5±0.12	3±0.08	3.5±0.04
7	AS1+AgNPs	2.5±0.08	3.4±0.02	3±0.08	4.5±0.12
8	BS1+AgNPs	3±0.04	2.9±0.08	3.5±0.12	3.5±0.08

Table 2: Zone of inhibition of plant extracts with antibiotics.

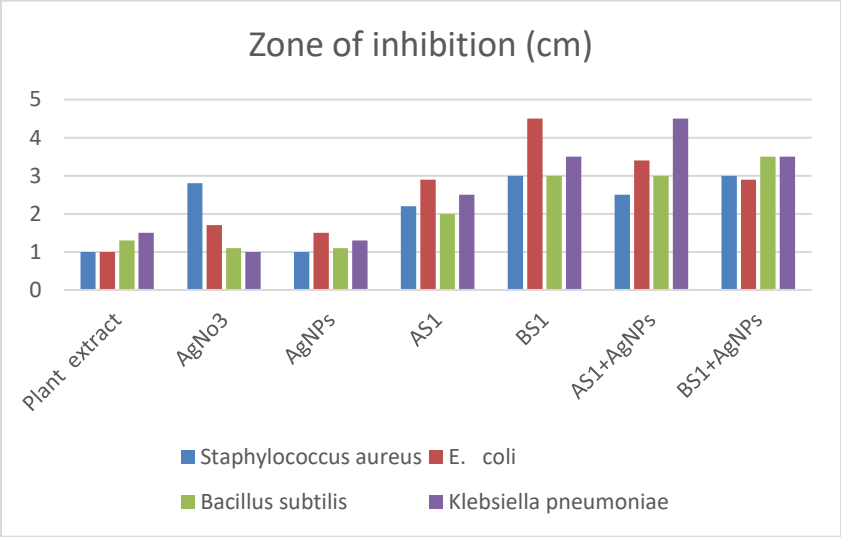

Fig.8 Zone of inhibition of plant extracts with antibiotics.

CONCLUSION

Nano particles that were synthesized showed more antibacterial activity than plant extract alone. A good synergistic effect of commercial antibiotics was observed with synthesized nanoparticles. It can be concluded that antibiotics plus AgNPs showed more inhibitory activity than antibiotics alone. The inhibition was more against pathogenic bacterial strains. As the increase of multidrug resistant microorganisms is on the rise the Green synthesized silver nanoparticles are a better alternative to commercial synthetic antibiotics. Thus, the present work carried gives a good alternative approach to treat infectious diseases.

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