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BIOLOGICALLY SYNTHESIZED SILVER NANOPARTICLES OF *CURCUMA CAESIA* ROXB. RHIZOME EXTRACT AND EVALUATION OF THEIR ANTIBACTERIAL ACTIVITY AGAINST MDR BACTERIA

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Keywords:

MDR, Curcuma caesia, Silver nanoparticles, DPPH, Antibacterial activity

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ABSTRACT: Multidrug-resistant (MDR) bacterial infection is severe health concerns in the field of medicine. The researcher used green synthesized silver nanoparticles to overcome from MDR bacterial infection. Present study revealed successful synthesis of nanoparticles from *Curcuma caesia* aqueous rhizome extract by use of silver metal as a capping agent. Synthesized silver nanoparticles were identified by various methods like UV-Vis spectroscopy, Transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and Scanning electron microscope (SEM). Silver nanoparticles having an average size of 31.92 ± 2.8 nm by TEM. The synthesized silver nanoparticles were discovered as an effective source of nanomedicine against MDR strains of *Klebsiella pneumonia*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The antibacterial activity of nanoparticles measured in the range from 11.4 ± 0.83 mm to 17.73 ± 0.91 mm. The highest zone of inhibition was reported against *Pseudomonas aeruginosa i.e.,* 17.73 ± 0.91 mm at a concentration of 30 µg/µl. Silver nanoparticles showed increased total phenolic and total flavonoid content, additionally with higher DPPH activity as compared to the aqueous rhizome extract. Present results support the green synthesis of nanoparticles that have promising advantages as antimicrobial activity against MDR and better antioxidant activity compare to aqueous extract.

INTRODUCTION: MDR (Multidrug-resistant) bacterial infection in recent years growing very rapidly, and it is the leading concern in the field of medicine as it increases mortality rate [1]. Even though the concern is for both types of bacteria, gram-positive and gram-negative. But due to absence of new effective antibiotics against gram negative bacteria attention toward them is too high. Current day antibiotics have lost their competence in handling these infections. So to overcome from MDR infections, researchers are trying to develop novel drugs or substitute. Nanotechnology offers as a promising approach for the development of novel and effective antibacterial agents by exploring its properties as nanoparticle having a high surface area to volume ratio [2, 3, 4, 5].

In this respect, nano-biotechnology is an exclusive multidisciplinary field that enables the use of nanoparticles in biomedical settings through green approaches [6]. The green synthesis of nanoparticle is cost-effective and less toxic, and it can be used as a drug [7]. Nowadays, these are extensively used as a promising bactericidal agent against MDR [8].

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Therefore, researchers are shifting towards nanoparticles synthesis, specifically silver nano-particles to resolve the problem of MDR infections [9]. *Duran et al.,* (2007) [10] reported that the use of silver metal for synthesis of nanoparticles could be useful in many medical treatments like abrasions, fungal diseases, burn cure, dental supplies and as it having low cytotoxicity with high thermal stability.

Curcuma is a large genus belonging to the family Zingiberaceae. *Curcuma caesia* Roxb is a native of North-East and Central India. It is often well-known as black or kali haldi. It is a rhizomatous herb having bluish-black rhizome color with very intense camphoraceous aroma. *C. caesia* rhizomes are historically used in several disorders like leucoderma, asthma, bronchitis, piles, tumors, *etc*. [11]. The great extent of work has been conducted on the chemical constituents of the plant and concluded that the oils of the plant possess antibacterial, antifungal properties and numerous pharmacological activities like anti-diabetic, antioxidant, analgesic, anti-cancer, antiulcer, antiviral, antitumor and antipyretic effects [12]. Recently it was reported that *C. caesia* exhibit antibacterial activity against MDR of *Myco-bacterium tuberculosis* [13]. So, considering the significance of nanoparticles and their synthesis from the medicinal plant, the present study is undertaken on "green synthesis" of silver nanoparticles by using *Curcuma caesia* rhizome extract. Antibacterial activity of synthesized silver nanoparticles has been measured against MDR strains.

MATERIALS AND METHODS:

Preparation of Aqueous Plant Extract: The rhizome was collected from the Indian Council of Agricultural Research (ICAR)-Indian Institute of Spices Research, Kerala with Accession No: 1154 (Voucher no: 266608) and grown in the botanical garden of Maharshi Dayanand University, Rohtak, Haryana. The fresh rhizomes of *Curcuma caesia* were collected in November 2017 from botanical gardens and washed with running tap water for the removal of adhering impurities. Rhizome material was cut into pieces, and 50 g of fresh rhizome was boiled in 500 ml of distilled water in 1000 ml flask for about 10 min followed by allowing it to cool down at room temperature and filtered through Whatman filter paper no 1.

Green Synthesis of Silver Nanoparticles: AgNPs were synthesized *via* Singh *et al.,* (2014) [14] method with minor modifications. Plant extract and 1mM solution of Silver nitrate were mixed in a ratio of 3:7 respectively and heat-treated at 60 ºC on magnetic stirrer for about 20-30 min. The initial indication of synthesis of the nanoparticle is by a change in color from white to dark brown. Early characterization for AgNPs was performed using a UV-visible spectrophotometer. The solution was subjected to a centrifuge for 15 min at 13000 rpm on room temperature, followed by washing to remove unbounded capping materials and lyophilized for further characterization and use.

Characterization of Silver Nanoparticles: Initial characterization of AgNPs was performed through a UV-visible spectrophotometer of Shimadzu UV-2450 spectrophotometer, Japan. Absorbance was measured after 24-48 h of AgNPs synthesis between the ranges of 300 to 600 nm. Fourier transform infrared spectroscopy (FTIR) offers a suitable, rapid, cost-effective, and reproducible technique to identify the biomolecules which are involved in the reduction of silver nitrate to silver. FTIR spectroscopy analysis was performed using Alpha FTIR-ATR (Bruker, Germany). The percentage transmittance FTIR spectrum was recorded between the wavelength ranges of 4000 cm^{-1} to 500 cm^{-1}. All the analysis was performed thrice for confirmation of spectra. Zeta potential is a technique for calculating the surface charge on nanoparticles in solution (colloids). Zeta potential indicates the dispersion and stability of the synthesized silver nanoparticles were measured. Nanoparticles having zeta potential values more than +25 mV or less than -25 mV usually have high degrees of stability. The surface charge was measured using the laser zeta meter (Malvern zeta seizer 2000, Malvern). From Scanning Electron Microscopy (SEM) technique, surface morphology of synthesized nanoparticles were studied. First lyophilized nanoparticles were coated on stabs, and then images were taken by scanning electron microscope EVO18 Zeiss (CARL ZEISS, Germany) at 20 kV voltage. The size and shape of AgNPs were estimated by transmission electron microscopy (TEM) method from the lyophilized nanoparticles dissolved in methanol. A drop of methanol nanoparticles solution placed on a copper grid and stand to dry at room temperature.

Images were acquired through Tecnai, G 20 (FEI) at 200 KV with diverse magnification. SEM and TEM analysis was carried at AIIMS, New Delhi via availing the facility of SAIF.

Antibacterial Assay: Antimicrobial activity of green synthesized nanoparticles was carried out by disc diffusion method [15]. Antibacterial activity of synthesized AgNPs of *Curcuma caesia* rhizome was tested against three multidrug resistance (MDR) bacterial strains and also with respective ATCC bacterial strain. All MDR and ATCC strains were Gram-negative. The bacterial strains used were *Escherichia coli* (MDREC1) (ATCC25922), *Klebsiella pneumonia* (MDRKP2) (ATCC 700603) and *Pseudomonas aeruginosa* (MDRPA3) (ATCC 27853).

MDR strains were obtained from PGIMS (Microbiology Department), Rohtak, Haryana, India. A stock solution of nanoparticle was prepared in $1/10^{th}$ diluted DMSO. Streptomycin discs of 10 µg/disc (Himedia Laboratories Pvt. Ltd. India) as a standard. Plates were incubated at 37 ºC for 24 h. The zone of inhibition was calculated by using Hi Antibiotic Zone Scale TM-C from Himedia (Himedia Laboratories Pvt. Ltd. India).

Total Phenolic Content and Total Flavonoid Content: Total phenolic content and total flavonoid content were estimated using the Folin-Ciocalteu method and Aluminium chloride colorimetric assay [16], respectively, with slight modification. Gallic acid and quercitin were used as a standard for the determination of phenolic and flavonoid contents, respectively. The absorbance was measured at 750 nm and 600 nm for phenolic and flavonoid content, respectively.

DPPH Free Radical Scavenging Assay: Antioxidant activity was determined with the help of DPHH free radical scavenging assay following the method of Clarke *et al.*, (2013) [17]. Ascorbic acid was used as the reference standard and the absorbance was measured at 517 nm on UV-VIS Spectrophotometer. The Lower values of absorbance show higher free radical scavenging activity.

Statistical Analysis: The result of antibacterial assay was presented as a mean and standard deviation (S.D) of three replicate. The bar diagram is made by using graph pad prism 5.

RESULTS:

Synthesis of CcAgNPs: The CcAgNPs were successfully synthesized from aqueous rhizome extract of *Curcuma caesia* with 1mM solution of silver nitrate. The color change from off white to dark brown, as illustrated in **Fig. 1**. This change of color indicates the formation of CcAgNPs, which is due to the reduction of Ag^+ ion.

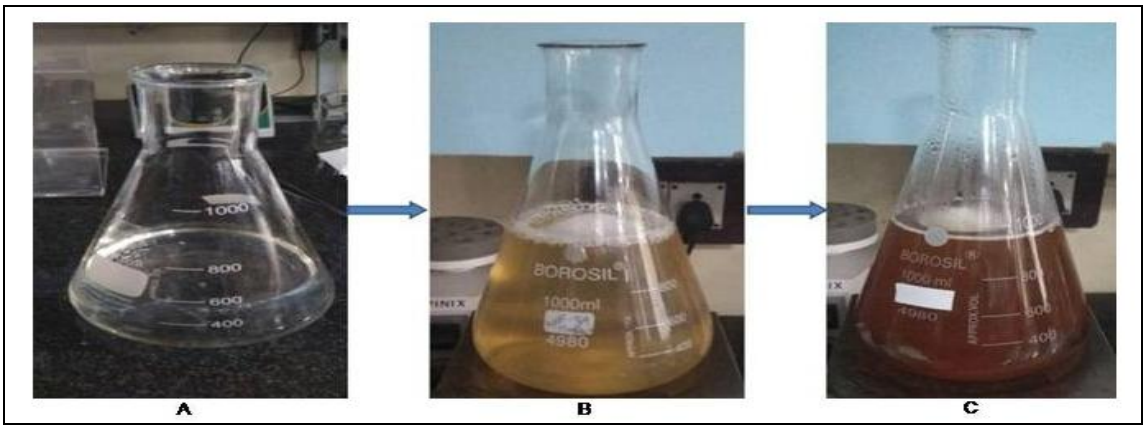

FIG. 1: IMAGE SHOWING THE SYNTHESIS OF SILVER NANOPARTICLES OF *C. CAESIA* BY CHANGING COLOUR (A) SHOWS COLOUR OF AQUEOUS EXTRACT (B) MIXTURE OF EXTRACT AND SILVER NITRATE AND (C) BROWN COLOUR INDICATING NANOPARTICLE SYNTHESIS

Characterization of CcAgNPs: Biosynthesis of CcAgNPs from aqueous solution of $AgNO_3$ with the aqueous rhizome extracts of *C. caesia* was initially confirmed by UV–Vis spectroscopy (Shimadzu). The change in color of the reaction mixture from off white to brown is due to the excitation of surface plasmon resonance (SPR) vibration of CcAgNPs.

Spectrophotometric analysis of brown color colloidal solution was monitored from 300 nm to 600 nm, and a strong peak specific to synthesized nanoparticle was observed at 420-430 nm **Fig. 2**.

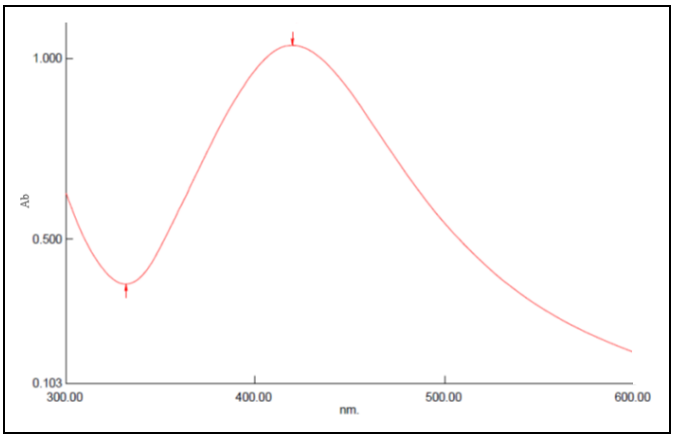

FIG. 2: GRAPH OF UV SPECTRUM OF SILVER NANOPARTICLES OF *C. CAESIA*

Analysis of rhizome mediated silver nanoparticles synthesis showed the size of CcAgNPs in the range of nanometer scale, with an average size of 31.92 ± 2.8 nm **Fig. 3** by TEM.

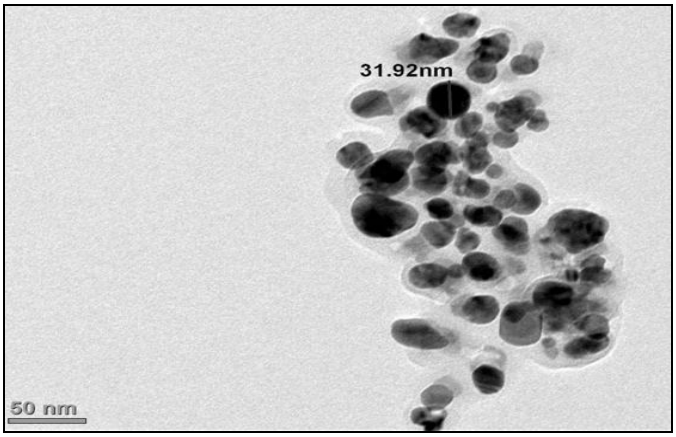

FIG. 3: IMAGE OF TEM OF SILVER NANOPARTICLES OF *C. CAESIA*

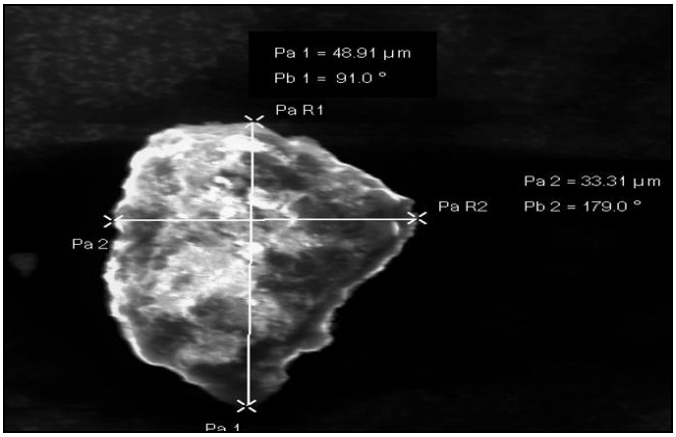

FIG 4: IMAGE OF SEM OF SILVER NANOPARTICLES OF *C. CAESIA*

The surface morphology of CcAgNPs was spherical in shape as analyzed by scanning electron microscopy (SEM) **Fig. 4**.

EDX characterization **Fig. 5** showed the absorption of the silver signal at near about 3 KeV along with carbon and oxygen signal. The average charge of the CcAgNPs was −22.8 + 5.22 mV by Zeta potential **Fig. 6**.

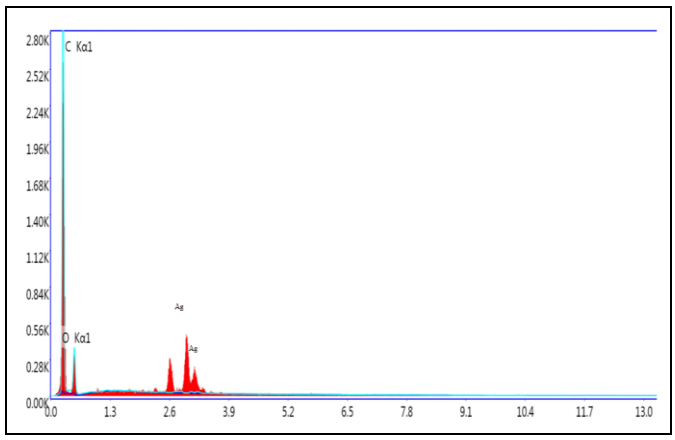

FIG. 5: IMAGE OF EDX OF SILVER NANOPARTICLES OF *C. CAESIA*

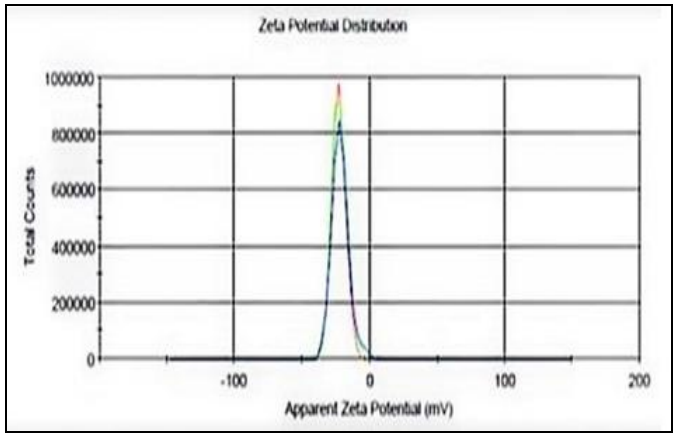

FIG. 6: ZETA POTENTIAL GRAPH OF SILVER NANOPARTICLES OF *C. CAESIA*

It is well reported that plant functional groups were involved in capping and reduction process during the synthesis of silver nanoparticle, which can be analyses by FTIR. This enable we to recognize the functional group involved in the CcAgNPs synthesis. The FTIR spectrum **Fig. 7** clearly shows the change of peak in CcAgNPs compares to aqueous rhizome extract. Shifting of a peak during the synthesis of CcAgNPs is depicted in **Table 1**.

Antibacterial Assay: Initially, CcAgNPs were tested against ATCC bacterial strain, namely *Escherichia coli* (ATCC 25922), *Pseudomonas*

aeruginosa (ATCC 27853) and *Klebsiella pneumonia* (ATCC 700603) strain **Table 2** and with the interpretation of results we further tested on MDR strains.

TABLE 1: FTIR SPECTRA OF C. CAESIA NANOPARTICLES AND AQUEOUS RHIZOME EXTRACT

Wave numbers (cm^{-1})	Chemical bond	Phytoconstituents	Peaks observed	
			CcAgNPs	Aqueous rhizome extract
3550-3200	O-H Stretching	Alcohol	3268	3269
3000-2800	N-H Stretching	Amine salt	2927	2927
2260-2190	C≡C Stretching	Alkyne	-	2249, 2208
2200-2000	N=C=S Stretching	Isothiocyanate	2200, 2170, 2116, 2024	2172, 2147, 2119, 2050
2000-1800	C=C=C Stretching	Allene	1999, 1978, 1953, 1919	1988, 1933
1800-1600	C=C Stretching	Conjugated alkene	1627	1627
1600-1500	N-O Stretching	Nitro compound	1518	1516
~1450	C-H Bending	Methyl group	1451	1455
1390-1310	O-H Bending	Phenol	1386	1396
1250-1020	C-N Stretching	Amine	1233	1227
1205-1124	C-O	Tertiary alcohol	1149	1151
1085-1050	C-O	Primary alcohol	1077	1077
1200-1000	S=O Stretching	Sulfoxide	1077, 1001	1077
1000-800	=C-H Bending	Alkene	929, 853	-
600-400	C-Br Stretching	Alkyl halide	563, 534, 516	562, 534, 505

Abbreviations: CcAgNPs, *Curcuma caesia* silver nanoparticle

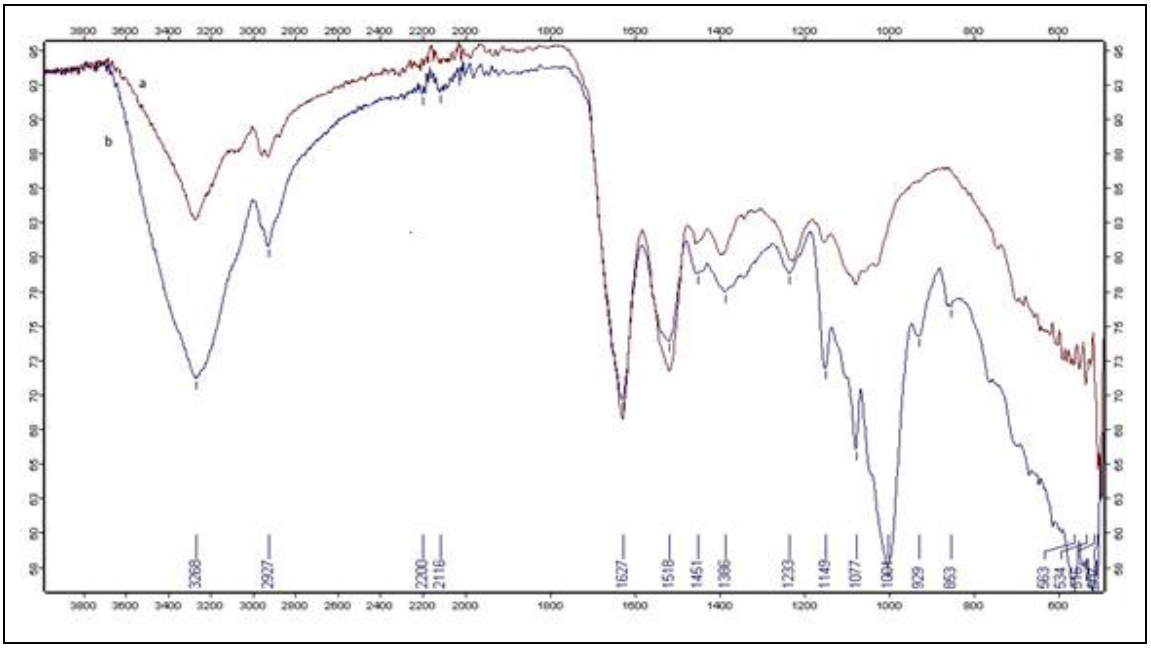

FIG. 7: IMAGE OF FTIR ANALYSIS OF SILVER NANOPARTICLES OF *C. CAESIA* (A) REPRESENT THE FTIR OF AQUEOUS RHIZOME EXTRACT AND (B) SHOWS THE SILVER NANOPARTICLES OF *C. CAESIA* FTIR

TABLE 2: ZONE OF INHIBITION OF SILVER NANOPARTICLE OF *C. CAESIA* AGAINST ATCC BACTERIAL STRAINS

S. no.	Bacterial Strain	Zone of inhibition(mm)				
		Standard	100 µg/ml	150 µg/ml	200 µg/ml	25 0µg/ml
1	*K. pneumonia*	22.7 ± 0.55	10.6 ± 0.25	10.6 ± .403	12.44 ± 0.35	14.452 ± 0.32
2	*P. aeruginosa*	22.0 ± 0.45	11.8 ± 0.46	13.03 ± 0.61	13.51± 0.25	15.8 ± 0.09
3	*E. coli*	20.7 ± 0.81	9.8 ± 0.40	10.6 ± .4	11.2 ± 0.69	11.62 ± 0.68

Note: Values are mean ± SD of three independent sets of experiments. Abbreviations: SD-standard deviation: mm-millimeter

The Multidrug-resistant strain of *Escherichia coli* (MDREC1), *Pseudomonas aeruginosa* (MDRPA3), and *Klebsiella pneumonia* (MDRKP2)) were tested at different concentration of CcAgNPs, *i.e.*, 20, 25 and 30 µg/µl to determine the efficacy of nanoparticles by disc diffusion method. The zone of inhibition of CcAgNPs against different MDR strains was shown in **Table 3** and **Fig. 8**.

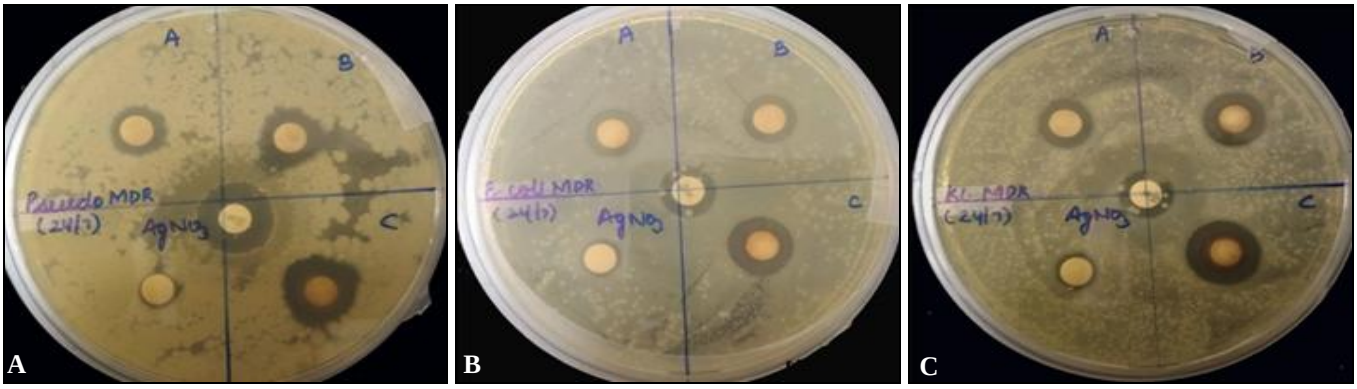

FIG. 8: ZONE OF INHIBITION OF SILVER NANOPARTICLES OF *C. CAESIA* AGAINST MDR STRAINS

P. aeruginosa was found to be most susceptible where zone of inhibition was found 17.73 ± 0.91 mm at 30 mg/ml concentration of CcAgNPs. Antibacterial efficacy was concentration-dependent. As the zone of inhibition increase with an increase in concentration, to observe the interaction of bacterial cells with CcAgNPs, TEM imaging was done on *P. aeruginosa* cultured overnight with CcAgNPs. TEM imaging **Fig. 9** clearly showed that the considerable amounts of CcAgNPs were pierced the cell membranes and entered in bacterial cells. As compared to the control cells, nanoparticle treated *P. aeruginosa* cell showed asymmetrical appearance which further support that the increased in cell membrane permeability caused by CcAgNPs. The untreated cell with an intact cell wall of bacteria, the rupturing of the cell wall, and the leakage of cellular material of bacterial cell in the extracellular matrix due to nanoparticle is depicted in **Fig. 9A**, **9B,** and **9C,** respectively.

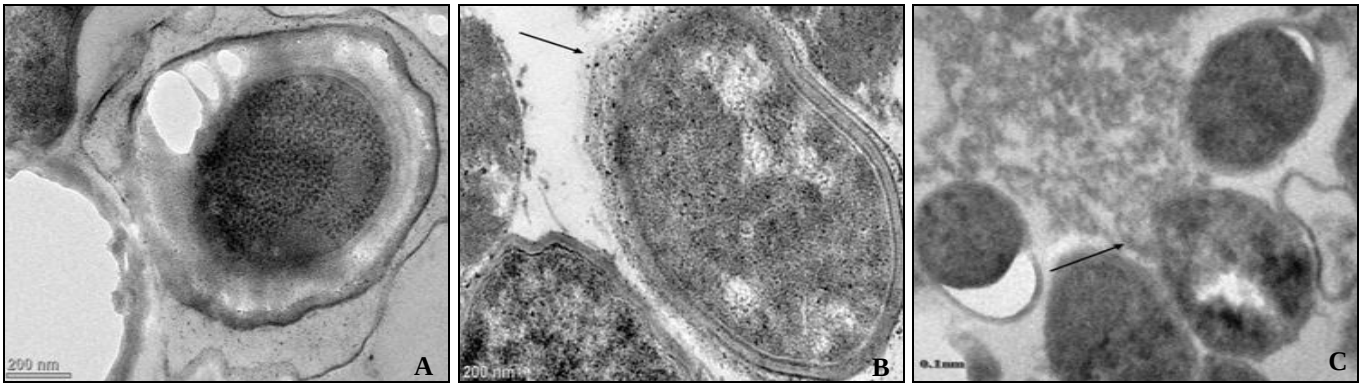

FIG. 9: TEM IMAGE OF *PSEUDOMONAS AERUGINOSA* BEFORE AND AFTER EXPOSURE TO AGNPS (A) UNTREATED CELL WITH INTACT CELL WALL (B) RUPTURING OF CELL WALL AND (C) INDICATE THE LEAKAGE OF CELLULAR MATERIAL OF BACTERIAL CELL IN EXTRACELLULAR MATRIX DUE TO NANOPARTICLES

TABLE 3: ZONE OF INHIBITION OF SILVER NANOPARTICLE OF *C. CAESIA* AGAINST MDR

S. no.	Bacterial Strain	Zone of inhibition(mm)				
		Standard	20 µg/µl	25 µg/µl	30 µg/µl	$AgNO_3$
1	*K.pneumonia (*MDRKP2)	13.6 ± 0.57	11.9 ± 0.05	13.4 ± 1.15	16.33 ± 0.57	8.66 ± 0.57
2	*P.aeruginosa (*MDRPA3)	15.7 ± 0.85	12.0 ± 0.43	13.2 ± 0.35	17.73 ± 0.90	8.33 ± 0.57
3	*E. coli* (MDREC1)	12.5 ± 0.51	11.4 ± 0.83	11.6 ± 1.06	14.16 ± 0.37	8.73 ± 1.10

Note: Values are mean ± SD of three independent sets of experiments. Abbreviations: $AgNO_3$ silver nitrate solution; SD-standard deviation; mm- millimetre

Total Phenolic Content and Total Flavonoid Content: The total phenolic content and total flavonoid content of CcAgNPs was found to be 78.443 ± 0.540 mg of GAE/mg as compared to aqueous rhizome extract (61.007 ± 0.972 mg of GAE/mg) and 14.233 ± 0.411 mg of QE/mg were observed in the aqueous rhizome extract compare to 17.663 ± 0.220 mg of QE/mg respectively **Fig. 10**.

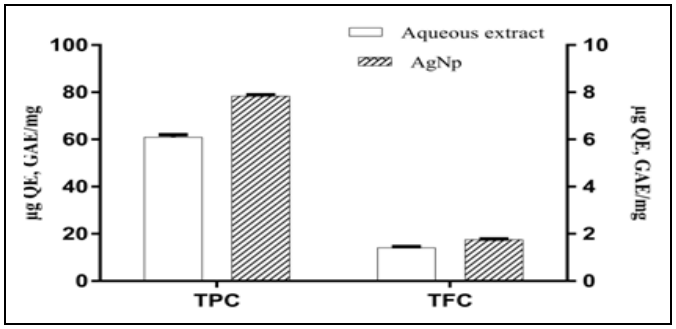

FIG. 10: BAR DIAGRAM SHOWING TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT OF SILVER NANOPARTICLES OF *C. CAESIA* WITH STANDARD DEVIATION

DPPH Radical Scavenging Assay: The anti-oxidant potential of CcAgNPs and aqueous rhizome extract were determined by calculating DPPH activity at different concentration *i.e.*, from 20 µg/ml to 100 µg/ml **Fig. 11**.

The IC_{50} valve of CcAgNPs and aqueous rhizome extract were 55.43 ± 1.49 and 58.01 ± 4.36, respectively.

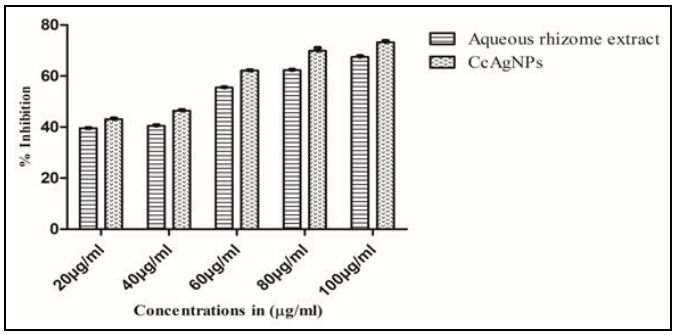

FIG. 11: BAR DIAGRAM SHOWING DPPH ACTIVITY OF SILVER NANOPARTICLES OF *C. CAESIA* WITH STANDARD DEVIATION

DPPH assay result showed a significant difference between CcAgNPs and aqueous rhizome extract. Anti-oxidants are capable of scavenging DPPH free radicals due to their capacity to give hydrogen and integrate electrons.

DISCUSSION: Green synthesis of nanoparticle has established significant consideration due to emergent need to improve biologically kindly technologies in material synthesis. The secondary metabolites of plants are used as effective medicines to control the growth of microorganisms and have less toxicity. These metabolites have substantial therapeutic applications against human pathogens. Many studies show that for the search of novel antimicrobial agents, various plant extracts were evaluated. *C. caesia* was reported to have antimicrobial efficacy, anti-diabetic and anti-mutagenic activity [18, 19, 20].

The magnificence of the present study is that green synthesized nanoparticle from *C. caesia* is effective against MDR strains in terms of novelty. We have synthesized silver nanoparticle from *C. caesia*, a species of Zingiberaceae family, which is well known for its efficacy. Studies revealed that *C. caesia* contains mainly camphor, ar-turmerone, borneol, and many more phytochemicals [21, 22]. It was also well-identified facts that phytochemicals are directly involved in the reduction of silver and development of nanoparticles. The precise mechanism of reduction varies from plant to plant because of the presence of different phytochemicals. FTIR confirmed the presence of various functional groups from IR radiation characteristic absorption peak presence. A strong peak near 420 nm showing the absorption spectra of CcAgNps characterizes the formation of nanoparticles. EDX spectrum also shows the absorption peak of silver. Further TEM, SEM, showing the size and zeta potential contribute to the firmness of CcAgNps.

The precise mechanisms by which silver nanoparticles work as bactericidal, is still under consideration. It was noticed that nanoparticles making pits and holes in cell membranes of bacteria and gather inside bacterial cells where they can damage cellular machinery and are interrupted with cell function [23]. The discharge of silver ion (Ag^+) is also an important mechanism for the bactericidal effect.

The interaction between positively charged Ag^+ and the negatively charged bacterial cell surface disrupt the selective permeability and structure of the bacterial cell membrane [5], resulting in protein leakages [24]. After penetrating the bacterial cell, Ag^+ interacts with DNA and ribosomes, which resulted in DNA damage and disrupted the protein translation [25]. TEM images revealed that the interaction of CcAgNPs with the cell membrane resulted in the rupturing of the cell membrane and secretion of cellular material in extracellular matrix **Fig. 9**.

It is well documented that the phenolic compounds may contribute directly to anti-oxidative action [26].

However, antioxidant activities are attributed to the phenolic contents in plants probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [27]. The synthesized CcAgNps were capped by the plant metabolites that prohibited their aggregation. Natural capping offers the further advantage of the stability in the green chemical synthesis [28]. Nanoparticles can act as a promising free radical scavenger [29].

The present study also shows that CcAgNps having higher antioxidant potential as compared to aqueous rhizome extract with higher DPPH activity. The outcomes of the study suggest that the green synthesized silver nanoparticles (AgNPs) can be used as natural antioxidants and as antimicrobial agents against MDR strains for health prospective after analyzing the cytotoxicity.

CONCLUSION: In the present study, a simple and rapid approach was applied for silver nanoparticles synthesis from aqueous extract of *Curcuma caesia* rhizome. The aqueous rhizome extract act as both capping and a reducing agent. The change of silver ions into silver nanoparticles takes place within 25 minutes at a temperature of 40 °C.

The functional groups of plant extracts were identified from FTIR, which shows the reduction of silver ions. Various methods were used for the characterization of silver nanoparticles, namely SEM, TEM, and UV-Visible spectrophotometer. The dose-dependent antioxidant activity and antibacterial activities against three MDR bacterial strains revealed from green synthesized silver nanoparticles.

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CONFLICTS OF INTEREST: The authors declare that they have no competing interests.

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