Short Communication

Phytogenic synthesis of silver nanobactericides for anti-biofilm activity against human pathogen *H. pylori*

Ashwini Prasad[1] · Syed Baker[2,3] · M. N. Nagendra Prasad[3] · Aishwarya Tripurasundari Devi[3] · S. Satish[4] · Farhan Zameer[5] · Chandan Shivamallu[1]

Abstract

The present study reports the phytogenic synthesis of silver nanobactericides using *Acorus calamus* L. and their anti-biofilm activity against clinically isolated *H. pylori*. The synthesis was confirmed with change in the color of the reaction mixture to brown. The increased in the color intensity was periodically monitored with UV–visible spectroscopy which displayed maximum absorption at 410 nm. The biomolecular interaction was studied with FTIR spectral measurements of silver nanobactericides which revealed the presence of broad absorbance band appearing at 3361 is due to OH group and the prominent peak at 1634 correspond to an amide group. X-ray diffraction (XRD) displayed Bragg's intensities at 2θ angle reflecting (111), (200), (220) and (311) of the face centered cubic (fcc) structure of silver which was compared with standard XRD pattern. The morphological characteristics of nanobactericides were studied using Transmission electron microscopy (TEM) analysis which revealed the polydispersity of nanoparticles with size ranging from 5 to 60 nm. The anti-biofilm activity of silver nanobactericides against *H. pylori* was measured using crystal violet and ruthenium red assays which revealed 350 µg/mL to be more effective. The obtained activity was validated with standard antibiotics amoxicillin. Overall, the results obtained in the present investigation are promising enough to reveal the efficacy of silver nanoparticles to inhibit the biofilm production.

Keywords *Acorus calamus* L. · Anti-biofilm · *H. pylori* · Crystal violet assay · Ruthenium red assay

1 Introduction

The emergence and growing rate of drug resistance has resulted in scarcity of potent antibiotics thus affecting all forms of lives [1, 2]. The drug resistant pathogens have high virulence rate which elevate the mortality and morbidity rates [3]. One of the favorable habitats for drug resistant pathogens to explore is human body and hospitals which are often unrecognized [4]. In most of the cases, the normal flora is being treatment without diagnosing the real causative agents. Apart from ESKAPE pathogens which are recognized as the source of drug resistant pathogens, there are untraced pathogenic microbes which are often more dangerous [5, 6]. One such pathogen includes *H. pylori* which is regarded as one of the most common causes of microbial infections among different communities. According to World health Organization (WHO), *H. pylori* is ranked as Class I pathogen responsible for various gastric cancers which has been documented with different epidemiological studies which highlights the

✉ Chandan Shivamallu, chandans@jssuni.edu.in | [1]Department of Water & Health, Faculty of Life Sciences, JSS Academy of Higher Education & Research, Sri Shivarathreeshwara Nagara, Mysore 570015, India. [2]Department of Microbiology, Krasnoyasrk State Medical University named after Prof. VF. Voino-Yasenetskiy, Partizana-Zheleznyaka Street, 1, Krasnoyarsk, Russia 660022. [3]Department of Biotechnology, JSS Science and Technology University, Sri Jayachamarajendra College of Engineering, Mysore 570015, India. [4]Biotechnological Laboratory, Department of Studies in Microbiology, University of Mysore, Manasagangothri, Mysore-6, India. [5]Department of Biochemistry, School of Basic and Applied Sciences, Dayananda Sagar University, Shavige Malleshwara Hills, Kumaraswamy Layout, Bangalore 560078, India.

SN Applied Sciences (2019) 1:341 | https://doi.org/10.1007/s42452-019-0362-2

Received: 8 January 2019 / Accepted: 11 March 2019 / Published online: 14 March 2019

pathogenicity and its ability to induce cancer. *H. pylori* is a Gram –ve bacterium which colonizes gastric mucosa and is reported to inhabit and affect nearly half of the world's population [7, 8]. Morphologically, it is spiral or helical shaped which drives by flagella and moves and disturbs the stomach lining [9, 10]. The disruption caused by *H. pylori* is also associated with release of gastric acid, gastrin, cytokines and chronic inflammation process [11]. The symptoms may vary from among individual and degree of infection which ranges from nausea, vomiting, loss of appetite, bleeding which may lead to anemia, weakness and fatigue [12]. The recent up gradation of scientific domain with materials functioning at nano-scale has transformed new era of nano-revolution [13, 14]. The progress of nano-related products has influenced all spheres of lives [15]. There are innumerable commercialized products already gained success at global market [14, 16]. There are numerous methodologies to synthesize nanoparticles and most of these methods are not feasible to produce nanoparticles for medical applications [17]. Hence there is new paradigmatic shift towards producing nanoparticles using plants [17–20]. One such plant includes *Acorus calamus* L. The selected plant is rhizomatous herb of moist habitats of Asian countries and some parts of America and Europe [21]. Scanty reports on this plant have resulted to have its application in ancient medicine especially in Ayurvedic formulation [22]. To best of our knowledge less explorative studies has been carried on *Acorus calamus* L. towards evaluating its potential against *H. pylori* and synthesis of silver nanobactericides. Based on these considerations, the present study was designed and executed towards employing *Acorus calamus* L. to synthesize silver nanobactericides and their evaluation against clinically isolated *H. pylori*. Scientific studies report the effect of silver nanobactericides on wide range of microbial pathogens [23–26]. These properties can be highly valuable against treatment of drug resistant pathogens. To best of our knowledge, scanty reports are available on activity of silver nanoparticles against biofilm of pathogens like *H. pylori*. Hence the present study reports the brief communication to synthesize silver nanoparticles using *Acorus calamus L.* and their activity against *H. pylori*.

2 Materials and methods

2.1 Selection of plant species and preparation of extract

Plant species were selected from abundant growing area of Mysore, Southern India. Initially plants were processed by washing under running tap water followed by double distilled water. Later, plants were treated with 5% of sodium hypochlorite and washed with sterile water and dried aseptically in hot air oven at 50 °C. The dried plant source was powdered in a mixer and was sieved to obtained fine powder which was stored until further experiments.

2.2 Synthesis of nanoparticles from selected plants

The processed plant material was boiled for 30 min to obtain aqueous extract. The aqueous extract obtained was evaluated for synthesis of silver nanoparticles by treating it with 1 mM silver nitrate solution. The synthesis was initially monitored with visual observation followed by analysis under UV–visible spectroscopy in the spectral range of 200–800 nm at different time intervals. The synthesized silver nanoparticles were characterized using FTIR analysis to reveal the phyto-constitutes and functional groups. The crystalline nature of nanoparticles was studied using XRD. The morphological characteristics of nanoparticles were analyzed using TEM [25, 27].

2.3 Anti-biofilm activity of silver nanoparticles

The silver nanoparticles were assessed for anti-biofilm activity against *H. pylori* as per the protocol described by Filoche et al. (2005) with slight modification. In brief, the cultured biofilm was freshly added with 200 µLHAMF'12 media which was supplemented with tests samples varying different concentration of plant extract and silver nanoparticles ranging from 5,50,200,400,500 µg/mL. The test samples were incubated for 48 and 72 h. The activity of antibiofilm was measured by crystal violet and ruthenium red dye reduction assay [28].

3 Results and discussion

3.1 Plant preparation for synthesis of nanobactericides

The plant extraction was obtained by heating of plant materials for 3 h on magnetic stirrer. The mixture was sieved using a muslin cloth followed by centrifugation at 8000 rpm for 20 min to remove plant debris. The clear supernatant was collected and aseptically maintained and evaluated for future studies. The aqueous extract was used to synthesize nanobactericides which was initially screened and optimized to achieve the rapid synthesis process by studying the individual parameters like pH, temperature, concentration and ration of metal salts. The initial synthesis was confirmed with change in the color of reaction process from pale yellow to dark brown color. The synthesis of nanoparticles was rapid and completed within

20 min of incubation time. The synthesis was maximum at alkaline pH and elevated temperature above 70 °C. These results are incongruence with previous findings highlighting the worthiness of the parameters [23, 26, 29].

3.2 Characterization of nanobactericides UV–visible spectroscopy analysis

The synthesis was further confirmed with UV–visible spectroscopy of synthesized nanobactericides with prominent absorption peak occurring in the range of 300–500 nm with maximum absorbance at 424 nm (Fig. 1). The peak was sharp and it was observed that the intensity of the reaction mixture increased with the increased in the temperature. Further, the synthesis was highest with alkaline pH compared to other pH and metal concentration at 1 mM displayed more synthesis compared to 0.5, 1.5 and 2 mM. Similarly, the ratio of the metal salt: plant extract was also studied which showed 7:3 as optimal ratio to synthesize maximum nanobactericides. Similar findings have been well reported with plant mediated synthesis of nano-silver which reported the temperature dependent property for rapid synthesis [24]. Also, maximum absorbance at 424 nm has been reported which might be due to the phyto-reduction of metal salts. The increased intensity attributed deep brown color which is due to surface plasma resonance owing to optical properties of nano-silver [30]. The surface plasma resonance is the conduction of electrons on the metallic surface which undergoes oscillation upon excitation of light resulting in unique absorption properties in comparison with its bulk components.

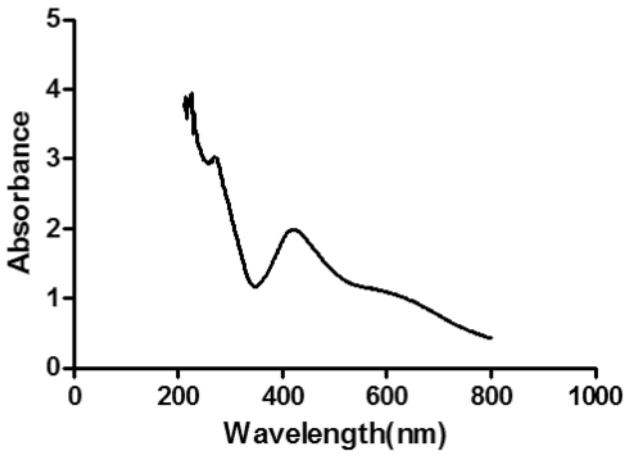

Fig. 1 UV–visible spectroscopic analysis of silver nanobactericides from *Acorus calamus* L.

3.3 FTIR analysis of silver nanobactericides

The scientific studies related to plant mediated synthesis of silver nanobactericides reports the interaction of phyto-constituents which influences the optical and applicative properties of nanobactericides [25]. The interaction of phyto-constituents and the reduction of silver nitrate to produce nanobactericides were studied using the FTIR analysis which displayed broad absorbance band appearing at 3361 due to OH group and the prominent peak at 1634 corresponds to an amide group (Fig. 2). Perusal of studies envisions the spectral analysis of FTIR to determine the bio-molecular interaction of organic molecules corresponding to phyto-constituents and obtained results coincides with earlier findings [31–33]. In the present investigation, the FTIR analysis reveals the reduction of metal salts and capping of phyto-molecules with silver nanobactericides. Earlier studies also confer the role of capping agents to attenuate the biological properties to the synthesized nanobactericides [33]. Unlike the other conventional methods which require the external agents in the synthesis protocol to stabilize the nanobactericides, biological mediated synthesized nanobactericides are reported to be stabilized with the biological entities which participate in the reduction process of metal salts [25]. The prior studies on *Acorus calamus* L. has already reported to bear chemical

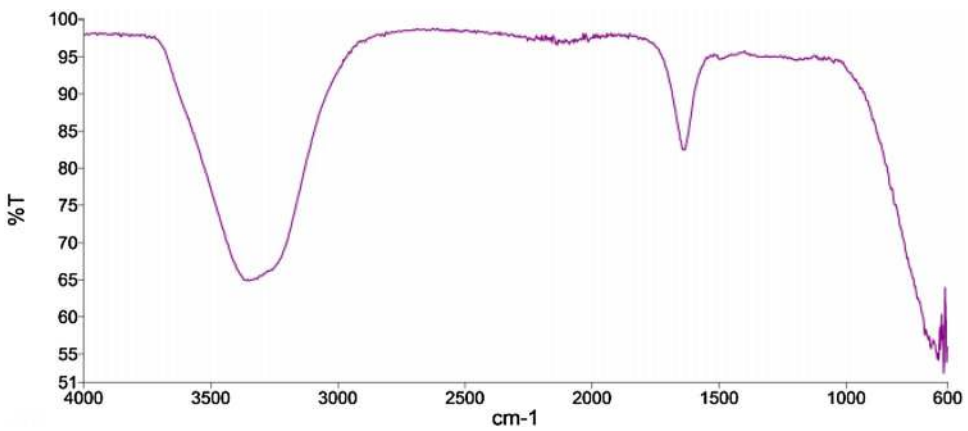

Fig. 2 FTIR analysis of silver nanobactericides synthesized by *Acorus calamus* L.

diversity of phyto-components which still under the exploration. These results are in accordance with the previous reports highlighting the role of carbonyl, aliphatic, amide, and aromatic groups facilitating the synthesis and stabilization of nanoparticles [34, 35].

3.4 X-ray diffraction analysis

X-ray diffraction is ideal tool to study nanomaterials with at least one dimension in the size ranging 1–100 nm. It probes the structure of nanomaterials and measures the atomic spacing which provides the arrangements of atom at interfaces. In the present investigation, X-ray diffraction displayed Bragg's intensities at 2θ angle reflecting (111), (200), (220) and (311) of the face centered cubic (fcc) structure of silver with peaks occurring at 31.6°, 38.74°, 44.90° and 66.4° (Fig. 3). The obtained results is in well agreement with the earlier reports of plant mediated synthesized nanobactericides which reports the lattice indexed of silver nanobactericides and the additional peaks marked as star might be due to the capping agents and functional moieties attached to nanobactericides. The obtained results coincide with earlier scientific studies which provide the insight on the X-ray diffraction of plant mediated synthesis. For instance, according to study conducted by Syed et al. [24] the diffraction pattern was measured between 10° and 80° at 2θ angle which displayed fcc structure of silver. The nanoparticles were synthesized from plant *Chamerion angustifolium* inhabiting Siberian region which displayed additional peaks due to bioorganic phase in plant extract. Numerous scientific studies confer the presence of additional studies [35, 36].

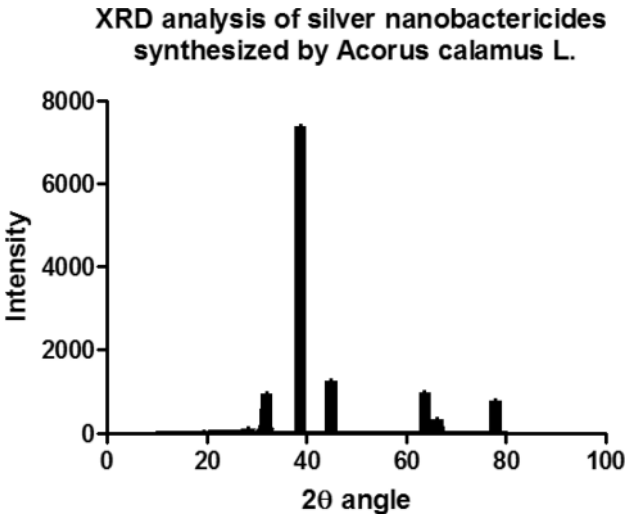

Fig. 3 XRD analysis of silver nanobactericides synthesized by *Acorus calamus* L. *Note* The star indicates the presence of phyto-components bound to nanobactericides

3.5 TEM, DLS and zeta potential analysis

The morphological characteristics of nanobactericides were studied using TEM analysis of which revealed the poly dispersity of nanobactericides with different size and shape. The size of the nanobactericides ranged from 5 to 60 nm with average size of the nanobactericides was found to be 20 nm (Fig. 4). It has been well demonstrated that size and shape of the nanobactericides play vital role for applicative properties and influences the activity [37]. The majority of silver nanobactericides were spherical and near to spherical which were well dispersed and separated as shown in the Fig. 4. The DLS study was conducted and histogram was constructed which was in accordance with the TEM analysis as shown in the Fig. 5a which demonstrate the size distribution based on the size pattern with average size between 20 and 30 nm. In addition zeta potential value was determined to measure the stability which displayed negative value −9.5 mv as shown in the Fig. 5b which indicated the stability of nanoparticles. This negative value might be due to the

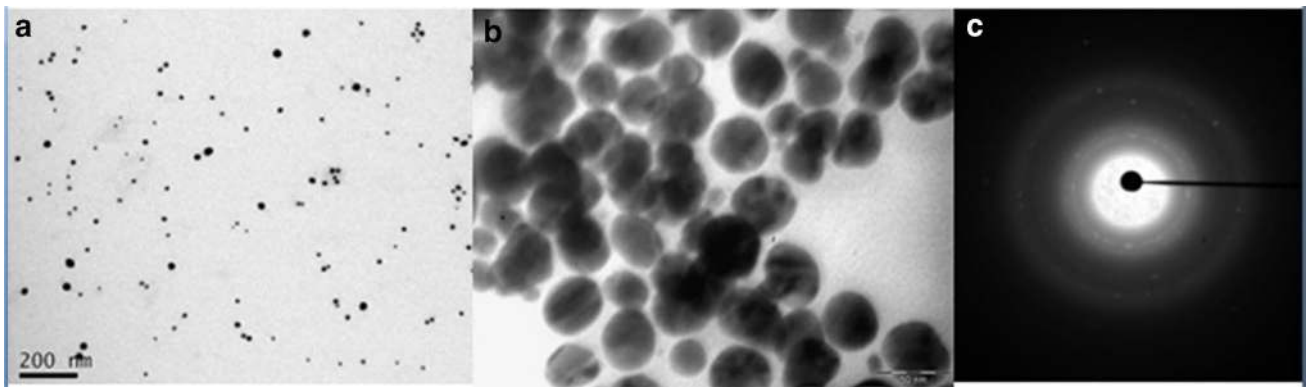

Fig. 4 TEM analysis of silver nanobactericides synthesized by *Acorus calamus* L. **a** TEM analysis at 200 nm scale. **b** TEM analysis at 50 nm scale. **c** Diffraction pattern of single pattern

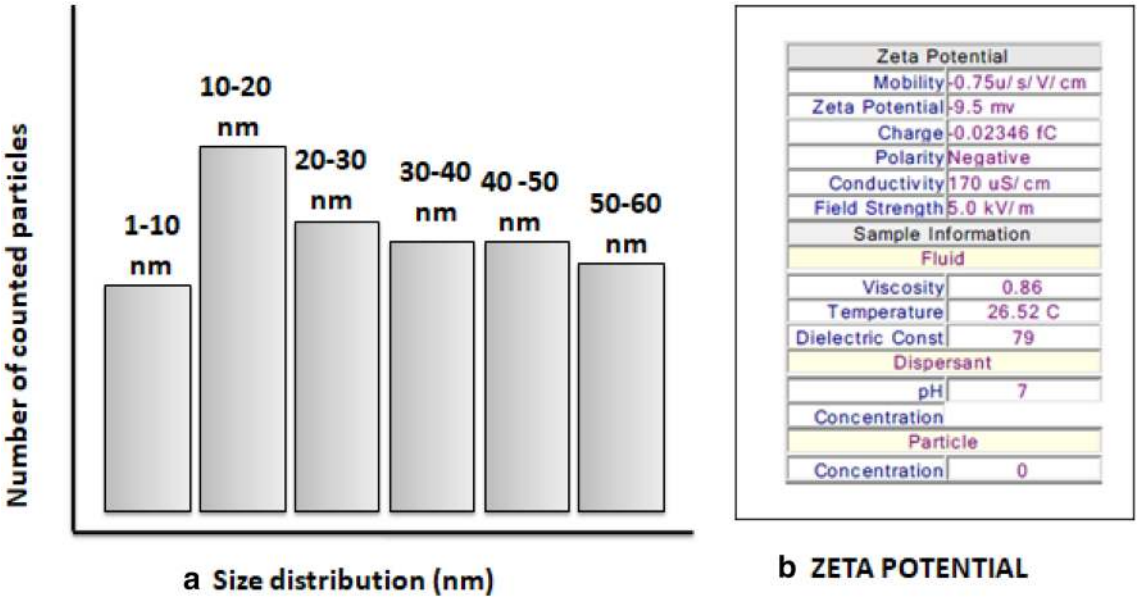

Fig. 5 Size distribution analysis and Zeta potential of silver nanobactericides synthesized by *Acorus calamus* L.

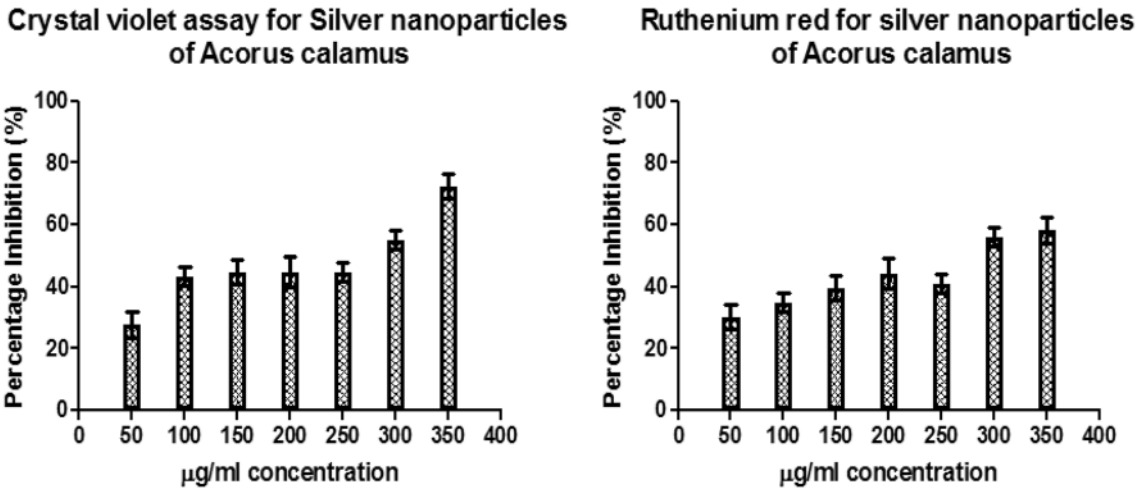

Fig. 6 Anti-biofilm potential of silver nanobactericides synthesized from *Acorus calamus*, on *H. pylori* biofilms. **a** Crystal violet assay for assessing biofilm biomass expressed in terms of percentage inhibition. **b** Ruthenium red assay for exopolymeric substances expressed in terms of percentage inhibition

capping phyto-components to attribute the stability [38]. This result coincides with the FTIR analysis which indicates the presence of functional groups bound to nanoparticles.

3.6 Anti-biofilm activity analysis of silver nanobactericides

In the present investigation, anti-biofilm activity of silver nanobactericides synthesized from *Acorus calamus* L. was assessed against *H. pylori*. The activity was measured using in crystal violet assay with different concentration of nanobactericides and compared with the antibiotic. The crystal violet assay evaluates the strength of the bio film based against the density of the cells. It was observed that, the activity was dose dependent and antibiofilm activity was increased as the concentration of nanobactericides were increased and highest activity at concentration 350 µg/mL with crystal violet assay. In the case of ruthenium red assay, the highest activity was obtained at 350 µg/mL. The activity was compared with standard amoxicillin at concentration 50 µg/mL as control for validation (Fig. 6). The quantification assay clearly suggested efficacy of silver

nanobactericides to act efficiently against eradicating the formation of biofilm. The obtained results are in accordance with the previous findings which report the silver nanoparticles to suppress the growth of pathogens and prevent biofilm formation.

4 Conclusion

The results obtained in the present investigation are promising enough to report plant mediated synthesis of nanobactericides and their efficacy for anti-biofilm activity against *H. pylori*. To best of our knowledge, scanty reports are available on evaluation of nanomaterials for activity against clinically isolated *H. pylori*. Further study will be valuable to reveal the activity of synthesized silver nanobactericides against different pathogenic bacteria which are significantly resistant to available to standard antibiotics.

Acknowledgements Authors acknowledge the Head of the Institute, JSS Academy of Higher Education, Mysuru for the facilities. C. S. greatly acknowledges the funding support from DST DST-SERB (YSS/2015/001135/LS (Ver-I)). Authors are also thankful for the collaborating partners such as Krasnoyarsk State Medical University named after Prof. V. F. Voino-Yasenetskiy and University of Mysore for their participation and timely assistances.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Lobanovska M, Pilla G (2017) Penicillin's discovery and antibiotic resistance: lessons for the future? Yale J Biol Med 90:135–145
2. Sharma C, Rokana N, Chandra M (2018) Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. Front Vet Sci 2018:00237
3. Kraker MEA, Stewardson AJ, Harbarth S (2016) Will 10 million people die a year due to antimicrobial resistance by 2050? PLOS Med. https://doi.org/10.1371/journal.pmed.1002184
4. Raspopovic K, Jankovic S, Opancina V (2016) Factors affecting inappropriate prescription of antibiotics and the emergence of antibiotic resistance in patients in primary health care. Medicinski Casopis 50:85–90
5. WHO publishes list of bacteria for which new antibiotics are urgently needed. World Health Organization 2017
6. Pendleton JN, Gorman SP, Gilmore BF (2013) Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther 11:297–308
7. Magana M, Sereti C, Ioannidis A, Mitchell CA, Ball AR, Magiorkinis E, Chatzipanagiotou S, Hamblin MR, Hadjifrangiskou M, Tegos GP (2018) Options and limitations in clinical investigation of bacterial biofilms. Clin Microbiol Rev 31(3):84–86
8. Salih BA (2009) Helicobacter pylori infection in developing countries: the burden for how long? Saudi J Gastroenterol 15:201–207
9. Mitchell HM, Li YY, Hu PJ, Liu Q, Chen M, Du GG, Wang ZJ, Lee A, Hazell SL (1992) Epidemiology of *Helicobacter pylori* in southern China: identification of early childhood as the critical period for acquisition. J Infect Dis 166:149–153
10. Khalifa MM, Sharaf RR, Aziz RK (2010) *Helicobacter pylori*: a poor man's gut pathogen? Gut Pathog 2:2
11. Hadley C (2006) The infection connection: *Helicobacter pylori* is more than just the cause of gastric ulcers-it offers an unprecedented opportunity to study changes in human microecology and the nature of chronic disease. EMBO Rep 7(5):470–473
12. Argent RH, Hale JL, El-Omar EM, Atherton JC (2008) Differences in *Helicobacter pylori* CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion. J Med Microbiol 57:1062–1067
13. Porter AL, Youtie J (2009) How interdisciplinary is nanotechnology? J Nanopart Res 11(5):1023–1041
14. Baker S, Karthik N, Bhat P, Bisht N, Prasad A, Satish S, Prasad MNP (2018) Phyto-biologic bimetallic nanoparticles bearing antibacterial activity against human pathogens. J King Saud Univ Sci
15. Shrivastava S, Dash D (2009) Applying nanotechnology to human health. J Nanotechnol 12:240–243
16. Baker S, Rakshith D, Kavitha KS, Santosh P, Kavitha HU, Rao Y, Satish S (2013) Plants: emerging as nanofactories towards facile route in synthesis of nanoparticles. Bioimpacts 3(3):111–117
17. Kuppusamy P, Yusoff MM, Maniam GP, Govindan N (2016) Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications—an updated report. Saudi Pharm J 24:473–484
18. Jain S, Mehata MS (2016) Medicinal plant leaf extract and pure flavonoid mediated green synthesis of silver nanoparticles and their enhanced antibacterial property. Sci Rep 7:158–167
19. Shaik MR, Khan M, Kuniyil M, Al-Warthan A, Alkhathlan HZ, Siddiqui MRH, Shaik JP, Ahamed A, Mahmood A, Khan M, Adil SF (2018) Plant-extract-assisted green synthesis of silver nanoparticles using *Origanum vulgare* L. extract and their microbicidal activities. Sustainability 10:913
20. Baker S, Satish S (2012) Endophytes: toward a vision in synthesis of nanoparticle for future therapeutic agents. Int J Bio-Inorg Hybd Nanomater 1:67–77
21. Dušek K, Galambosi B, Hethelyi EB, Korany K, Karlová K (2007) Morphological and chemical variations of sweet flag (*Acorus calamus* L.) in the Czech and Finnish gene bank collection. Hort Sci (Prague) 34:17–25
22. Devi NS, Kishir R, Sharma GJ (2012) Microrhizome induction in *Acorus calamus* Linn—an important medicinal and aromatic plant. Hortic Environ Biotechnol 53(5):410–414
23. Azmath P, Baker S, Rakshith D, Satish S (2015) Mycosynthesis of silver nanoparticles bearing antibacterial activity. Saudi Pharm 01:008
24. Syed B, Bisht N, Bhat PS, Karthik RN, Prasad A et al (2017) Phytogenic synthesis of nanoparticles from *Rhizophora mangle* and their bactericidal potential with DNA damage activity. Nano-Struct Nano-Object 10:112–115
25. Syed B, Nagendra Prasad MN, Satish S (2016) Synthesis and characterization of silver nanobactericides produced by *Aneurinibacillus migulanus* 141, a novel endophyte inhabiting *Mimosa pudica* L. Arab J Chem. https://doi.org/10.1016/j.arabjc.2016.01.005
26. Baker S, Kumar KM, Santosh P, Rakshith D, Satish S (2015) Extracellular synthesis of silver nanoparticles by novel *Pseudomonas veronii* AS41G inhabiting *Annona squamosa* L. and their bactericidal activity. Spectrochim Acta A Mol Biomol Spectrosc 136:1434–1440
27. Shankar S, Ahmad A, Sastry M (2003) Geranium leaf assisted biosynthesis of silver nanoparticles. Biotechnol Prog 19:1627–1631

28. Sara Filoche K, Chris Sissons H, Soma K (2005) Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. Oral Microbiol Immunol 20(4):221–225
29. Kavitha KS, Baker S, Rakshith D, Kavitha HU, Yashwantha Rao HC, Harini BP, Satish S (2013) Plants as green source towards synthesis of nanoparticles. Int Res J Biolog Sci 2(6):66–76
30. Yong Song J, Kim-Beom S (2009) Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioprocess Biosyst Eng 32:79–84
31. Devi TSR, Gayathri S (2010) FTIR and FT-Raman spectral analysis of paclitaxel drugs. Int J Pharm Sci Rev Res 2:106–110
32. Urbaniak-Domalaga W (2012) The use of spectrometric technique FTIR-ATR to examine polymer surface. Advance Aspects of Spectroscopy. Rijeka (Croatia): Intech Open Science (2012)
33. Fan M, Dai D, Huang B (2012) Fourier transform infrared spectroscopy for natural fibres. In: Salih, Salih Mohammed (eds) Fourier transform—materials analysis. InTech. 2012
34. Ahmed S, Ikram S (2015) Silver nanoparticles: one pot green synthesis using *Terminalia arjuna* extract for biological application. J Nanomed Nanotech 6:4
35. Madhavan RV, Rosemary MJ, Nandkumar MA, Krishnan KV, Krishnan LK (2017) Silver nanoparticles impregnated poly (ε-caprolactone) scaffolds: optimization of antimicrobial and noncytotoxic concentrations. Tissue Eng Part A 17:439–449
36. Joy Prabu H, Johnson I (2015) Plant-mediated biosynthesis and characterization of silver nanoparticles by leaf extracts of *Tragia involucrata*, *Cymbopogon citronella*, *Solanum verbascifolium* and *Tylophora ovate*. Karbala Int J Mod Sci 1:237–246
37. Syed B, Nagendra-Prasad MN, Mohan K, Dhananjaya BL, Satish S (2017) Endo-symbiont mediated synthesis of gold nanobactericides and their activity against human pathogenic bacteria. Environ Toxicol Pharmacol 52:143–149
38. Raja S, Ramesh V, Thivaharan V (2017) Green biosynthesis of silver nanoparticles using *Calliandra haematocephala* leaf extract, their antibacterial activity and hydrogen peroxide sensing capability. Arab J Chem 10:253–261