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Rauwolfia tetraphylla mediated synthesis of herbal nano powders by ball milling and GC-MS analysis, evaluation of their antimicrobial activity

Vasa Padmaja, Gorrepati Rosaiah*, Kakumanu Babu

Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India

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ABSTRACT

Synthesis, characterization and evaluation of biological applications of nanoparticles is of immense importance in recent years in the field of Nanotechnology. Due to increasing demand of nanoparticle synthesis from medicinal plants now a days, in the present study, it is aimed to produce bio compatible, non-toxic, homogenous nanopowders by Ball milling, one of the top-down approaches from root, stem and leaf of *Rauwolfia tetraphylla*, an important medicinal plant of Apocynaceae. *Rauwolfia tetraphylla* L., an endangered species, is rich in various phytochemicals and often used as a substitute of *Rauwolfia serpentina* and therefore aimed to produce nanopowders from different parts of this plant. These biosynthesized nanopowders were characterized by UV-Visible Spectroscopy, Scanning Electron Microscopy, X-Ray Diffraction and Fourier Transform Infrared Spectroscopy. Further bioactive components of root, stem and leaf nanopowders were analysed by Gas Chromatography-Mass Spectrometry(GC-MS), and different compounds were identified by comparison of GC-MS spectrum with library searches. Methanolic root, stem and leaf nanopowders were evaluated for antibacterial and antifungal activity. Antibacterial activity was assessed against human pathogenic bacteria *Klebsiella pneumoniae, Salmonella typhi, Vibrio cholerae, Corynebacterium diphtheriae, Vibrio parahemolyticus.* Human pathogenic fungi *Aspergillus fumigatus, Candida albicans, Mucor hiemalis* were selected for evaluating antifungal activity. The present study confirms the good antibacterial and antifungal activity of *Rauwolfia tetraphylla* synthesized nanopowders. As GC-MS analysis revealed different phytoconstituents, other pharmacological activities need to be evaluated.

*Corresponding Author

Name: Gorrepati Rosaiah
Phone: +91 9490218205
Email: gorrepati_r@yahoo.co.in

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INTRODUCTION

Nanotechnology, an emerging field of Science and Technology, has attracted the attention of researchers in recent years as it has diverse applications. Nanoparticles, the critical components of nanotechnology with a size less than 100 nm, exhibit novel and enhanced properties in terms of size, distribution and morphology compared to their bulk counterparts (Banerjee *et al.*, 2014). Nanoparticles can be synthesized by physical, chemical and biological methods. The chemical methods include the use of hazardous chemicals such as silver, gold, palladium, copper oxide, zinc

oxides (Yadi *et al.*, 2018). Among noble metal nanoparticles, silver nanoparticles are of great interest due to their unique properties and a wide range of applications. Due to their exceptional characteristics, AgNps have multi-functional bioapplications such as antibacterial, antifungal, antiviral, anti-inflammatory, antiangiogenic and antitumour properties (Zhang *et al.*, 2016).

Although AgNps exhibit promising antimicrobial activity, recent studies have reported toxic effects of AgNps at a cellular level which include generation of reactive oxygen species (ROS), DNA damage and cytokine induction. Due to the toxic effects of metal nanoparticles, there is a need to develop eco-friendly, non-hazardous, biocompatible herbal nanoparticles, which exert less side effects (Stensberg *et al.*, 2011). The biological approach of nanoparticles synthesis also called "Green synthesis", utilizes various micro organisms and plants as nano precursors (Zhang *et al.*, 2016). Nanoparticles with defined size and shape having potential biomedical applications can be synthesized from plants. Ball milling is one such method which produces homogenous nanoparticles (Karthik *et al.*, 2017) with eco friendly and avoids the use of organic solvents.

Rauwolfia tetraphylla L. is an ever green shrub and is a critically endangered species of ethnobotanical significance from Apocynaceae. It is native to West Indies and is commonly known as Wild Snake Root, Devil Pepper, Be Still tree (Mahalakshmi *et al.*, 2019). The whole plant, as well as different parts, are widely used in traditional medicine due to the presence of different bioactive secondary metabolites, especially alkaloids (Iqbal *et al.*, 2013). In traditional medicine, *R. tetraphylla* L. is used in the treatment of hypertension, cardiovascular diseases, as a tranquillizing agent in intestinal problems to stimulate uterine contractions in case of difficult delivery (Jyothi *et al.*, 2012). Till now, very little focus was exerted on the synthesis of herbal nanoparticles from *R. tetraphylla*, although different metal nanoparticles were synthesized from this plant. In the present study, we have concentrated on the synthesis of herbal nanoparticles from different parts of *R. tetraphylla* and to evaluate the different bioactive compounds present through GC- MS. In addition, an effort was also made to determine the potential antibacterial and antifungal activity of synthesized herbal nanoparticles.

MATERIALS AND METHODS

Collection of plant material

The plant *Rauwolfia tetraphylla* was collected in Srikakulam between $18^0 17^{|} 49.1064^{||}$ N and $83^0 53^{|} 48.4152^{||}$ E in Srikakulam District of Andhra Pradesh, India.

Synthesis of herbal nanoparticles

From the collected *Rauwolfia tetraphylla* plants, roots, stems and leaves were separated. To remove the dust particles, all the parts were washed twice with tap water, followed by washing with double distilled water for multiple times. Then all the parts were dehydrated under shade for 3 weeks at room temperature. The samples of root, stem and leaf were then cut into small pieces and grinded into a coarse powder with a mixture grinder, and the powders were stored in airtight containers for future use. Then the coarse powders were made into fine nanopowders by using Ball Mill (PM 200 RETSCH) with a steel vial of 125 ml volume, 7mm size zirconium balls at the department of nanotechnology, Andhra University, Visakhapatnam. Ball milling of root, stem and leaf coarse powders were done at 500 rpm for about 25 hours. Then the formation of nanoparticles was ascertained by SEM.

Characterization of herbal nano particles

UV–Visible spectroscopy (UV–VIS)

The initial characterization of nanopowders was done by UV-VIS Spectroscopy. For optical analysis, root, stem and leaf nanopowders were dissolved in carbinol and placed in a quartz cuvette at room temperature. The absorption spectra of all herbal nanoparticles was measured using UV-Visible Spectrophotometer UV-2400 PC series, Shimadzu operated at a wavelength range of 200-800 nm at a resolution of 1.0 nm.

Scanning electron microscopy (SEM)

The prepared herbal nanoparticles were subjected to SEM analysis to identify the morphology and microstructure of nanoparticles. Field emission scanning electron microscope – FE–SEM–JSM–7100 F/JEOL India Pvt., Ltd., Bangalore, with a resolution of 1.2 nm at 30 KV and 32 nm at 1 KV, was used for carrying SEM analysis.

X-ray diffraction studies (XRD)

The XRD pattern of synthesized nanopowders was analyzed using XPERT–PRO Diffractometer (PANALYTICAL) at a voltage of 45 KV and a current of 40 mA. The face centre cubic crystalline nature of synthesized nanoparticles can be known from XRD. The dried powder was coated on an XRD copper grid, and Cu K alpha radiation of 1.5406 angstorms in the range of 2 theta from 10^0 to 91^0 was used for reording the spectrum. From the width of XRD peaks, the crystallite domain size of nanoparticles is calculated

Table 1: XRD data of studied herbal nanoparticles of *R.tetraphylla*

Plant Part	Peak Position (2Θ)	Average Crystalline Size
Root	27.064330	26.02 nm
Stem	38.622260	17.95 nm
Leaf	31.714910	10.57 nm

Table 2: FTIR Spectroscopical data of *R. tetraphylla*

Wave Number (cm^{-1})	Functional Groups
Root	
2341.66,2359.02(strong, sharp peaks)	CO_2
1047.38,1039.67(strong, broad peaks)	Si-OR
1641.48,1629.90(weak, sharp peaks)	N-H bend(amine)
3398.65,3414.12,3421.83 (strong broad peaks)	Intermolecular Alcohol O-H stretch
3857.76, 3844.26(weak sharp peaks)	N-H stretch
Stem	
2343.59, 2359.02	CO_2
3446.91, 3421.83(strong, broad peaks)	Intermolecular Alcohol O-H stretch
2922.5(weak, sharp peak)	Asymmetric C-H stretch
1629.90 (weak broad peak)	C=C stretching
1053.17,1037.74(weak, broad peak)	=C-H(bending in-ring plane)
Leaf	
3423.76, 3439.19(strong, broad peaks)	Intermolecular Alcohol O-H stretch
2924.18(strong, sharp peak)	Alkane C-H stretch
1639.55,1629.90 (strong, broad peaks)	C=C stretch alkene
1442.80(weak, broad peak)	CH_2, CH_3 bend
1020.38(strong, sharp peak)	Flouro Alkanes C-X bend

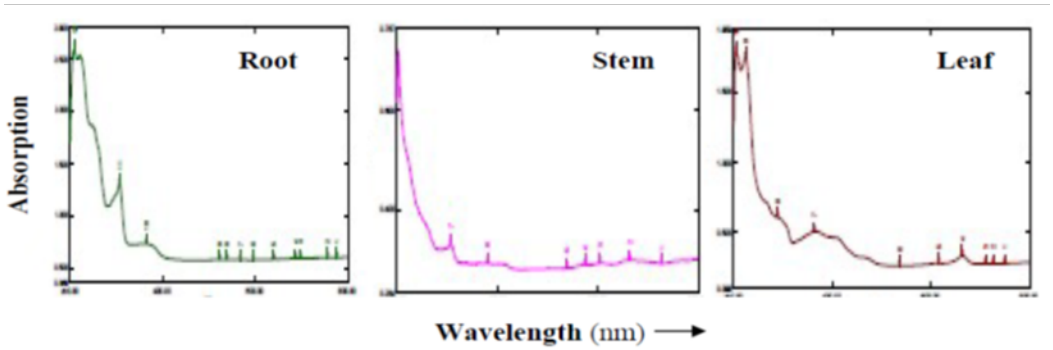

Figure 1: UV-VIS Spectrum of root, stem and leaf nanoparticles

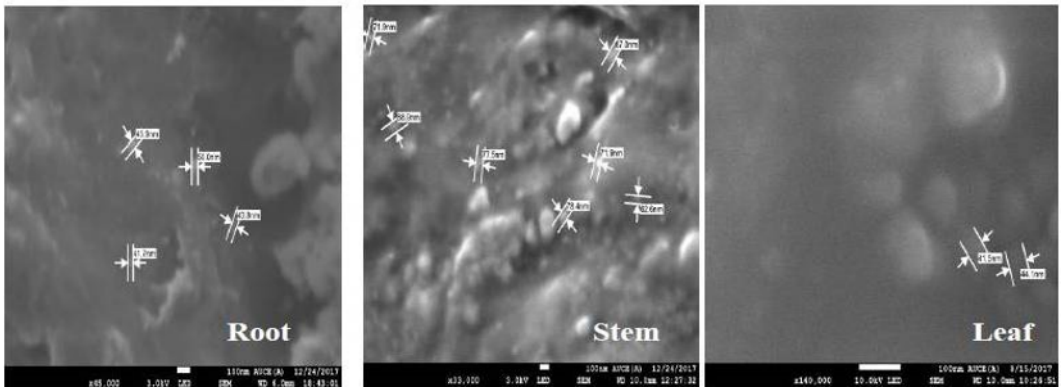

Figure 2: SEM analysis of *R. tetraphylla*

Table 3: Compounds identified through GC-MS Analysis

Plant part	Retention Time	Area%	Matching	Name of the Compound
Root				
1	1.253	97.78	99	Isopropyl Alcohol
2	1.952	0.08	93	2-pentanone,3-methyl-
3	27.243	0.08	95	Argon
4	25.620	0.05	96	Argon
5	27.125	0.05	97	Argon
6	26.133	0.04	94	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
7	26.892	0.04	98	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
8	26.716	0.15	96	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
9	23.150	0.04	98	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
10	19.992	0.04	100	Trideuteroacetonitrile
11	27.525	0.03	100	Argon
12	25.712	0.03	97	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
13	25.250	0.03	92	Beta ionone epoxide
14	23.400	0.03	97	1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic Acid
Stem				
1	1.235	98.62	99	Isopropyl Alcohol
2	32.108	0.07	85	Isobutanonitrile
3	2.137	0.06	93	Isobutyyl Ester Of Nitrous Acid
4	32.050	0.06	86	(4E,8Z)-4,8-pentacosadienol
5	33.950	0.05	88	(+,_)-3-tert-butoxy-4-(1,5-dimethyl hex-4onyl)cyclobut-3-ene-1,2-dione
6	31.750	0.04	82	Propargyl alcohol
Leaf				
1	1.244	86.21	99	2-Propanol
2	2.984	11.32	98	DMSO
3	2.147	0.07	95	Isobutyl ester of nitrous acid
4	33.275	0.11	87	3-methyl-2-(7,8-dimethyl-trans-tridec-7-ene-3,11-di-ynyl)-2-cyclopentanone
5	27.258	0.04	87	1,1,2-triflouro-2,5-bis(triflouro methyl)hexane
6	33.317	0.05	84	Acetonitrile
7	29.783	0.04	83	1,1-dibromo-2-(trimethyl sylyl) cyclo propane

Table 4: Zone of Inhibition observed in different bacteria

↓Test Organism Plant Part →		Zone of Inhibition (mm)		
	Plant Extract (μl)	Root	Stem	Leaf
K. pneumoniae	50	10±0.0	11±0.1	10±0.1
	100	11±0.1	12±0.1	12±0.0
	150	12±0.1	12±0.1	14±0.2
	200	14±0.1	14±0.1	16±0.1
S. typhi	50	11±0.1	10±0.0	10±0.0
	100	12±0.1	11±0.1	12±0.0
	150	12±0.1	11±0.0	14±0.2
	200	14±0.2	12±0.0	16±0.1
V. cholerae	50	11±0.1	11±-	10±0.0
	100	11±0.1	12±0.0	12±0.0
	150	12±0.0	13±0.1	13±0.1
	200	14±0.1	15±0.1	15±0.2
V. parahaemolyticus	50	11±0.1	-	10±0.0
	100	12±0.1	10±0.0	10±0.1
	150	12±0.1	11±0.1	12±0.1
	200	13±0.2	12±0.0	14±0.1
C. diphtheria	50	10±0.1	10±0.1	10±0.1
	100	11±0.1	12±0.1	11±0.1
	150	12±0.1	12±0.0	12±0.0
	200	13±0.1	13±0.1	14±0.0

Table 5: Zone of Inhibition observed in different fungi

↓Test Organism Plant Part →		Zone of Inhibition (mm)		
	Plant Extract (μl)	Root	Stem	Leaf
A. fumigatus	50	12±0.1	12±0.1	11±0.1
	100	14±0.0	13±0.2	12±0.1
	150	14±0.0	14±0.1	13±0.1
	200	16±0.1	15±0.0	14±0.1
C. albicans	50	12±0.1	13±0.0	11±0.2
	100	13±0.1	14±0.1	12±0.0
	150	15±0.1	16±0.3	13±0.0
	200	16±0.2	16±0.2	14±0.1
M. hiemalis	50	12±0.0	12±0.1	12±0.0
	100	13±0.1	14±0.1	16±0.1
	150	14±0.0	15±0.0	18±0.2
	200	15±0.1	16±0.1	20±0.2

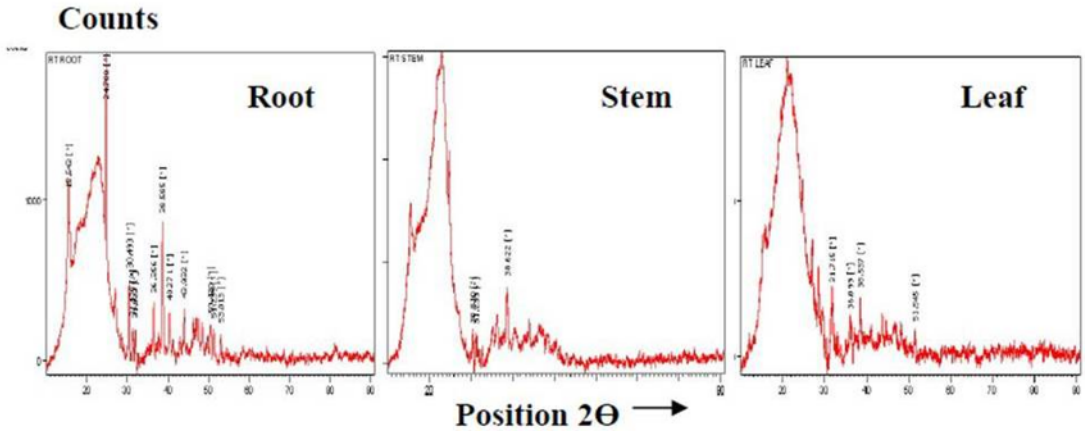

Figure 3: XRD Analysis of *R.tetraphylla*

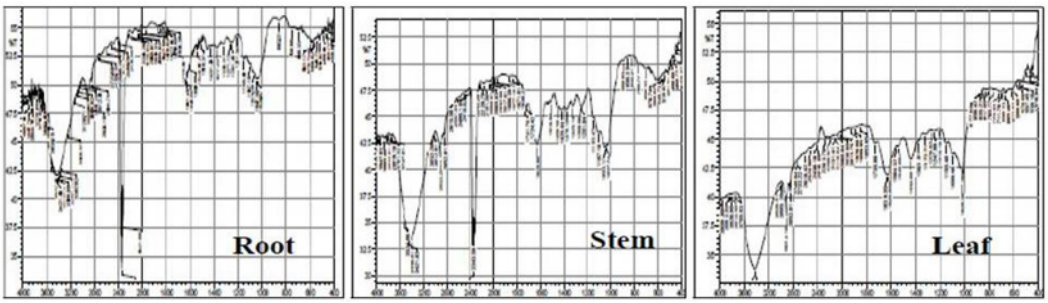

Figure 4: FTIR analysis of *R. tetraphylla*

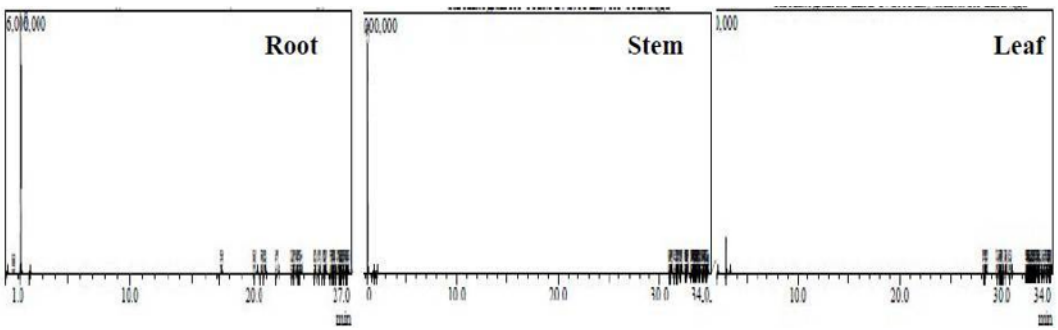

Figure 5: GC-MS chromatogram of herbal nanoparticles

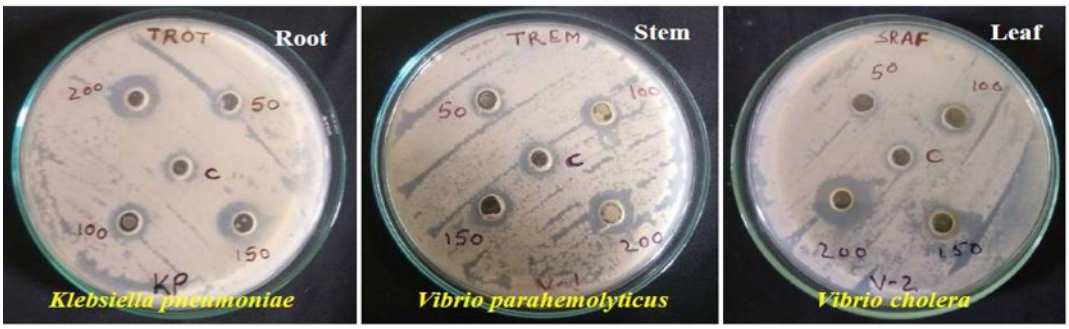

Figure 6: Antibacterial activity of nanoparticles on test bacteria MIC

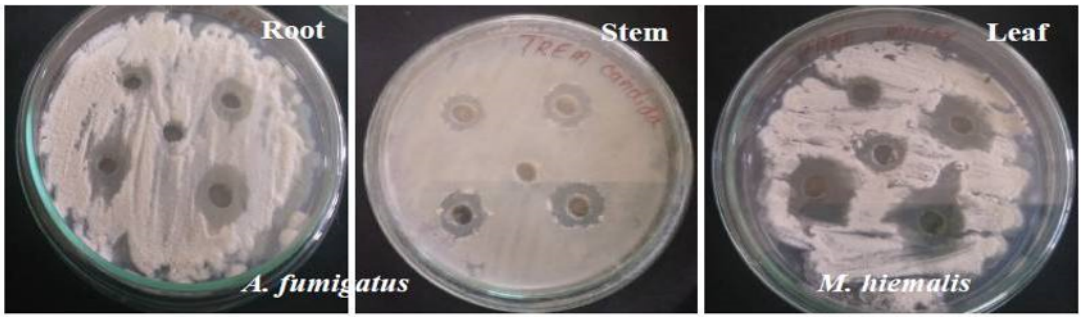

Figure 7: Antifungal activity of nanoparticles against *A.fumigatus* and *M. hiemalis*

using the Debye – Scherrer formula.

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

Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, i.e., 1.5406 angstorms, β is the full width at half maximum (FWHM), and θ is the diffraction angle.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of *R. tetraphlla* nanoparticles were obtained using FTIR SHIMADZU Spectrometer (IR Affinity 1S) in the frequency range from 400 to 4000 cm^{-1} at a resolution of 4 (1/cm) by an average of 10 scans per sample using KBr pellet. By mixing a 200:1 ratio of KBr, nanoparticles, the pellet was obtained, which was used for FTIR analysis in transmittance mode.

Gas chromatography-Mass spectroscopy (GC-MS)

GCMS QP 2010 SHIMADZU was used for performing a GC-MS analysis of the methanolic extract of the sample. 30 m X 0.25 mm ID fitted with DB – 5 methyl phenyl siloxane column was used for performing chromatography. The carrier gas was Helium with a constant flow rate of 1ml/m, and 1 microlitre of the sample was injected. The oven temperature was programmed from 80 ^{0}C for 2 minutes, with an increase of 260 ^{0}C for 10 minutes. The total running time of GC was 28 minutes. By comparing the mass spectra and retention indices of compounds of methanolic extract of different parts of *Rauwolfia tetraphylla nanopowders* with published literature and The National Institute of Standard and Technology (NIST) library data base, the name, molecular weight, molecular formula and structure of the components of the test materials were ascertained.

Antibacterial activity

The human pathogenic bacterial cultures, namely gram-positive *Corynebacterium diphtheriae* and gram-negative *Klebsiella pneumoniae, Salmonella typhi, Vibrio cholerae and Vibrio parahaemolyticus* were obtained from Dept. of Microbiology, Andhra University, Visakhapatnam. The synthesized nanopowders of *Rauwolfia tetraphylla root*, stem and leaf were tested for antibacterial activity against the above test bacteria by agar well diffusion method. Four various concentrations of herbal nanopowders, i.e. 50 μl, 100 μl, 150 μl, 200 μl were used, and antibiotic chloramphenicol (30 μg/ml) was used as positive control and DMSO (30 μl/ml) was used as a negative control. The diameter of zone of inhibition (ZOI) greater than 8 mm were measured for antibacterial activity and studies performed in triplicate and mean values for each bacteria was recorded.

Antifungal activity

The fungicidal activity of synthesized nanopowders from *Rauwolfia tetraphylla* root, stem and leaf were studied against human pathogenic fungi viz. *Aspergillus fumigatus, Candida albicans, Mucor hiemalis* by using agar well diffusion method. Antifungal activity was determined using 4 various concentrations of herbal nanopowders (50 μl, 100 μl, 150 μl, 200 μl. The antifungal drug Nystatin was used as a positive control. The diameter of zones of inhibition (ZOI) greater than 8 mm recorded for antifungal activity. Studies were performed in triplicate and mean values for each fungus was recorded.

Minimum inhibitory concentration (MIC)

MIC is defined as the minimum concentration of extract that causes 20% inhibition of test micro organisms under defined conditions (Wiegand *et al.*, 2008; Bussmann *et al.*, 2010). The incubated cultures were serially diluted to the density of 2×10^4 cells per ml. Hemocytometer was used for cell counting. 100 μL of cell culture was inoculated in tubes having 2 ml of broth. To each tube, different concentrations of extract from 50-200 μL were added, and the experiment was carried in triplicate. With every experiment, growth control was run parallel and all experimental tubes were incubated for 24-48 hours. Then optical density (OD) was measured with a spectrophotometer at 600 nm. The percentage of bacterial inhibition by each extract was computed using the following equation.

Inhibition (%)=

$$\frac{O.D\ in\ Control - O.D\ in\ Test\ Set}{O.D\ in\ Control} \times 100$$

RESULTS

Herbal nanoparticles characterization studies

UV-VIS spectral analysis

The presence of nanoparticles was confirmed by UV Visible spectrophotometry, which is a simple method. UV-Visible spectral analysis of root nanoparticles showed surface plasmon resonance peaks at 365.50 nm and 307 nm. Stem nanoparticles produced a single narrow peak at 307 nm, and leaf nanoparticles produced peaks near 664 nm, broad peak at 363.50 nm, 289.50 nm, narrow peaks at 224 nm and 206 nm (Figure 1).

SEM analysis

The micrograph clearly shows herbal nanoparticles. SEM images reveal an almost spherical structure of root, stem and leaf nanoparticles. The size of root, stem and leaf nanoparticles were 42-50 nm, 63-89 nm and 41.5- 44.1 nm, respectively (Figure 2).

XRD analysis

The XRD pattern of *Rauwolfia tetraphylla r*oot, stem and leaf nanoparticles is depicted in Figure 3. The XRD pattern confirms the crystalline nature of nanoparticles. The crystallite domain size is calculated using the Scherrer formula. Leaf nanopowders have smaller nanoparticles of size 10.57 nm. The size of the nanoparticles formed was calculated and presented in Table 1.

FTIR analysis

The presence of functional groups in nanopowders is confirmed by FTIR analysis. The FTIR spectroscopical data and functional group identification of root, stem and leaf nanopowders were presented in Table 2 and Figure 4.

GC-MS analysis

The GC-MS analysis of root, stem and leaf nanopowders shows various chemical compounds. The methanolic mixture of root nanopowder showed chemical compounds such as isopropyl alcohol, 2-pentanone,3-methyl-, argon, 1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylicacid, trideuteroacetonitrile. The methanolic herbal nanopowders of stem contains the following chemical compounds isopropyl alcohol, isobutanonitrile, isobutyl ester of nitrous acid (4E,8Z)-4,8-pentacosadienol, (+,_)-3-tert-butoxy-4-(1,5-dimethyl hex-4onyl)cyclobut-3-ene-1,2-dione, propargyl alcohol. The leaf nanoparticles extracted with methanol recorded the chemical constituents 2-propanol, DMSO, isobutyl ester of nitrous acid, 3-methyl-2-(7,8-dimethyl-trans-tridec-7-ene-3,11-di-ynyl)-2-cyclopentanone, 1,1,2-triflouro-2,5-bis(triflouro methyl)hexane, acetonitrile, 1,1-dibromo-2-(trimethyl sylyl) cyclo propane, acetonitrile, 1,1'-Bibicyclo(2.2.2)Octyl-4-Carboxylic acid. The GC-MS chromatogram and various chemical compounds identified in methanolic root, stem and leaf nanopowders were depicted in Table 3, Figure 5.

Evaluation of antibacterial and antifungal activity

Herbal nanoparticles synthesized from different parts of *R. tetraphlla* exhibited significant antibacterial and antifungal activity with degrees of variation. The methanolic herbal nanopowder extract of *R. tetraphylla* root showed ZOI between 13-14 mm against all test bacteria, which is on par with positive control chloramphenicol (14 mm). Similarly, stem and leaf nanopowder extracts showed more ZOI than positive control against all test bacteria. The maximum ZOI of stem nanopowder was 15 mm against *Vibrio cholerae* and leaf nanopowder was16 mm against *V. parahemolyticus* (Table 4). Antibacterial activity of nanopowders on test bacteria is depicted in Figure 6.

MIC

The concentration of plant extract required to prevent the growth of 50% of organisms tested is referred to as MIC 50. The MIC of methanolic root nanopowder extract was observed at 200 μl/ml for *Klebsiella pneumonia*e, *Salmonella typhi, Vibrio cholerae, Vibrio parahemolyticus*. The MIC 50 of *Rauwolfia tetraphylla* stem nanopowders is 150 μl/ml for *Klebsiella pneumoniae*, *Vibrio cholerae*, 200 μl/ml for *Vibrio parahemolyticus* and 200 μl/ml for *Corynebacterium diphtheriae*. The MIC 50 of *R. tetraphylla* leaf nanopowders is 150 μl/ml against *Klebsiella pneumoniae, Salmonella typhi, Vibrio cholerae, Corynebacterium diphtheriae*.

Antifungal activity

The good anti fungal activity was shown by *R. tetraphylla* root nanopowders against all tested fungi with ZOI between 15-16 mm, which is more than control Nystatin, while stem nanopowders showed ZOI between 10-13 mm and leaf nanopowders showed ZOI between 14-20 mm (Table 5). The antifungal activity of nanopowders on test fungi is depicted in Figure 7.

MIC

The MIC of methanolic root nanopowder extract was observed at 150 μl/ml for *Aspergillus fumigatus* and *Candida albicans*, 200 μl/ml for *Mucor hiemalis*. The

MIC_{50} of stem nanopowders was 150 μl/ml for *Candida albicans, Mucor hiemalis, Aspergillus fumigatus*. The MIC of leaf nanopowders was 100 μl/ml for *Mucor hiemalis,* 200 μl/ml for *Candida albicans.*

DISCUSSION

Herbal nanoparticles were synthesized from different parts of *Rauwolfia tetraphylla* by a novel method in the present study. The ball milling method, one of the top-down approaches, was adapted for nanoparticle synthesis, which is eco-friendly. The morphology of nanoparticles appeared almost spherical under SEM with a size range between 42-89 nm. In the present study, the crystallite nature of nanoparticles was confirmed by XRD, and the root, stem and leaf nanoparticle size from XRD analysis was calculated as 26.02 nm, 17.95 nm and 10.57 nm, respectively, and tally with the results of sliver nanoparticles derived through bioreduction in *Rauwolfia tetraphylla* (Vinay *et al.*, 2019) The presence of nanoparticles was confirmed by UV-Visible spectral analysis and root, stem and leaf nanoparticles produced various absorbance peaks the broad peak at 363.50 nm, 289.50 nm and narrow peaks at 224 nm and 206 nm respectively. These results correlate with the results produced by herbal nanoparticles synthesized by ball milling from *Tridax procumbens* leaf (Karthik *et al.*, 2017). Spectra with two absorption maxima in the range of 230-290 nm and 300-350 nm in the UV-Visible spectrum indicates the presence of flavanoids (Ranjana and Mendhulkar, 2015). The FTIR spectroscopic analysis of a methanoic extract of root, stem and leaf nanopowders reveals the presence of certain functional groups in the infra red region. Root nanoparticles showed absorbance peaks at 2341.66, 2359.02 cm^{-1} which confirms the presence of CO_2, 3090.07cm^{-1} confirms aromatic C-H stretch, 3421.83, 3414.12,3398.65 cm^{-1} confirms intermolecular bonded alcohol O – H stretch of alcohols, phenols (Jyoti *et al.*, 2016) and 3857.76, 3844.26 cm^{-1} confirms N-H stretch. The absorbance peaks of Stem nanoparticles observed at 2343.59, 2359.02 cm^{-1} confirms CO_2, 3446.91, 3421.83 cm^{-1} confirms intermolecular bonded alcohol O-H stretch of phenols, alcohols, hydrogen-bonded flavanoids, tannins (Sangeetha *et al.*, 2016; Nandiyanto *et al.*, 2019), peak at 2922.5cm^{-1} confirms asymmetric C–H stretch and peak at 1629.905cm^{-1} confirms C=C stretching, weak, broad peak at 1053.17,1037.74 cm^{-1} confirms =C-H bending in-ring plane (Karthik *et al.*, 2017; Coates, 2006). The absorbance peak of leaf nanoparticles at 3423.76, 3439.19 cm^{-1} confirms the presence of intermolecular bonded alcohol O – H stretch. A peak at 2924.18 cm^{-1} confirms the presence of C–H bending mode, and a peak at 1020.38 cm^{-1} confirms the presence of flouroalkanes C–X bend, peak at 1639.55,1629.90 cm^{-1} confirms C=C stretch of alkene (Jyoti *et al.*, 2016; Coates, 2006). The peaks observed between 800-1700 cm^{-1} in the herbal nanoparticles studied indicate the presence of flavanoids (Kumar *et al.*, 2018)

Twenty-one peaks were identified in the GC-MS chromatogram of root nanopowder, indicating the presence of 5 different compounds besides a number of peaks with narrow retention time. Stem nanopowder showed 6 peaks in the GC-MS chromatogram, indicating the presence of 6 different compounds and leaf nanopowder showed 9 peaks indicating the presence of 9 different compounds besides a number of peaks with narrow retention time. The reported compounds from GC-MS were not identified elsewhere in this species and other species (Thinakaran *et al.*, 2009; Hussain *et al.*, 2015; Sivaraman *et al.*, 2017). The chemical compounds identified through GC-MS analysis in root, stem and leaf nanopowders were presented in Table 3. Argon identified in root nanopowder possess neuro protective ability (Nowrangi *et al.*, 2014). Methane sulfinyl bis (DMSO) identified in leaf nanopowder possess antibacterial activity (Ansel *et al.*, 1969)

The inhibitory effect of root, stem and leaf nanopowders on test bacteria and fungi was more than standard antibiotic. The highest zone of inhibition of *R. tetraphylla* root nanopowder was 14 mm against *Klebsiella pneumoniae*, *Salmonella typhi, Vibrio cholerae*. The highest zone of inhibition of stem nanopowder was 15 mm against *Vibrio cholerae*. The highest zone of inhibition of leaf nanopowder was 16 mm against *Klebsiella pneumoniae*, *Salmonella typhi*. Compared to a zone of inhibition of standard fungal antibiotic Nystatin, whose ZOI is 12 mm, root nanoparticles showed the highest ZOI against *Aspergillus fumigatus, Candida albicans* (16 mm). Stem nanoparticles showed the highest ZOI (16 mm) against *Candida albicans,Mucor hiemalis*, and leaf nanopowders showed the highest ZOI (20 mm) against *Mucor hiemalis*, although all nanopowders are potent inhibitors of all tested fungi. In the MIC assay, the inhibition percentage of bacterial and fungal growth increased with an increase in the concentration of nanopowder extract. The root nanopowder extract showed MIC_{50} at a conc. Of 150 μl/ml against *Vibrio cholerae*, Stem nanopower extract showed MIC_{50} at a conc. of 150 μl/ml against *Klebsiella pneumoniae, Vibrio cholerae,* whereas leaf nanopowder extract showed MIC_{50} at a conc. of 150 μl/ml against *Klebsiella pneumoniae*, *Salmonella typhi, Vibrio cholerae and Corynebacterium diphthe-*

riae. The root nanopowder extract showed MIC_{50} at a conc. of 150 μl/ml against *Aspergillus fumigatus, Candida albicans.* Stem nanopowder extract at a conc. of 150 μl/ml against all three tested fungi and leaf nanopowder extract at a conc. of 100 μl/l against *Mucor hiemalis*. These nanoparticles exhibited excellent antibacterial and anti-fungal properties compared to metal nanoparticles synthesized from the same species (Jakaria *et al.*, 2016; Kalaiarasi *et al.*, 2013).

CONCLUSIONS

In the present work, an eco-friendly method of ball milling was adapted without using harsh chemicals for the synthesis of nanoparticles from different parts of *R. tetraphylla,* a medicinal plant which has long been used in traditional Indian medicine. The obtained nanopowders from different parts of this plant were characterized by different analytical techniques like UV-VIS Spectroscopy, SEM, XRD analysis and FTIR analysis. The GC-MS analysis of methanolic nanopowders revealed different phytoconstituents in different parts reported for the first time in this plant with potential biological activities. This observation helps in the exploration of herbal nanoparticles application in other pharmacological activities.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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