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A novel approach towards eco-friendly green synthesis of copper nanoparticles from bunium persicum and their biomedical applications

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***Abstract*---**Nanotechnology is getting attention profoundly rooted in current living scenario which should license a consequential, scrupulous message deeply rooted in unceasing partaking and altercation among contributors and inhabitants. Therefore, the aim of present investigation deals with synthesis and characterization of copper nanoparticles (CuNPs) by aqueous extracts of leaves of Bunium persicum. Phytochemical screening confirmed presence of carbohydrates, proteins, flavonoids and alkaloids which had major role as reducing and sustainable agent for formation of CuNPs. Further these particles were characterized by UV- Vis, FTIR, SEM, TEM, XRD and DLS. FT-IR showed presence of various function groups like alcohols, amines and hydroxyl which were engaged in reduction and capping during formation of nanoparticles. Studies by XRD, SEM and TEM confirmed that nanoparticles appear spherical and crystalline in nature when morphology was studied and their dimension ranged between 30 to 70 nm. UV spectra showed peaks at 525 nm while they were crystalline in structure. Further various pharmacological activities were performed like antimicrobial by agar well diffusion, antioxidant by various assays like Lipid peroxidation [LPO], catalase and peroxidase and antiplatelet by Prothrombin, activated partial thromboplastin time and Lactate hydrogenase release assay (LDH).

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***Keywords*---**Copper nanoparticles, Bunium persicum, Antimicrobial, Antioxidant, Antiplatelet, LDH.

Introduction

Nanotechnology has been gaining keen interest among scientific community in recent scenario. Therefore, people will be able to understand the innovation in science and technology in this field which could affect their livelihood beyond genuine developments. There after impulsive passion or wariness these highly ground breaking technical innovation which can assure that nanotechnology is getting attention profoundly rooted in current livelihood which should license a consequential, scrupulous information relying on an unceasing partaking and altercation among contributors and inhabitants [1]. So, green synthesis of nanoparticles through plant mediated route is one of the crucial phases for reduction and sustainability of various metallic elements in their ionic form. The green synthesis approach for metallic nanoparticles fabrication has been quite effective, non-toxic, reliable, cost-effective, biocompatible and environmentally friendly substitute to chemical and physical methods, which use harmful, toxic, and costly chemicals in addition to high energy, pressure and temperature [2, 3].

There are various eco-friendly approaches for synthesis of nanoparticles like irradiation, Tolensis, Polyoxomethanate and Polysaccharide [4]. Generally physical and chemical assays applied in designing of nanoparticles are costly which possess toxic and hazardous chemicals which are major threats in ecological and biological systems [5]. Generally, through chemical synthesis routes there is synthesis of some harmful compounds which are fascinated at active site and thus cause limitation for their application in medical science [6]. Therefore, an environmentally and economically attuned protocol is required for synthesis of nanoparticles. The antimicrobial properties of copper rely on quantity of copper and allotment of unconstrained copper. Copper has been found motionless when in metallic phase but when it interacts with humidity in skin and injury lesions it converts and becomes in ionic phase. Cu+2 is extremely imprudent as it adheres to tissue proteins and results in morphological variations in the cell wall some prokaryotes including nuclear membrane and finally causes deformation and apoptosis.

It has been reported that oxidative strain has a crucial part in progressive cell death. ROS is directly engaged in the disease-causing phase of different metabolic disorders, ultimately leads to many disorders like neurodegenerative, respiratory, cardiovascular and cancer diseases [7]. The increase in ROS is also correlated with ageing, however, though it is difficult to prove its direct role. Further, during maturation of cells, the increased amount of ROS is also responsible for mutilation of mitochondria and disruption in oxidative cycle at cellular level [8]. Since ROS and NO free radicals has been distorted due to nanoparticles representing stress signal transformation so nanoparticles are widely recommended drugs to act as free radical scavengers.

Platelets are those components of blood which do not have nucleus which appears rounded when observed morphologically with size between 2 and 3 µm and are important constituent of human blood. Platelets has crucial task in sustaining the body metabolism in response to environment and avoid clotting of blood when wounded and cures the individual from bereavement [9]. They have inflammatory roles which affects both inherent and artificial immune communications [10]. Although abnormal concentration of platelets can cause several disorders in biological routes which results in many disorders finally causing death. Extreme triggering in platelets results in atherosclerosis including Alzheimer disease. Thrombosis is deeply rooted in vain and respiratory embolism often cause high rate of apoptosis [11]. Thus, antiplatelet agents are very crucial in curing many disorders generally related to cardiovascular, cerebro vascular, and tangential arterial routes.

It has been assumed that nanoparticles prominently resist platelet stimulation when insisted by integrin-, in dose dependent way. Prior administration of platelets with enhancing dose of nanoparticles results in systematic reticence of renunciation caused due to clotting of fibrin. During this process, main proteins of platelets undergo progressive removal of phosphate group on tyrosine. Administration of platelets by nanoparticles reduce the limitation of phosphorylation. CuNPs also reduced the polymerization of actin protein by stimulation of thrombin- which discharge intracellular Ca2+ acting as a crucial controller of cell communication pathways. There are lot of applications of CuNPs, which cleaves into grades of invigorating and novel features. There some transport mechanism inside the body which have some ions which aids in regulating the metabolism involved in homeostasis. CuNPs are also having prominent role of acting as antimicrobial agents as they combat against variety of clinically important microbe [12]. They also have application in various fields like electrical, photothermal, agricultural, photocatalytic and biomedical. CuNPs are gaining lot of focus among scientific communities because of their medicinal importance which can be designed at low cost. Besides this CuNPs also possess other pharmacological applications like free radical scavengers and antiplatelet agents thus acting as thrombolytic drugs recommended in regulating the frequency of blood clotting or curing them at large scale which is crucial in saving individual life [13].

Bunium persicum [BOISS]B. Fedtsch is a prominent flavor adding species among the resources of diverse medicinal plants perusing typical fragrance m, cosmopolitan in Asian continent. Besides this, it is recommended as flavouring agents in various curries including in flavoring food stuffs and beverages. Seeds are recommended as additive in edible items like bread, cooking, rice and yogurt for its carminative and as muscular suppress agent. Besides this plant possess various activities like antibacterial, antifungal [14], antiallergic [15] and Anti-inflammatory [16].

Materials and Methods

The chemicals used in preparation of copper nanoparticles were purchased from Hi-media, Jaipur, India. Doubly distilled water was consumed as solvent for aqueous extracts. In vitro assay reagents for antimicrobial, antioxidant and antiplatelet activity were purchased from Sigma Aldrich, India.

Phytochemical screening of leaves of Banium persicum

Leaves of experimental plant were macerated and soxhlet isolated successively with petroleum ether [60-80^0 C], C_6H_6, $CHCl_3$, C_2H_5OH and water having duration of 1-2 days. Thereafter all sequential extracts were filtered, dehydrated *in vacuo* and screening for the extractive values. Finally applying recommended assays, extracts were screened to analyze the occurrence of carbohydrates, proteins, flavonoid, alkaloids. Prior to this procedure, tested samples were solubilized again in their respective particular chemicals and fragmented in aliquots to execute some confirmative assays which are described as follows:

Carbohydrates
Fehling' Test

To two mL of above reaction mixture, similar ratio of freshly made Fehling's solution [processed by adding mixture A: 7.0 g $CuSO_4 . 7H_2O$ in 100 mL deionized water and B: 24.0 g KOH and 34.6 g $C_4H_4KNaO_6$ in 100 mL deionized water] was aggregated then mixture kept on heat in incubator. The appearance a tarnished brown appearance confirmed the occurrence of carbohydrates.

Benedict Test

To 2 mL reaction mixture 0.5 ml of Benedict reagent [prepared by amalgating 17 g of Na3C6H5O7 and 10 g of Na_2CO_3 in 75 mL of deionized water, thereafter filtered and further, 17.3 g of $CuSO_4.7H_2O$ dissolve in 100 mL deionized water] was mixed and thereafter whole solution was incubated on a water bath. A chronological variation in visual appearance [blue-green-orange] confirmed occurrence of carbohydrates.

Proteins
FeCl3 Test

To 2 mL of the aliquot, 5% aqueous $FeCl_3$ solution was slowly added and the presence of proteins was indicated by the development of a blue colour changing to olive green as a result of addition of more $FeCl_3$ solution.

Phenanzone Test

To the sample [2mL], 0.2 g solution acid phosphate was added followed by heating, which was subsequently brought to room temperature and filtered. And to this, 2% phenanzone solution was added resulting in the formation of a bulky and coloured precipitation thus evidencing the presence of tannins.

Flavonoids
Shinoda' S Test

To 2 mL of the solution a piece of Mg^{++} ribbon and concentrated HCl was added drop by drop. The resulting pink/scarlet crimon or occasionally green/blue colour indicated the presence of flavonoids.

Alkaloids

In test sample 2% HCl was added and further heated at for 120 min which was chilled and filtered. A pattern of the white impetuous after mixing of 2-3 drops of the below mentioned reaction mixtures to 2 mL of the above solution confirmed that alkaloid was present.

1. **Modi mayer's reagent*:*** - 3.95g KI was added in 1.35 g of $HgCl_2$ and finally quantity was increased to 100 mL by distilled water.
2. **Wagner's reagent: -** 2.00g KI was added in 1.27g I_2 and finally quantity was increased to 100 mL by distilled water.
3. ***Bouchardt's reagent:* -** 4.0 g KI was added in 2.0 g I_2 and finally quantity was increased to 100 mL by distilled water.

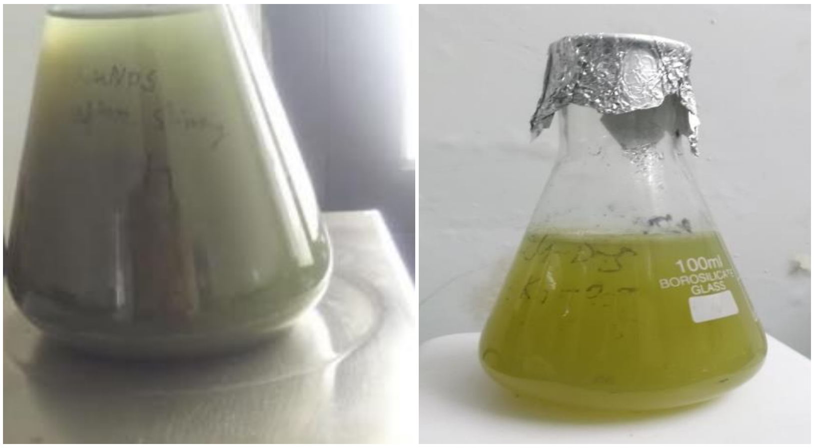
Preparation of Aqueous Extracts of *Banium persicum*

The synthesis of nanoparticles via green route particles were processed by biologically by aqueous extract of ***Banium persicum***. The leaves were cleaned thoroughly with deionized water and sustained at ambient heat for 48 hrs to remove moisture which further spliced into small pieces and thereafter grinded for further use. For making aqueous extract, 10 g grinded leaves of ***B. persicum*** were added in autoclaved 100 ml of distill water [ratio: 1:10] and kept on the rotator shaker around $37^{0}C$ for 48 hrs. Afterwards, the aqueous solution was riddled with Whatman filter paper and were kept at 4C°.for further experiment.

Synthesis of Copper Nanoparticles

100 ml of $CuSO_4$ [mol wt. 249.67 g·mol−1, odorless, concentration 3.6 g/cm3, b.p. 650 °C] with dilutions of 1, 1.5, 2, 4 and 10 mm added to a 2.5, 5, 10, 20 and 40 mL of filtrates. The reaction mixture kept on the shaker for 1hr at rotation of 200 rad. The variation in light to murky green colour in the solution will confirm formation of CuNPs.

The pure nanoparticle fine particles were harvested by lyophilisation and stored at 37 ^{o}C for further experiments. Thereafter, the absorbance peaks of nanoparticles recorded at 200–800 nm. Since these nanoparticles are reactive in light so, all glassware were covered with aluminum foil. These synthesized nanoparticles were carried out for characterization by Ultraviolet visible spectroscopy [UV-Vis], Scanning electron microscopy [SEM], Transmission electron microscopy [TEM], Dynamic light scattering [DLS], Fourier-transform infrared spectroscopy [FT-IR] and X –Ray diffraction [XRD] and further tested for their biomedical applications.



(a) (b)

Fig.1 Change in colour from light (a) to dark green (b) showing synthesis of Copper Nanoparticles

Bioefficacies
Anti-microbial activity
Antibacterial Activity

This activity was done as per the established protocol [17]. Clinical laboratory isolates of different bacterial colonies viz. *Escherichia coli* [MTCC no 443], *Bacillus subtilis* [MTCC no 10619], *Staphylococcus aureus* [MTCC no 3381] were isolated from the mother cultures at SMS Medical College, Jaipur India [**MTCC- Microbial Type Culture Collection]*. These strains revived in Nutrient agar medium obtained from sigma –aldrich which was autoclaved at 15 lbs. psi for half an hour] and kept at room temperature for 2 days. The clinical isolates were further preserved on the media with similar composition at regular intervals of 48h. Revived strain were prepared in saline solution prior to experimentation.

Antifungal activity

This activity was also done using established protocol [17]. Clinical laboratory isolates of different fungal colonies of Trichoderma *reesei* [ATCC no 26921], *Aspergillus niger* [ATCC no 16888], *Penicillium funiculosum* [ATCC no 8725] *and Fusarium oxysporium* [ATCC no 62705] has been collected from SMS Medical College, Jaipur India [**ATCC- American Type Culture Collection*]. They were further revived on PDA media at 28^0C for two days. Gel puncture was used to create wells [approx 6mm deep] in media which were of different size. In these wells nanoparticles at different concentration (from 60 to 120μg/ml) along with standard antibiotics were loaded. Plates were gestated at 37°C. After 48 hrs activity in the form of inhibition zone was calculated (in mm). Finally, activity index was calculated as

ACTIVITY INDEX = Sample Zone/ Zone of Control Drug

Antiplatelet activity

Activated Partial Thromboplastin time [APTT]

Chemical Required

- Activated cephaloplastin which is used in form of APTT reagent
- $CaCl_2$
- 3.2% buffered Tri-sodium citrate solution

Blood samples were collected from veins in unsoiled vessel having 1 part of 3.2% buffered Tri-sodium citrate reagent which was urgently amalgated with heparin to delay formation of spume. Again, the whole solution was sonicated for 15 min at 3000 g while plasma was detached into another tube. During experiment it is recommended to use freshly isolated plasma. Opaque, Haemolysed, lipaemic plasma is not recommended. APTT reagent was mixed slowly spinning prior further experiment. 100µl of APTT reagent and 0.025 mol/L $CaCl_2$ was mixed and desiccated on water bath for 3 min at 37^0 separate test tubes. Further, 100µl of amalgated $CaCl_2$ mixed thoroughly in pre heated test tube having solution of plasma and APTT reagent and instantaneously clotting time was noted by stop watch. All components were amalgated thoroughly and the stopwatch was ended when we observed fibrin strand that caused formation of gel clot. The duration consumed for formation of clot was calculated almost 0.1 sec. This was taken as control and nanoparticles in various dilutions [1 to 10 µg/mL] were mixed as substituent for the APTT reagent to determine the efficacy at various dilutions of our sample.

Prothrombin Time [PT]

Both sodium citrate [0.11mol/l] along with isolated blood samples were assorted in the ratio of 1: 9 with care, to delay the formation of spume. The blood samples were sonicated at 1800rpm for 17 min. Serum was separated from platelets rich plasma. 100µl of citrated plasma was added. Nanoparticles in various dilutions [1to 10 µg/mL] were mixed. To calculated the coagulation time stopwatch was set on the coagulation analyzer [13].

Determination of Cytotoxicity using LDH dehydrogenase

The cytotoxic potential in present research were investigated by calculating LDH release factors. Platelets administrated with Triton X- 100 was taken as reference sample in correlation with optimum injuries present in cell. Subsequently after heating PRP at 37^0C for 5min along with CuNPs, it was rotated on shaker for 60 sec at 500 rad. For further studies on LDH release, 25µL of supernatant was unruffled and amalgated with 100µL of NADH solution [prepared by adding 0.03% of NADH in 15 ml of phosphate buffer] and 25µL of pyruvate reagent [22.7mM pyruvic acid in phosphate buffer]. Decrease in optical density around 340 nm was observed as the reaction changing NADH to NAD+ was noted down to calculate LDH release factor in supernatant. This release was calculated as ratio of total enzyme potentiality observed in platelets utterly degraded with 0.2% Triton X- 100 [18]. The experiment has been done thrice and the observations are denoted as average ratio ± standard variation.

Antioxidant Activity

Various antioxidant assays like LPO [19], Catalase and Peroxidase [20] were carried out using established protocols.

Statistical Analysis

All aforementioned protocols carried away in triplicates and the obtained data were uttered as mean ± Standard Deviation [SD]. Statistical implication between the groups was calculated by 1 way ANOVA. A p-value > 0.05 was implicated as statistically significant**.**

Results

Phytochemical Screening of plant extracts

Physico-chemical screening of various metabolites [carbohydrates, proteins flavonoids and alkaloids] of leaves of *B. persicum* had been analysed (Table 1). On sequential extraction, all the major groups of metabolites tested. Among the different solvents used for sequentially extraction, the total extractability was maximum in alcohol, while in C_6H_6 and $CHCl_3$ it was minimum and they confirmed the presence of phytochemicals like flavonoids, alkaloids, sugars and proteins.

Table1. Phytochemical screening of leaves of *Bunium persicum*

Parameter		Organic Solvents Used				
		Pet. Ether	Benzene	Chloroform	Alcohol	Water
Physical Appearance	Plant Parts	Yellow Green Sticky	Bright Red Oily	Yellowish Orange Oily	Red Brown Sticky	Brown Dusty Viscous
Carbohydrates	Leaves	-	+	++	++	++
Proteins		++	-	+	++	++
Flavonoids		-	++	+++	++	+++
Alkaloids		+	+ +	+	+ +	+++

- absent; + trace amount; ++ moderate amount; +++significant amount

Visual confirmation of prepared Copper nanoparticles

Preliminary confirmation of the formation of CuNPs is observed when there is colour change in the reaction. The initial colour was light green which changed into a dark green confirming the reduction of $Cu2^{+}$ to Cu^{0} thus synthesis of nanoparticles. This variation is because of surface plasmon resonance [SPR] mechanism as it includes excitation of electrons in electron transport chain in the reaction mixture.

Characterization
UV–Visible spectroscopy

Using this technique, the uniform peak observed around 525 nm denotes in response of particularity of CuNPS occurring because of prominent excitation binding energy. It has been recommended that the band width enhance on reduction in particle size. There is contrast between band width and the wavelength of absorption spectra. The increased shift in spectra for the synthesized CuNPs is correlated with high reduction in particle size. Free electrons are triggered by acquiring visible light and shifted to a higher energy level but they do not have stability in an excited state and move towards at base energy level and concomitantly a photon is released. Thereafter resonance frequency of surface plasmon in these nanoparticles relies on morphology, size and laboratory environments at where they are designed (Fig.2).

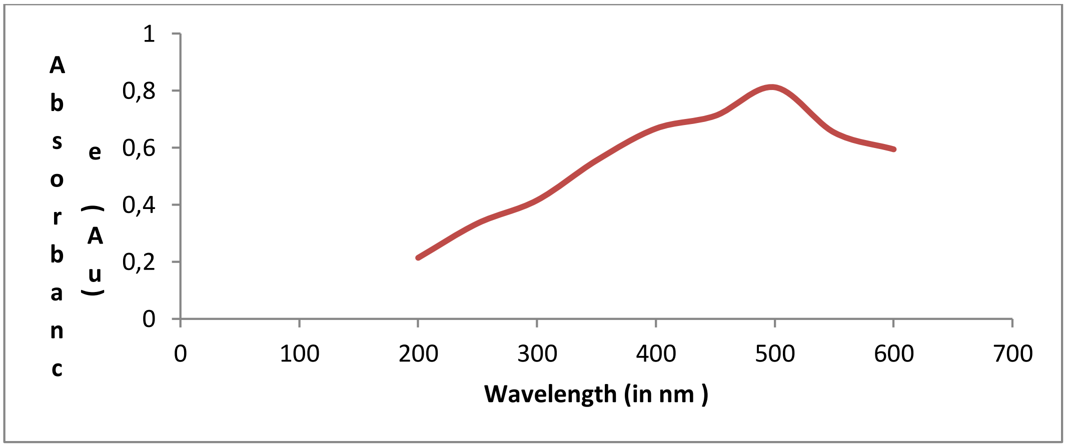


Fig. 2 UV-spectroscopy of copper nanoparticles

FT-IR

This technique was applied to study about prominent phytochemicals present in *Bnium persicum* extract which are engaged in reduction, capping and proficient sustainability of synthesized CuNPs. The assimilation of bands at 3900, 3600, 3500, 3000, 2800, 2700, 2400, 2100, 1800, 1700, 1650, 1600, 1550, 1500, 1400, 1350,1200, 1100, 1050, 1000, 850 and 600 cm−1 were found. The prominent peaks at 3900 cm−1 correspond to – O-H stretching confirms presence of OH group. The band at 3600 cm−1 confirms alcohol O–H stretching vibration. The spectra at 3500 cm−1 confirm the occurrence of N-H stretching vibration of primary amine. 3000 cm−1 because of O-H stretching. Furthermore, peaks assigned at 2800 C-H stretching alcohol and 2700 cm−1 were due to O-H stretch having Alcohol. The peak at 2400 Cm^{-1} denotes O=C=O stretching amine. The peak at 1550, and 1500 corresponds at N-O stretching alkene. The spectra at 1400 cm−1 corresponds to C-H bending Alkene. Other prominent bands at 1350 C-H corresponds stretching Alkyne, 1200 denotes O-H stretching Aldehyde and 1100 cm−1 were characteristic of S=O aromatic ester. Peaks at 1050 and 1000 cm −1 C-O, C=C Secondary Alkoxy C-O were observed. The peak at 850 cm−1 shows presence of alkyne C-CI bond bearing halo compounds. Finally, the spectra at 600 cm−1 shows C-I stretching bearing halogen groups. Presence of these different moieties have crucial role synergistically and have importance in reduction, capping and stability during synthesis of CuNPs . Thus, it has been concluded that different functional groups like –OH [hydroxyl] –C=O [carbonyl] and C–H [Methylene] have been found in the aqueous extracts of leaves of *Banium persicum* which were engaged for formation of CuNPs thus assisting the reduction of Cu^{+2} to Cu^{0} (Fig.3).

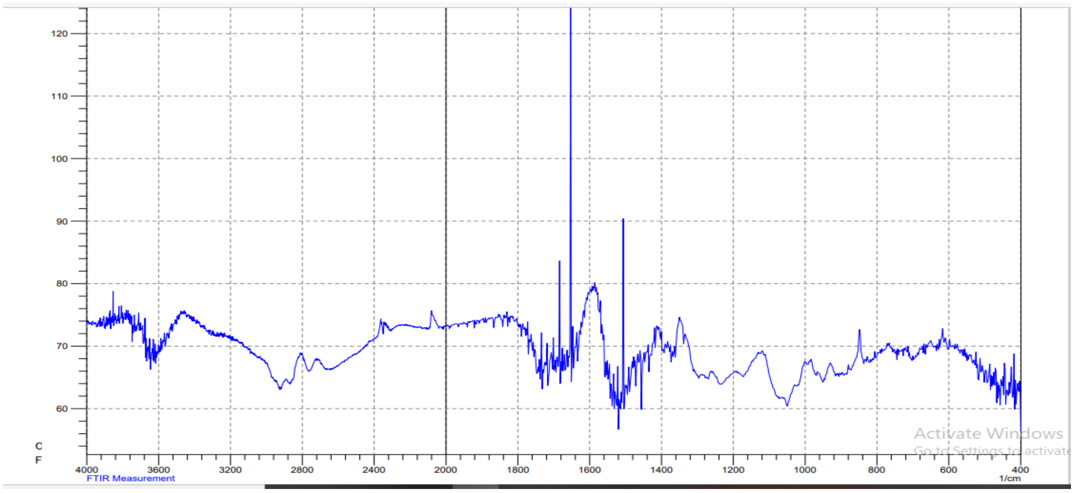
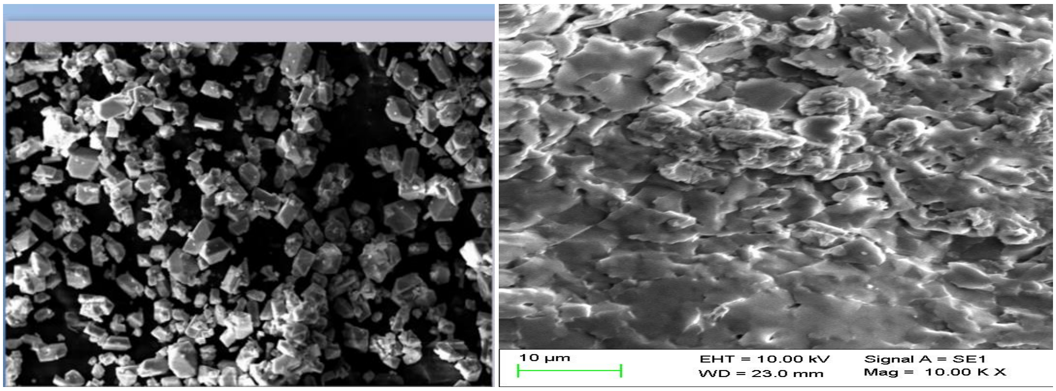


Fig. 3 FT-IR spectra of copper nanoparticles

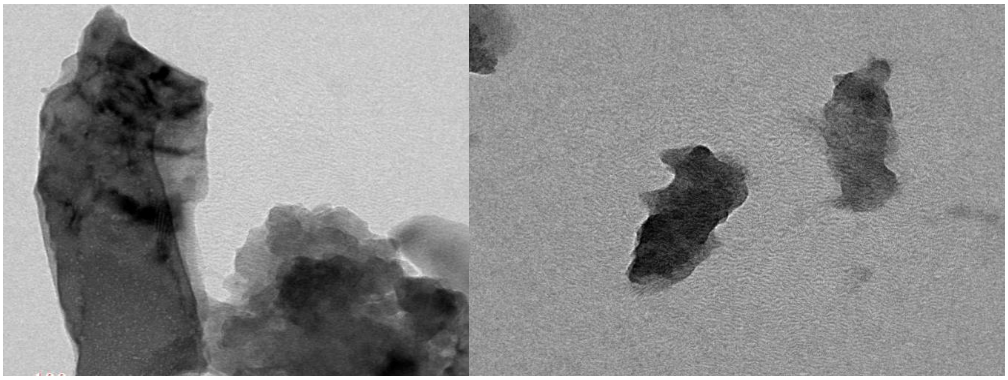
SEM and TEM

Further various microscopy tools were applied to confirm the surface and the lattice structure of the prepared CuNps. Using this technique, we found that the biosynthesized CuNPs have spherical type of morphology when seen at different resolutions including their optimum dispersed condition. Using TEM, it was observed that the particles are in nanoscale and synthesized with uniform structure. The dimensions of CuNPs were in range of 10 and 100 nm bearing average size 50 nm (Fig. 4 and 5). It has been reported that variation in physical and chemical conditions like pH, experimentation tenure, temperature, molar dose level of $CuSO_4$ and volume of plant extracts can directly effects the dimensions of nanoparticles.



(a) (b)

Fig. 4. Showing SEM Images of Cu-NPs



(c) (d)

Fig. 5 showing TEM images of copper nanoparticles

DLS

This technique is applied to determine surface and size of nanoparticles which acts as vital micronutrient or catalyst of the biochemical and ecological systems. Finally, it was observed that the mean dimension of CuNPs in favorable environment was in range of 10to100nm (Fig. 6)

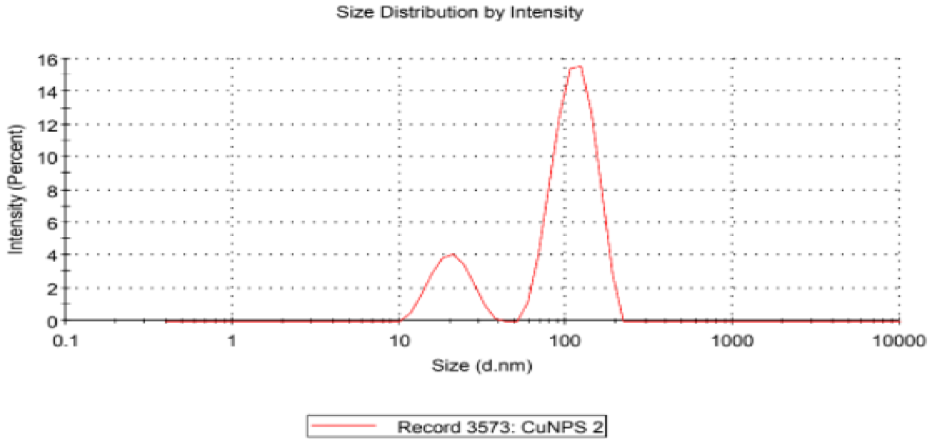


Fig.6. DLS of copper nanoparticles

XRD

Finally, diffraction patterns using X ray was applied to study crystalline nature and the phase pattern of the CuNPs. These kinds of diffraction patterns are denoted by various peaks and were confirmed with JCPDS [Joint Committee on Powder Diffraction Standards] file. It was observed that synthesized nanoparticles had face centered cubic structures and was crystalline in nature. The occurrence of picks on planes which are calculated in 2Θ are related to presence of natural products found in aqueous extracts of leaves of *B. persicum* which possess crystalline nature (Fig.7)

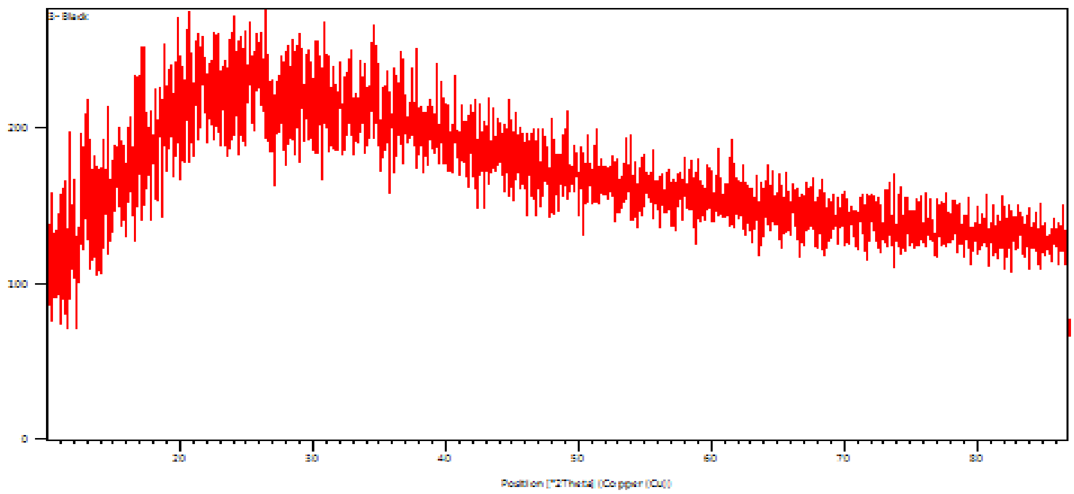


Fig. 7. XRD Pattern of Copper nanoparticles

Bioefficacies

Anti-microbial activity

This assay of CuNPS was carried away by well diffusion method against medicinally pathogenic microbes. Coating of nanoparticles on sterile disc generally results in vagueness, so there are issues related to uniform distribution, in order to overcome this problem, well diffusion method was given priority over this protocol. In the present investigation CuNPs revealed potent anti-bacterial efficacy against *E. coli* at dose level of 120 µg/ml where zone of inhibition was found to be 14mm. Further at dose level of 100 µg/ml the zone was found to be 13mm, at 80 µg/ml it was found 12mm and minimum zone of inhibition was found 10mm at 60 µg/ml. Finally, against *S. aureus* highest assay was observed at 100 µg/ml having zone of 13mm and lowest zone was [10mm] at concentration of 120 µg/ml. Against *B. subtilis* dose level of CuNPs at 120 µg/ml had zone of 14mm which reduced with decrease in concentration and minimum at dose level of 100 µg/ml [IZ-10mm] (Table 2).

Table 2. Antibacterial activity of copper nanoparticles (Zone of inhibition in mm)

Concentration (in µg/ml)	*E. coli*	*S.aureus*	*B. subtilis*
60	10±0.01 AI-0.55	12±0.02 AI-0.54	Nil
80	12±0.02 AI-0.66	12±0.02 AI-0.54	11±0.01 AI 0.55
100	13±0.04 AI-0.72	13±0.04 AI-0.59	10±0.01 AI-0.50
120	**14**±0.05 **AI-0.77**	10±0.01 AI-0.45	14±0.04 AI-0.70
Ciprofloxacin (Control antibiotic)	18±0.08	IZ-22±0.06	IZ-20±0.20

AI = Activity Index which is calculated as Zone of Test sample /Zone of standard.

Against different fungal colonies tested, maximum efficacy has been found against *C. albigans* at 100 µg/ml [IZ-15mm] and minimum at 80 µg/ml [IZ-10mm]. When it was studied against *T. reesei* highest zone was observed at 60 µg/ml [IZ-15 mm] while least at concentration of 120 µg/ml [IZ-10mm]. It was observed that *A. niger* and *F. oxysporium* was found to be completely resistant as no zone was observed at any concentration. Increased efficacy of CuNPs in dose dependent manner is directly correlated due to interaction with the cytoplasmic constituents (Table 3).

Table 3 Antifungal activity of copper nanoparticles (Zone of inhibition in mm)

Concentration (µg/ml)	*C. albicans*	*T. reesei*	*A. niger*	*F. oxysporum*
60	Nil	15±0.15 AI-0.41	NIL	NIL
80	10±0.01 AI-0.25	Nil	NIL	NIL
100	15±0.15 AI-0.37	10± AI-0.38	NIL	NIL
120	Nil	**16±0.06 AI-0.44**	NIL	NIL
Ketokenazole	40±0.2	36±0.19	32±0.2	28±0.17

(Control antibiotic)

IZ – Inhibition zone in mm
I AI = Activity Index which is calculated as Zone of Test sample /Zone of standard (Fig:8-9).

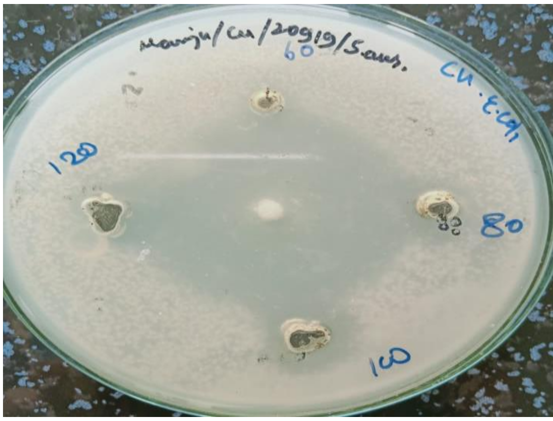


Fig. 8 Antibacterial activity of CuNPs against *E. coli.* Fig. 9 Antifungal activity of CuNPs against *Trichoderma reesei*

Antiplatelet activity

PT Assay

In this assay dose level of nanoparticles protracted the duration of clotting in contrast to control. Maximum activity was found at dose level of 7µgmL^{-1} 22.89 times of control and 56.46 times in contrast to standard], which was highest and thus it was observed that they showed variation in dose dependent manner (Table 4).

Table 4 Clotting time of copper nanoparticles at different concentrations assayed by PT

Concentration (µg/ml)	Clotting time (Sec.)	Standard**	Control*
1	167±2.21	11.13	4.51
2	210±2.42	14	5.67
3	250±2.48	16.66	6.75
4	641±4.21	42.73	17.32
5	237±2.21	15.8	6.40
6	155±1.71	10.33	4.18
7	**847**±5.21	**56.46**	**22.89**
8	116±1.21	7.73	3.13
9	113±1.14	7.53	3.05
10	115±1.19	7.66	3.10

Standard value for PT [Plasma + Thromborel (PT reagent); 1:2] -=15 sec ; Control = (Plasma+Distilled water); = 37 sec
***s times higher than standard; * c times higher than control*
Values are presented as means± SE (n=3)

APTT Assay

Here also the dose level of nanoparticles protracted the duration of clotting. Highest efficacy was observed at dose level of 1µgmL^{-1} [39.76 times of control and 46.72 times in contrast to standard] and minimum at 10µgmL^{-1} [1.61 times of control and 1.9 times contrast to standard] which reduced with increase in concentration (Table 5).

Table 5 Clotting time of copper nanoparticles at different concentrations assayed by AP PT Assay

Concentration (in µg/ml)	Clotting time (sec.)	Standard**	Control *
1	**1869**±5.21	**46.72**	**39.76**

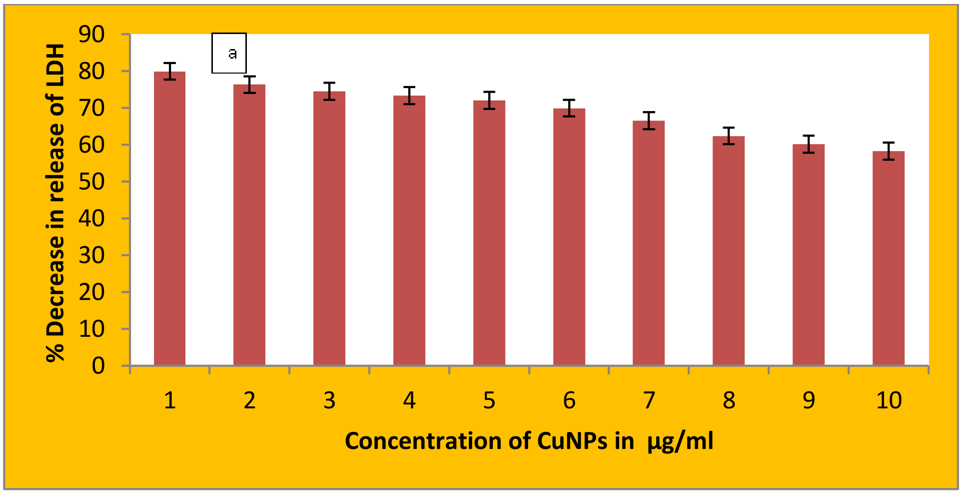
3114

2	215±2.38	5.37	4.57
3	385±2.87	9.62	8.19
4	280±2.61	7	5.95
5	1572±4.21	39.3	33.44
6	334±2.21	8.35	7.10
7	104±2.21	2.6	2.21
8	321±2.21	8.02	6.82
9	298±2.21	7.45	6.34
10	76±2.21	1.9	1.61

Standard value for APTT [Plasma+Activated cephaloplastin +$CaCl_2$; 1:1:1] - 40 sec
Control - (Plasma+Distill water+$CaCl_2$) - 47 sec.
***s times higher than standard; * c times higher than control*
Values are presented as means± SE (n=3)

Cytotoxicity using LDH dehydrogenase

The average percentage reduction in release of LDH in PRP without nanoparticles has been observed around 80.21 which confirms to PRP without treatment of CuNPs and Triton X-100.Post administrating the ADP triggered platelets at various dose level of CuNPs, the ratio of LDH discharge showed some variations, while mean data of this ratio reduction in LDH discharge were 79.91, 76.3, 74.48, 73.3, 72.01. 69.91, 66.52, 62.36, 60.09, 58.28 for 1µg/ml, 2 µg/ml, 3 µg/ml, 4 µg/ml, 5 µg/ml, 6 µg/ml, 7 µg/ml, 8 µg/ml, 9 µg/ml and 10 µg/ml treatment dose of CuNPs respectively. Finally, these observations proves that CuNPs did harm membrane veracity of the platelets [Fig 10].



a**P < 0.001; b P < 0. 001. Differences are considered significant at P < 0.001
Fig. 10 showing Effect of CuNPs on the % decrease in LDH release from platelets

Antioxidant Assay

In LPO assay it was observed that activity was dose dependent. Maximum efficacy was found at 10µg/ml [1.395 nmol/g]. Maximum catalase efficacy has been found at 6 µg/ml [12.88 nmol/g] and maximum peroxidase activity was noted down at 8 µg/ml [0.428]. Thus, it was concluded that these enzymatic activities were not concentration dependent so increase in the production levels of ROS was under the influence of nanoparticles independent of their dose levels [Tables 6-8].

Table 6 Lipid Peroxidation Assay of Copper Nanoparticles

Concentration (in µg/ml)	Activity (nmol/g)
	0.515±0.07
1	0.634±0.08
2	0.678±0.09
3	0.696±0.09
4	0.712±0.11
5	0.765±0.15
6	0.883±0.22
7	0.902±0.35
8	0.956±0.41
9	**1.395**±0.5

Values are presented as means ± SE (n=3)

Table 7 Catalase activity of Copper Nanoparticles

Concentration (in µg/ml)	Activity (in U/g/mn)
1	6.34±1.1
2	4.58±0.9
3	9.73±1.23
4	10.75±1.31
5	11.75±1.45
6	**12.88**±1.52
7	9.85±1.24
8	8.88±1.01
9	7.96±1.15
10	5.04±0.7

Values are presented as means± SE (n=3)

Table 8 Peroxidase activity of Copper Nanoparticles

Concentration (in µg/ml)	Activity (in µg/mn)
1	0.234±0.02
2	0.331±0.03
3	0.356±0.03
4	0.319±0.04
5	0.228±0.02
6	0.386±0.03
7	0.391±0.04
8	**0.428**±0.04
9	0.400±0.04
10	0.413±0.04

Values are presented as means± SE (n=3)

Discussion

The application of plant mediated synthesis of nanoparticles has many beneficial effects prior to microorganisms which includes easy conduction of methodology, voluntarily scalability and deterrence of preservation of microbial cultures. However, the key point relies in choosing of plant resources which is very important as they possess prominent reducing compounds which synthesize perfect dimension and morphology of nanoparticles. This plant mediated pathways has several advantages over some of the physicochemical assays engaged in synthesis of nanoparticles. Plant is rich source of natural products and enzyme which can be manipulated for designing nanoparticles. The key phytochemicals found in plants are flavonoids, flavonoids glycosides and phenylpropanoid glycosides. In our present investigation similar metabolites were identified in aqueous extracts of leaves of *B. persicum.* Physical methods of nanoparticles producing needs large expenditure of energy while chemical mediated routes usually produce toxic compounds and produce particles which are useless in biological applications. Therefore, there is urge for biosynthesis of nanoparticles by plants mediated routes.

Antibiotic resistance is key point of attention in the public health as antibiotic is exploited or not appropriate for target. This enhanced multidrug resistance increases medical cost due to protracted handling counting admittance and convalescence, so there is urge of novel antibiotic compounds and use of efficient and broad-spectrum protocols to regulate wide spread of contamination caused due to harmful pathogens which possess multidrug resistance. Although these complications found in low ratio of patients and they are crucial because of risk ultimately results in mortality. With latest innovating technologies in nanotechnology as drug delivery agents it has attracted scientific communities significantly in biomedical applications.

CuNPs are is highly reactive metal as it possesses high ratio of surface area to volume that bypass them to profusely react within the cell membrane, and disrupting the biochemical constituents of tissues, results in apoptosis. Basic cause responsible for prominent aggregation with *E. coli* is correlated to the negative charge occurring at facade of cell, which leads electrochemical aggregation with the positive charges in nanoparticles. The probable mode of action projected for the antibacterial efficacy is undeviating dissemination or through endocytosis. The grip of these nanoparticles at active site of microbe relies undoubtedly on surface coarseness, electrochemical aggregation, chemical profile and solubility. So, the assault of free radicals to the outer membrane of various biomolecules like nucleic acids, proteins results in oxidative stress ultimately leading to apoptosis.

It has been reported that antibacterial and free radical scavenging potentiality of plant and biodegradable plant assisted synthesis of copper nanoparticles through different solvents of *Cissus arnotiana*. They reported that this plant mediated CuNPs had potent antibacterial efficacy against the *E. coli* which we also observed in our present investigation as synthesized nanoparticles gave prominent antibacterial activity as compared to antifungal assay.

Conclusion

In the current investigation, copper nanoparticles are prepared from leaves of *B persicum.* The secondary metabolites present in aqueous extract in the leaves were accountable for reducing and stabilizing agents for reduction and sustainability of CuNPs. This plant mediated synthesized nanoparticles were spherical in shape with a average dimension of 50 nm. They possessed dose dependent antimicrobial, free radical scavenging and antiplatelet efficacy. These nanoparticles could be used as prominent natural drugs as antimicrobial, antioxidant and antiplatelet agents. Still there is need of detailed research for understanding the molecular mode of action of these nanoparticles including *in vivo* studies.

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