IJPSR (2020), Volume 11, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 30 July 2019; received in revised form, 20 May 2020; accepted, 22 July 2020; published 01 August 2020

SYNTHESIS OF TUNGSTEN NANOPARTICLES FOR THEIR BIOMEDICAL APPLICATION

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Keywords:

Tungsten nanoparticles, Antimicrobial, Antiplatelet, Cytotoxicity

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ABSTRACT: The application of nanobiotechnology is an emerging area of nanoscience and nanotechnology. In the present studies, tungsten nanoparticles were synthesized and reduced chemically, and character-rization was done by UV, SEM, TEM, FT-IR, and XRD. The size of nanoparticles was found to be 20 nm. Further, these nanoparticles were teste for various biological applications. In antimicrobial activity, it was observed that potent efficacy was observed against *Streptomyces grisveus* (12 mm) at 80 µg/ml while in fungus maximum activity was observed against *Penicillium funicolusum* (24 mm) at the same dose. The antiplatelet activity of nanoparticles was investigated by Prothrombin (PT) and Activated Partial Thromboplastin Time (APTT). In both PT and APTT assay, maximum activity was observed at 40 µg/ml (295 sec and 80 sec, respectively). Cytotoxicity was also studied by MTT assay against various cell lines. Against MCF-7, potent activity was observed at 200 µg/ml, while in 3T3 it was observed at 500 µg/ml. Finally, it was observed that these nanoparticles have potent activity against tuberculosis at 1000 µg/ml. The result showed the nanoparticles are inexpensive and safe without any toxicity and consequently does not have any side effects.

INTRODUCTION: In recent years, metal nano-particles have been prepared with a broad range of applications in various fields, from chemistry to medicine. Mainly, as shapes, sizes, and compo-sitions of metallic nanomaterials are significantly linked to their physical, chemical, and optical properties [1-3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(8).4070-77

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(8).4070-77

The fabrication and characterization of tungsten oxide nanofibers using the electrospinning technique and sol-gel chemistry were successfully demonstrated [4]. They insisted the potential applications of the electrospun tungsten oxide nano-fibers as a sensor material for gas detection.

Ultrafine tungsten and tungsten oxide powders with controllable particle size and structure had been synthesized by a reverse micro emulsion-mediated synthesis method [5]. The interesting applications in various fields such as catalysis, electronics, illumination, and gas sensors were illustrated. One of the most crucial applications of the metallic nano-particles, in biomedical science is as anti-

microbial agents. The fatal activity of nanoparticles against broad spectrum of Gram-positive bacteria, Gram-negative bacteria and fungi has been recommended by many researchers [6]. The present investigation is focused on evaluating the antimicrobial activity of chemically synthesized tungsten nanoparticles. Therefore, the search for new antimicrobial drugs from nanoparticles has increased as a substitute to commercial drugs. Arterial thrombosis persuaded by aggregation of platelet is accountable for life-threatening disorders like unstable angina and reocclusion after angioplasty. Therefore suspension of platelet aggregation is a key step for the prevention and treatment of cardiovascular diseases [7]. There are certain chemical compounds in the market effective in preventing cardiovascular disorders, although they bear some toxicity. During the initial stage of thrombosis, damage in blood vessels causes the production of adhesive proteins (such as collagen and von Willebrand factor) and soluble agonists (such as ADP and thrombin) at the injury site; which further leads to platelet adhesion, activation, and aggregation, therefore leads to the formation of a platelet-rich thrombus. Heparin (HP) has a number of therapeutic potentials that can be enhanced when composited with nanoparticles.

In recent times cancer is one of the most life-threatening diseases responsible for casualties globally around the world 4 according to the WHO, the annual cancer cases are to rise from 14 million in 2012 to 22 million in the next two decades. Thus, the innovation of potent and effective anticancer drugs is one of the most targeted goals. Therefore, the exploitation of natural products is one of the most successful methods to identify novel agents [9]. To best of our knowledge, little research has been done to elucidate the impact of nanoparticles against drug-resistant TB. Although, the development of novel TB drugs remains paramount to surmounting the TB epidemic, modifying new drugs in a nanoparticle-based delivery system is a feasible, cost-effective, and readily available alternative. Nanoparticle-based formulations may reduce drug regimen duration, reduce the frequency, and deliver medications more efficaciously, ultimately reducing patient default and improving completion rates. In turn, this holds significant potential in the reduction of DR-TB cases.

MATERIALS AND METHODS:

Synthesis of Tungsten Nanoparticles: 1 gm of Tungsten sulfide powder was dissolved into Aqua-regia solution (6 ml HNO_3 +2 ml HCL). Then it was heated at 75 ºC and further dried at 40-45 ºC and then kept in an incubator for 24 h. After incubation 50 ml of distilled water was added and filtered; thus, WS solution is obtained. 0.2 M solution of EDTA in 10 ml of distilled water was prepared, and it was kept on a magnetic stirrer. Further WS solution and sodium sulfide were added in EDTA solution drop by drop with the help of micropipette. Then it was kept on a magnetic stirrer for 24 h. Finally, sodium borohydride was added in the solution used as a reducing agent for the synthesis of nanoparticles.

Determination of Antibacterial and Antifungal Assay: Antibacterial activity of the synthesized nanoparticles was investigated by agar well diffusion method [10, 11].

Activity index = Zone of inhibition of sample / Zone of inhibition of standard

Antiplatelet Activity: Blood samples were collected from KCJ Diagnostic center, near SMS Medical College, Jaipur, and subjected to centrifugation. Centrifugation at 10000 rpm for 5.5 min, 0.2 ml platelet rich plasma was separated from the sample, dissolved in isotonic $CaCl_2$. Different hemostatic constraints *viz*. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured by using established protocol [12].

Anti-tuberculosis Activity:

Drug Preparation: Various clinical isolates of *M. tuberculosis* obtained from Magnum Diagnostic center and were subcultured on Middlebrook 7 H 11 agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) Suspensions were prepared in 0.04% (vol/vol) Tween 80-0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) so that their turbidities matched that of a McFarland no. 1 turbidity standard.

Suspensions were further diluted 1:25 in 7H9GC broth (4.7 g of Middlebrook 7H9 broth base (Difco, Detroit, Mich.), 20 ml of 10% (vol/vol) glycerol, 1 g of Bacto Casitone (Difco), 880 ml of distilled water and 100 ml of oleic acid, albumin, dextrose, and catalase. Isoniazid (INH), rifampin (RMP),

streptomycin (SM), and ethambutol (EMB) were obtained from Sigma. The anti-TB activity was done using Alamar Blue Method.

Anticancer Activity:

***In-vitro* Cytotoxic Assay - MTT Assay:** Assay is based on the ability of mitochondrial dehydrogenase enzyme present in viable cells to cleave the tetrazolium rings of the pale yellow MTT dye and from dark purple formazan crystals which are largely impermeable to cell membranes, results in its accumulation in the cells. The assay was done against MCF -7 (Breast Cancer cell lines) and 3 T3 (Normal fibroblast cell line) using protocol [13].

Statistical Analysis: Results were expressed as mean values with standard deviation (± SD) of three replicates and were subjected to analysis of variance (ANOVA) using Minitab release version [12], Windows 95. Significant levels were tested at $P < 0.05$.

RESULTS:

Characterization of Synthesised Nanoparticles:

Ultraviolet-visible Spectroscopy: Optical properties is one of the most important criteria in determining the formation of nanoparticles. Free electrons in these nanoparticles reduced by gripping visible light and transmitted to a higher energy level, but the electron is unstable in an excited state and returns to the base energy level, and as a result, photons are emitted. Simultaneously resonance frequency of surface plasmon in the metallic nanoparticles depends on shape, size, and environment maintained during the synthesis of nanoparticles. The UV-Vis spectrum of tungsten nanoparticles gave absorbance peaks around 350 nm, and it showed strong resonance at this wavelength. The UV-vis spectra also revealed that these nanoparticles remained stable even after 24 h. **Fig. 1.**

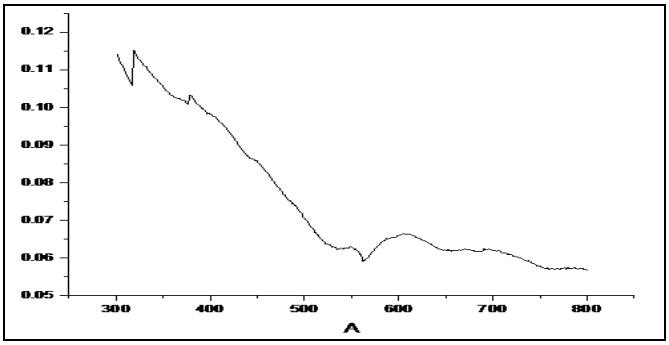


FIG. 1: UV SPECTRA OF REDUCED TUNGSTEN NANOPARTICLES

SEM and TEM: SEM technique was employed to determine the surface morphology and the topography of nanoparticles. The size of nanoparticles varied from 10 to 200 nm. SEM image exhibited that the chemically synthesized nanoparticles were mostly spherical in shape. The shape and size of the reduced nanoparticles were further analyzed by TEM. TEM image confirmed that most nanoparticles were spherical in shape and well dispersed, with an average size around 20 nm. The obtained results from the TEM image were in good agreement with the SEM data **Fig. 2** and **3.**

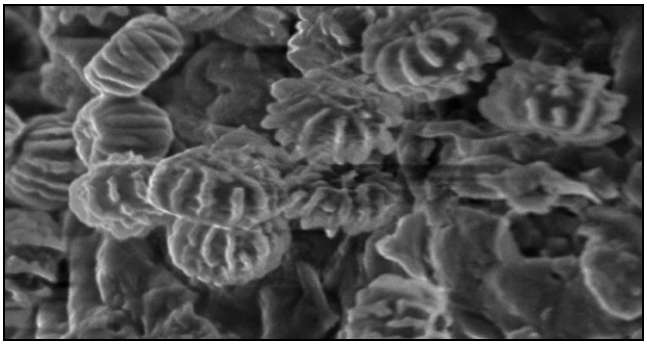


FIG. 2: SEM

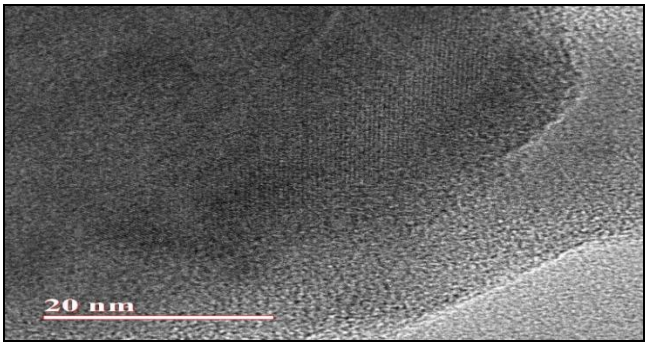


FIG. 3: TEM

FT-IR: FTIR spectroscopy was employed to determine the possible biomolecules and functional groups involved in reduction, capping, and efficient stabilization of newly synthesized nanoparticles **Fig. 4.** The absorption bands at 3357, 1577, 1395,1206, 1108, 1002,975,923, 861,769, 612,610 and 406 cm^{-1} were observed. The strong peaks at 3357 cm^{-1}correspondes to Hydroxy group, H-bonded OH stretch. The band at 1577 cm^{-1} was attributed to a secondary amine with NH bend. The peak at 1395 cm^{-1}correspondes to phenol or tertiary alcohol with OH bend.

The band at 1206 cm^{-1} corresponds to a secondary aromatic amine with CN stretches, while the band at 1108 cm^{-1} corresponds to alkyl substituted ether with C-O stretches, band at 1002 cm^{-1} shows the presence of aliphatic fluoro compounds. The band at 975 cm^{-1} corresponds to aromatic compounds with C-H in-plane bends while 923 cm^{-1} also represents the same band at 861 represents aromatic ring with di substitution while the band at 769 shows the presence of same compounds but with monosubstitution. Further, bands at 612 and 610 represent alkylene with C-H bands respectively, and 406 cm^{-1} represents C = O stretching vibration.

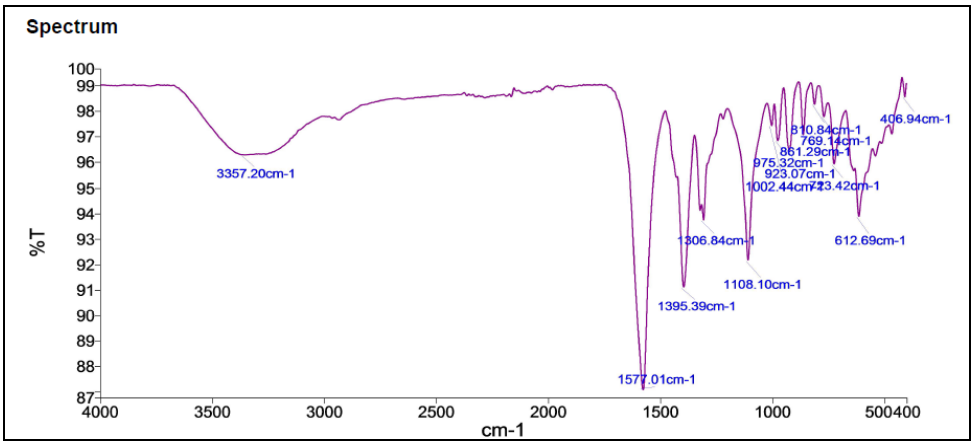
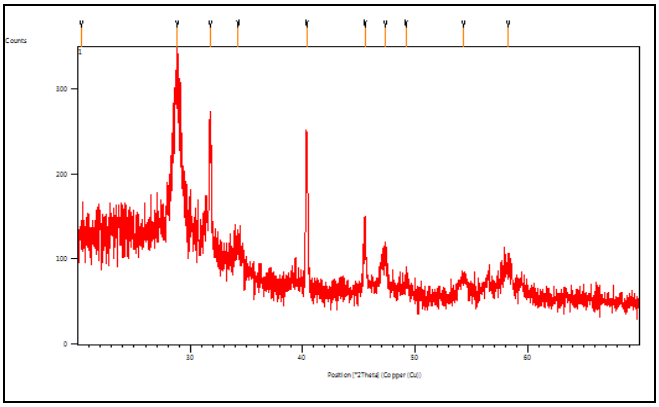


FIG. 4: FT-IR

XRD: X-ray diffraction (XRD) studies were carried out to confirm the synthesis of Tungsten nanoparticles and characterize crystallinity and the phase pattern of the nanoparticles. It was observed that 2^{Θ} (in degrees) were in the range of 25to 69.5 °C **Fig. 5**. These were compared with the JCPDS, Cu file no. 04-0836. The said 2θ values of peaks were in accordance with the standard of JCPDS. The XRD study confirms that the resultant particles were nanoparticles. Furthermore, it also confirms that the synthesized nanoparticles were free of impurities as no other characteristics XRD peaks were observed. The mean grain crystalline size of synthesized tungsten nanoparticles was calculated using the Debye Scherrer formula.

$$D = K\lambda / \beta \cos\Theta$$

Where, D is the average crystalline diameter size (Å), *K* is a constant (0.9), 'λ' is the wavelength of the X-ray used (*k* = 1.54 Å), 'β' is the angular line width at the half maximum of diffraction (radians) and 'Θ' is the Bragg's angle (degrees) [34].



Peak List

Pos.[°2Th.]	Height [cts]	FWHMLeft[°2Th.]	d-spacing [Å]	Rel. Int. [%]
20.3374	34.48	0.7872	4.36675	17.78
28.8382	193.87	0.3936	3.09597	100.00
31.7955	147.86	0.1968	2.81445	76.27
34.2694	28.05	0.4723	2.61672	14.47
40.3670	180.26	0.1968	2.23442	92.98
45.5440	77.87	0.1574	1.99175	40.16
47.3437	42.46	0.6298	1.92015	21.90
49.1648	13.66	0.9446	1.85321	7.05
54.2883	21.04	0.6298	1.68980	10.85
58.2530	28.85	0.7872	1.58388	14.88

FIG. 5: XRD OF SYNTHESIZED NANOPARTICLES

Antimicrobial Activity: The chemically synthesized nanoparticles showed potent antibacterial and antifungal activity at concentration ranging from 20 µg/ml to 80µg/ml on various clinical isolates. It was observed that against *E. coli* maximum zone was observed at 20 µg/ml (8 mm) while other dose level did not show any activity. Against *Bacillus subtilis* and *Pseudomonas aeruginosa* there was no

activity at any dose level as these were found to be resistant. Against Streptomyces *grisveus* it was observed that maximum zone was observed at 80 µg/ml 12 mm **Table 1**.

In the case of fungal strains no activity was observed against *Fusarium oxysporum.* Against *Penicillium funiculosum* maximum activity was observed at 40, 60, and 80 µg/ml (16, 22, and 24 mm respectively). When nanoparticles were tested against *Candida albicans* maximum activity was observed at 80 µg/ml 16 mm, which was at par with that of *Penicillium funiculosum*. *Trichoderma reesei* was found to be partially resistant to **Table 2**.

Antiplatelet Activity:

Thrombin Time (PT): All the concentrations of nanoparticles prolonged the clotting time as compared to control. Significant activity was observed at 40µgmL^{-1} (7.97 times of control and 19.66 times as compared to standard), which was maximum and increased in linear fashion **Table 3.**

Activated Partial Thromboplastin Time (APTT): In this assay, significant activity was observed at 40 µgmL^{-1} (1.70 times of control and 2 times as compared to standard), which increased slowly and was maximum **Table 4** with an increase in dose level.

TABLE 1: ANTIBACTERIAL ACTIVITY OF CHEMICALLY SYNTHESIZED TUNGSTEN NANOPARTICLE

Concentration (in µg/ml)	*E. coli*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Streptomyces grisveus*
20	IZ-8 AI-0.36	Nil	Nil	Nil
40	Nil	Nil	Nil	Nil
60	Nil	Nil	Nil	IZ-10 AI-0.45
80	NIL	Nil	Nil	IZ-12 AI- 0.54
Standard	IZ -22	IZ -22	IZ -22	IZ -22

Ciprofloxacin (as standard at 1mg/ml), iz- inhibition zone (in mm), ai- activity index (values are Mean of three replicates)

TABLE 2: ANTIFUNGAL ACTIVITY OF CHEMICALLY SYNTHESIZED TUNGSTEN NANOPARTICLES

Concentration (in µg/ml)	*Fusarium oxysporum*	*Penicillium funiculosum*	*Candida albicans*	*Trichoderma reesei*
20	Nil	Nil	IZ-10	Nil
40	Nil	IZ-16 AI-0.8	IZ-8 AI-0.4	Nil
60	Nil	IZ-22 AI-1.1	IZ-14 AI-0.7	Nil
80	Nil	IZ-24 AI-1.09	IZ-16 AI-0.72	Nil
Standard	IZ -20	IZ -20	IZ-20	IZ -20

Ketokenazole (as standard at 1 mg/ml), iz- inhibition zone, ai- activity index (values are Mean of three replicates)

TABLE 3: ANTIPLATELET ACTIVITY OF REDUCED TUNGSTEN NANOPARTICLES BY PROTHROMBIN TIME (PT)

S. no.	Concentration of Sample (in µg/ml)	Time	Standard	Control
1	10	119 sec	7.93	3.21
2	20	275 sec	18.33	7.43
3	30	162 sec	10.8	4.37
4	40	295 sec	19.66	7.97
5	50	160 sec	10.66	4.32
6	60	170 sec	11.33	4.59
7	70	185 sec	12.33	5.0
8	80	260 sec	17.33	7.02
9	90	75 sec	5.0	2.02
10	100	255 sec	17.0	6.89

Control-37 s. Standard PT (plasma + PT reagent)-15 s. Denotes potency of the sample at different concentrations when compared with standard and control

TABLE 4: ANTIPLATELET ACTIVITY OF REDUCED TUNGSTEN NANOPARTICLES BY ACTIVATED PARTIAL PROTHROMBIN TIME (APPT)

S. no.	Concentration of Sample (in µg/ml)	Time	Standard	Control
1	10	40 sec	1.0	0.85
2	20	50 sec	1.25	1.06
3	30	70 sec	1.75	1.48
4	40	80 sec	2.0	1.70
5	50	56 sec	1.4	1.19
6	60	63 sec	1.57	1.34
7	70	40 sec	1.0	0.85
8	80	73 sec	1.82	1.55
9	90	67 sec	1.67	1.42
10	100	50 sec	1.25	1.06

Control – 47 s.Standard PT (plasma + PT reagent) – 40 s. * Denotes Potency of the sample at different concentrations when compared with standard and control

Anticancer Activity (MTT Assay): The present study revealed the anticancer and cytotoxic potential of nanoparticles on breast cancer cells MCF-7, and the report was compared with cisplastin at various concentrations. It was noticed that nanoparticles with a concentration ranging from 10 to 200 µg/ml resulted in dose-dependent decrease in cellular viability of cancer cells with IC_{50} value of 44.79 µg/ml while cisplatin treatment revealed IC_{50} value of 9.47 µg/ml. The variation between the positive drug and samples is because the positive drug is pure and so it will require lower concentration to inhibit the growth of cancer cells. An alternatively higher concentration of samples resulted in more than 50% inhibition of cancer cells. Screening of cytotoxicity of nanoparticles on 3T3 cells revealed that it was marginally toxic to cells even at higher concentration. Overall in MCF-7, the viable cells were around 65% at 100 µg/ml which decreased to 37.29%, which reveals the fact that it is toxic at increasing concentration of the test sample. When compared to 3T3 cell lines, it was observed that the cells were viable at 41% at 500 µg/ml, which proved its non-toxicity **Tables 5-7.**

TABLE 5: SHOWING PERCENT CELL VIABILITY OF STANDARD (CISPLASTIN) (MCF7 BREAST CANCER CELL LINE)

Concentrations(µg/ml)	Viability
5	57.02 ± 0.43
10	52.12 ± 0.34
25	44.52 ± 0.26
50	41.09 ± 0.33
100	23.52 ± 0.12
250	19.36 ± 0.7
500	9.47 ± 0.5

TABLE 6: SHOWING CELL VIABILITY OF TESTED SAMPLE (REDUCED NANOPARTICLES) AGAINST MCF7 BREAST CANCER CELL LINE

Tested Concentrations (in (µg/ml)	Viability
10	88.46 ± 0.59
25	78.11 ± 0.44
50	65.87 ± 0.38
100	58.31 ± 0.28
200	37.79 ± 0.24
Control	100

TABLE 7: PERCENT CELL VIABILITY OF TESTED SAMPLE (REDUCED NANOPARTICLES) AGAINST 3T3 FIBROBLAST CELL LINE

Tested Concentrations (in µg/ml)	Viability
25	85.12 ± 0.79
50	74.43 ± 0.66
100	65.61 ± 0.58
250	57.89 ± 0.46
500	41.29 ± 0.31
Control	100.00

Anti-tuberculosis Activity: It was observed that synthesised nanoparticles showed potent activity to fight against *M. tuberculosis* at various concentrations in **Table 8.**

TABLE 8: ANTI-TUBERCULAR ACTIVITY OF REDUCED TUNGSTEN NANOPARTICLES

Tested concentrations(µg/ml)	
250 µg	++
500 µg	++++
1000 µg	+++

+++- moderate activity, ++++- Potent activity, +++++- Prominent activity

DISCUSSION: Nanosized particles, of either simple or composite nature, possess unique physical and chemical features and represent a demanding material in the innovations of novel nanodevices which can be used in numerous physical, biological, biomedical and pharmaceutical applications [14] Physical methods for synthesis of nanoparticles require high energy consumption and the chemical method usually leads to remaining some of the toxic reactions and non-use of generated particles in biological applications. So, attention has been focussed by many researchers for the synthesis of nanoparticles as therapeutic drugs.

The metal-based nanoparticles possess potent antimicrobial activity [15,] and keen researchers are engaged in innovating various nanoparticles as antibacterial agents. These nanoparticles have a unique benefit over traditional chemical antibiotics. Generally, the antimicrobial efficiency of drugs depends on the particular binding with the surface and the metabolism of agents into the microorganism. One of the most important challenges in the innovation of such drugs is that microorganisms have evolved drug resistance for many generations. So, to date, these antimicrobial drugs have been effective for therapy; but having various side effects. Therefore, an alternative way to overcome the drug resistance of various microorganisms is needed now desperately. Thus, in the present research potent, antimicrobial activity of synthesized tungsten nanoparticles were observed. Adenosine diphosphate is the main cause of platelets aggregation. The platelets are unexposed to ADP escape from such kind of mechanism. The ADP activated platelets without nanoparticle treatment reduce the clotting time in

terms of platelets aggregation. The reduction in aggregation of found to be higher in nanoparticles treated samples than the non-treated platelets. The cell viability assay is a crucial assay in nanotoxicology which reveals cellular response to a toxic material, and it can deliver information on cell death, survival, and metabolic activities [16] Cancer is an abnormal type of tissue growth in which the cells exhibit an uncontrolled division, relatively in an autonomous cell [17] The innovation and identification of new antitumor drug with minimum toxicity has become an essential goal in recent era [18].

Tuberculosis (TB) is one of the most threatening diseases caused by Mycobacterium tuberculosis. It generally targets lungs but can harm other organs as well. In India, almost 50% of patients are suffering, and one person dies from TB every minute [19]. However, due to the consumption of antibiotics, the challenge of multidrug-resistant TB has increased drastically. So, there is a need for the discovery of new anti-TB drugs that are safe, effective, and affordable. Thus, to the best of our knowledge, this is the first comprehensive report on various biological parameters of chemically synthesized nanoparticles.

CONCLUSION: Nanotechnology is very rapidly emerging in biological sciences as novel techniques are being developed to probe and manipulate the effect of single atoms and molecules against a wide range of microbes. The present research works a systematic and scientific approach to develop and investigate the nanoparticles and its biological activities against a range of routes. There is a great scope of the study of nanoparticles and as antifungal agents though having limited effectiveness. Our tactic is to assess the anti-microbial activity of tungsten nanoparticles to improve the effectiveness of drugs at low cost and its anticancer effect. Hence, we can also predict that tungsten nanoparticles have potent anti-microbial, antiplatelet, antiplatelet, anticancer, and anti-tuberculosis activity.

ACKNOWLEDGEMENT: The authors are thankful to Heads, Mewar University, and Seminal applied Sciences for providing the necessary facilities to carry out the research work related to the biological activities and Dr. Archana Mehta Magnum Diagnostic Lab for performing anti-tuberculosis activity.

CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest

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How to cite this article:
Sharma AK, Swami AK, Saran M and Mathur M: Synthesis of tungsten nanoparticles for their biomedical application. Int J Pharm Sci & Res 2020; 11(8): 4070-77. doi: 10.13040/IJPSR.0975-8232.11(8).4070-77.