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various biomedical applications. These procedures are simple, ecofriendly and serve as an alternative to complex chemical methods for preparation of nanomaterials.

Objective: In the present study, phytosynthesis of silver nanoparticles, to examine their antioxidant potential, toxic effects towards bacterial-, fungal-strains, brine shrimp nauplii and cancer cells was focused.

Method: Methanolic extract of *Euphorbia wallichii* roots was used for synthesis of silver nanoparticles. The synthesis was monitored and confirmed by UV-visible spectroscopy, Fourier Transform Infra-Red (FTIR) spectrometric analysis, Field Emission Scanning Electron Microscope (FESEM), Energy Dispersive X-ray (EDX) and X-ray powder diffraction (XRD).

Results: The synthesized particles were average 63 nm in size. Involvement of phenolic (46.7±2.41 µg GAE/mg) and flavonoid (11.71 µg QE/mg) compounds as capping agents was also measured. Nanoparticles showed antioxidant properties in terms of free radical scavenging potential (59.63±1.0%), reducing power (44.52±1.34 µg AAE/mg) and total antioxidant capacity (60.48±2.2 µg AAE/mg). The nanoparticles showed potent cytotoxic effects against brine shrimp nauplii (LD_{50} 66.83 µg/ml), proliferation and cell death of Hella cells as determined by MTT (LD_{50} 0.3923 µg/ml) and Tunel assays respectively. Antimicrobial results revealed that silver nanoparticles were found to be more potent against pathogenic fungal (maximum active against *A. fumigates*, MIC 15µg/disc) and bacterial strains (maximum active against *S. aureus*, MIC 3.33µg/disc) than the *E. wallichii* extract alone.

***Conclusion*:** These results support the advantages of using an eco-friendly and cost-effective method for synthesis of nanoparticles with antioxidant, cytotoxic and antimicrobial potential.

Keywords: Antimicrobial, Antioxidant, Cytotoxicity, *Euphorbia wallichii*, silver nanoparticles,

Running Title: Antioxidant and toxicity potential of silver nanoparticles

nanoscale level is rapidly growing in the field of science and technology [1]. Various methods are reported for the synthesis of nanomaterials from plant extracts [2], fungi and bacteria [3]. These methods are simple and serve as an alternative to complex chemical methods for the synthesis of nanoparticles. Nanoparticle synthesis of different metals such as gold, magnesium [4], zinc, copper, or titanium [5] are already reported. Silver nanoparticles are effective due to their good antiviral or antimicrobial efficacy against eukaryotic microorganisms [6], fungi [7] and pathogenic bacteria [8]. Silver nanoparticles have an important role in nanomedicine and nanotechnology. The remarkable advancement in understanding the organization of nanostructures ensures the dynamic and interesting role of nanotechnology in the future. Distinctive properties of colloidal silver such as chemical stability, catalytic conductivity and antimicrobial activities are of particular interest in a number of fields [9]. Different properties including smaller size and larger surface area of nanoparticles have enhanced their importance in various fields. These particles possess antimicrobial properties which might provide a solution to the increasing trend of antibiotic resistance exhibited by pathogenic bacterial strains and might prove to be remarkably valuable in biomedical field. Nanoparticles have been reported as active against various diseases. Silver nanoparticles are extensively being utilized in the different areas such as medical, bio-sensing, photonics, electronics, and potential optical receptors for biolabeling [10]. Plant extracts and purified bioactive phytochemicals serve as green reactants for the synthesis of silver nanoparticles [11]. There are numerous approaches for nanoparticle synthesis and these methodologies pose larger problems like toxicity and cost. Hence, use of plants for synthesis of nanoparticles provides ecofriendly and cost-effective option. *Camellia*

also reported to be used in the synthesis of silver nanoparticles [13].

Owing to better pharmacological responses, drugs obtained from plants constitute 25% of worldwide prescribed drugs [14]. *Euphorbia* genus is among the six largest genera of flowering plants, comprising 2160 species. *Euphorbia* consists mainly of perennial herbs, but also shrubs and trees [15]. Some species of the *Euphorbia* genus have been used in traditional medicines to treat tumors, cancers, migraine, gonorrhea, skin diseases, warts and intestinal parasites for hundreds of years. It has also been used for liver ailments, anti-inflammatory agent [16], and in the treatment of psoriasis [17]. Recently, different studies reported the use of plants of Euphorbia genus for synthesis of silver nanoparticles such as aqueous extract of *Euphorbia prostrate* leaves was used for the synthesis of silver nanoparticles and explored for their leishmanicidal activity [18]. Another study reported the larvicidal effect of the silver nanoparticle produced from leaf extract of *Euphorbia hirta* against cotton boll worm (*helicoverpa armigera*) [19].

Plants of this particular genus are used to cure rheumatism and bronchitis. *E. wallichii* roots have been used in Tibetan folk medicine for the treatment of edema and skin diseases such as cutaneous anthrax and furuncle [20]. It also showed significant cytotoxicity and phytotoxicity [21]. In the present study, silver nanoparticles were formed by reducing silver ions using *E. wallichii* extract as a reductant. Prepared nanoparticles were evaluated for antioxidant, cytotoxic and antimicrobial activity against various pathogenic bacterial and fungal strains.

Hela cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal-bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), penicillin and streptomycin were purchased from sigma Aldrich. All other chemicals and reagents were obtained from Sigma Aldrich until and unless mentioned.

2.2. Collection and extraction

E. wallichii plants were collected from Nathia galli, Khyber Pakhtunkhwa, Pakistan during June, 2012. The plant was identified by Prof. Dr Rizwana A. Qureshi, Department of plant sciences, Quaid-i-Azam University Islamabad, Pakistan. A voucher specimen (Specimen No. 125755) was previously submitted to the herbarium of medicinal plants of Quaid-i-Azam University, Islamabad. Roots were utilized for preparation of nanoparticles. Shade-dried plant material (0.5 kg) was macerated in methanol (2 liter) for three days. This process was repeated three times. Thereafter, the collected methanolic extract after each process was combined and filtered using filter paper (Whatman No. 1). The filtrate was then concentrated using a rotary evaporator (R-200 Buchi, Switzerland). Extracts were further dried in a vacuum oven at 40ºC (Vacucell, Einrichtungen GmbH). Finally, crude methanolic extract of *E. wallichii* plant was used for the synthesis of nanoparticles.

2.3. Synthesis of nanoparticles

Nanoparticles were synthesized from methanolic extract *E. wallichii* (EW). Silver nitrate ($AgNO_3$) was used as the source of the metal for EW nanoparticles (EWAgNPs).

changed. The nanoparticles were then separated by centrifuging (Ependorf Centrifuge 804) the solution at 5000 rpm for 3 min. The obtained nanoparticles were washed with deionized water and centrifuged again. The EWAgNPs were dried in a vacuum chamber (Vacucell) for 24 hours at 35ºC and then proceeded for characterization and biological evaluation.

2.4. Characterization of silver nanoparticles

The synthesized EWAgNPs were characterized by UV/Vis PDA spectrophotometric (Agilent 8453, Germany) analysis in the range of 300-620 nm. The shape and size were determined by using a field emission scanning electron microscope (FESEM) (JEOL JSM-6701F USA), while Fourier Transform Infra-Red (FT-IR) spectrometric analysis was carried out to check the presence of possible functional groups. The FT-IR spectra were recorded in the wavenumber frequency range of 4000-600cm^{-1} with a speed of 16 scans per spectrum using the bench-top Spectrum™ 65 FT-IR spectrometer equipped with the universal diamond ATR (Attenuated Total Reflectance) accessory (PerkinElmer Inc., USA). All the measurements were recorded in transmittance (%T) mode at room temperature. Energy dispersive X-ray (EDX, Oxford Aztec) was used to determine the elemental composition nanoparticles. XRD measurements were determined using Bruker D8 Advance brand*-2* configuration (generator-detector) X-ray tube copper S¼1.54Å and LYNXEYEPDS detector.

2.5. Determination of phenolic and flavonoid content: The involvement of phenolic and flavonoids in synthesized EWAgNPs and present EW were investigated as following:

2.5.1. Phenolic content

Phenolic content in extract was determined using Folin-Ciocalteu reagent method as

mixture was incubated at room temperature for 30 min. In the negative control, dimethyl sulfooxide (DMSO) was used instead of the sample. Absorbance was measured at 765 nm using the microplate reader (ELX 800, Biotek USA). Calibration curve of gallic acid standard was prepared. Then phenolic content involved in synthesized nanoparticle and in crude extract were measured from the standard curve equation and content was expressed as µg gallic acid equivalents (GAE).

2.5.2. Flavonoid content

Total flavonoid content was determined using the Aluminium chloride colorimetric method as described previously [25]. Aliquots of each sample solution (20 µL, 4 mg/mL DMSO) were mixed with 10 µL of aluminum chloride (10% w/v in H_2O) and 10 µL of potassium acetate (1 M) solutions. Consequently, distilled water was added to get a final volume of 200 µL. After 30 min of incubation (Incubator IC83 Yomato, Japan), absorbance was measured by using microplate reader (Bioteck, USA) at 415nm. Quercetin, a plant flavonoid was used as standard. Flavonoid content in plant crude extract and participated in nanoparticle synthesis were measured form calibration curve of standard. While, flavonoid contents were established in µg quercetin equivalent (QE).

2.6. Antioxidant assays. Antioxidant capacities of EWAgNPs were determined using different assays which are described below:

2.6.1. DPPH free radical scavenging assay

Antioxidant potential of EWAgNPs against α, α-diphenyl-β-picrylhydrazyl (DPPH) was determined using the method described by Clarke et al with some modification [24]. The

assays. After that the mixture was incubated at room temperature for 30 min, a change in color from violet to yellow was observed owing to the anti-oxidation potential of the sample. Here, ascorbic acid was used as a reference standard. The absorbance of the reaction mixture was measured on the microplate reader at 517 nm. The scavenging activity was calculated using the following equation:

% Scavenging activity = OD of control – OD of test sample / OD of control ×100

OD = optical density (absorbance)

2.6.2. Total antioxidant capacity

The Phosphomolybdenum method described by Banerjee and Narendhirakann was used for measuring the total antioxidant capacity [26]. The reaction mixture was produced by the addition of reagent solution [ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid (0.6 M)] and 100 µl of sample solution. This reaction mixture was incubated for 90 min at 95^{o}C. After incubation, the mixture was cooled and absorbance was recorded at 695 nm. Ascorbic acid was used as the standard. Antioxidant potential of EWAgNPs and methanolic extract was expressed as µg ascorbic acid equivalent per mg of EWAgNPs or extract (µg AAE/mg).

2.6.3. Reducing power assay

The reducing power of samples, EWAgNPs, was determined using the method described by Lue et al [27]. The reaction mixture was contained 100 µl of sample, 250 µl of phosphate buffer and 250 µl of potassium ferricyanide. The reaction mixture was then incubated for 30 min at 50^{o}C, after which 250 µl of trichloroacetic acid was added. It was centrifuged at 3000 rpm for 10 min. Supernatant (180 µl) was taken carefully and poured in

199 **2.7. Toxicity determination**

200 Toxicity of both EW and EWAgNPs were evaluated through MTT, TUNNEL and
201 Brine shrimp assays as described bellow

202 **2.7.1. Culture of Hella cells**

203 Hella cells were obtained from the Korean Cell Line Bank (KCLB), Republic of
204 Korea. The cells were grown in DMEM medium containing 10% heat-inactivated fetal
205 bovine serum, streptomycin (50 µg/mL), and penicillin (50 unit/mL). HeLa cells were grown
206 on glass cover slips in 24-well culture plates at density of 2.5×10^4 cells for tunnel assay or
207 1.5×10^3 per well in 96-well plate for MTT assay and incubated in CO_2 incubator (95% O_2,
208 5% CO_2) at 37°C in a humidified atmosphere. Medium was changed after two days. Cells
209 were allowed to grow in similar conditions to reach 70-75% confluence.

210 **2.7.2. MTT assay**

211 Toxic effect of samples was investigated against cell viability of hella cells by using
212 MTT assay as described previously [28]. Briefly, hella cells seeded 96-well plate was
213 exposed with different concentration (0-50µM/ml) of EW and EWAgNPs replacing with
214 previous medium and incubated for 24 h at 37ºC in CO_2 incubator. Then 10µl of MTT
215 reagent 1 (0.5 mg/ml) was added in each well and incubated for 4 h until purple formazan
216 crystals were developed. Followed by addition of 100 µl of MTT reagent 2 (Solubilization
217 buffer; 10% SDS with 0.01N HCl and DMSO) and overnight incubation in CO_2 incubator to
218 dissolve the formazan crystals. Finally, optical absorbance was measured at 570 nm using
219 microplate reader (Tecan, Maennedorf, Switzerland) and cells without treatment were

labeling (TUNEL) staining was performed to detect DNA fragmentation using a commercially available kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. HeLa cells were grown on glass cover slips in 24-well plate non-treated or treated with EW and EWAgNPs for 24 hours. The cells were washed with PBS, fixed for 15 min at room temperature with 4% paraformaldehyde. After fixing, cells were washed in PBS and incubated in a permeabilization solution (0.1% Triton X- 100 in 0.1% sodium citrate) on ice for 2 min. After that cell was washed in PBS and then incubated with a mixture containing terminal deoxynucleotidyl transferase and the reaction buffer containing rhodamine-dUTP for 60 min at 37 ◦C. Finally cells were washed PBS and mounted using prolong gold antifade reagent containing the nuclear staining *4'*,*6-diamidino* -2-phenylindole,dicloride (DAPI). Images were analysed using fluorescence microscopy.

2.7.4. Brine shrimp lethality assay

The cytotoxic effect of EWAgNPs was determined using the brine shrimp lethality assay by following the method of Bibi et al [29]. Brine shrimp (*Artemia salina*) eggs (Ocean Star Inc., USA) were hatched in sea water (3.8% Sea salt solution). Final concentrations of the sample solution of 500, 250, 125, 62.5, 31.62 and 15.6 µg/ml were used in this assay. Ten brine shrimp larvae were counted and transferred to vials (having 2 ml of sea water) using a pasture pipette. Then 5 µl from each stock of sample was added and volume in the vials was raised up to 5 ml with sea water to attain the final concentration of 500, 250, 125, 62.5, 31.62 and 15.6 µg/ml. These vials were left to incubate for 24 hours at a temperature of 28^{o}C. The numbers of surviving and dead shrimps were counted using pasture pipette and magnifying glass. The LD_{50} (50% lethal dose) was calculated using Table Curve 2D Version 4.07

2.8.1. Antibacterial assay

Five bacterial strains, two gram-positive (*Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538) and three gram-negative (*Bordetella bronchiseptica* ATCC 4617, *Escherichia coli* ATCC 15224, *Salmonella typhimurium* ATCC 14028) were used in this assay. Bactericidal activity of synthesized nanoparticles was determined by using the disc diffusion method as described by Kandile et al [8]. Bacterial cultures were refreshed in nutrient broth for 24 hours at 30^{o}C. Nutrient agar medium was used for the growth of bacterial strains. All chemicals used in the current study were of commercial grade and purchased from BDH (England) and Merck (Germany). Filter paper discs of 6 mm in size were prepared from Whatman no. 1 filter paper. Media, filter paper discs along with other apparatus required in this assay were sterilized using autoclave and the whole experiment was carried out in a microbiological safety cabinet. Solidified plates of nutrient agar were labeled and respective bacterial strains were inoculated. Cefixime-USP was used as the standard antibacterial drug and DMSO was used as negative control. For antibacterial activity, 5 µl of stock solution of sample (6mg/ml) was used per disc to load 30 µg of sample. These discs were placed on respective places on a petri plate. The petri plates were incubated for 24 hours at 37^{o}C. Zones of inhibition (mm) around the disc were measured after that the samples having ≥ 10 mm zone of inhibition were tested at lower concentrations by using standard three-fold micro broth dilution method [30]. A stock solution of samples and standard was serially diluted in 96-well microtiter plate with nutrient broth to obtain a concentration ranging from 10 µg/mL to 0.334 µg/mL. Inoculum was added so as to give inoculum size of approximately 5×10^2 CFU/ml in each well. Microtiter plates were then kept at 37°C for 24

2.8.2. Antifungal assay

Fungicidal activity of synthesized nanoparticles was measured by observing the growth response of five fungal strains (*Aspergillus fumigatus* FCBP 66, *Fusarium solani* FCBP 0291, *Mucor* species FCBP 0300, *Aspergillus niger* FCBP 0198, *Aspergillus flavus* FCBP 0064). Sabraud dextrose agar was used for maintenance of fungal cultures. A slightly modified method of Kandile et al [8] was followed for measuring the fungicidal capacities of EWAgNPs. Discs of 6 mm were prepared from Whatman no.1 filter paper and the experiment performed in triplicate and carried out in a microbiological safety cabinet. The sample solution was absorbed on discs and placed on the respective, labeled, solidified SDA plates. DMSO was used as negative control and terbinafine hydrochloride ≥ 98% (Sigma-aldrich, USA) was used as the standard antifungal drug. An amount of 30 µg for both EWAgNPs and EW were used per disc for antifungal activity by loading 5 µl of stock solution (6 mg/ml). Plates were incubated for 24 to 48 hours at 28^{o}C. Antifungal capacities of the samples were observed by measuring the inhibition zones in mm around the discs. Extracts producing an inhibition zone ≥ 10 mm in diameter were screened to determine minimum inhibitory concentrations (MICs) at lower concentrations ranging from 15 to 1.857 µg/disc by standard disc diffusion method. MIC was calculated as the lowest concentration of the extract around which a visible zone of growth inhibition was formed [30].

Statistical analysis

All the experiments were performed in triplicate, and results are expressed as mean ± standard deviation. Further, one-way analysis of variance (GraphPad Prism, version 5.01 for

3. Results and Discussion

3.1. Synthesis and characterization of silver nanoparticles

The present study was conducted for the green synthesis of silver nanoparticles (EWAgNPs) from the methanolic extract of *Euphorbia wallichii* (EW). The formation of nanoparticles was observed from the colorimetric change in the reaction solution from greenish to brownish color upon addition of 1% EW solution in 10 mM $AgNO_3$ solution at a 1:1 concentration. The change in color of the reaction mixture was due to the collective oscillations of free electrons in reduced EWAgNPs [31]. UV–visible spectrophotometric analysis of nanoparticles showed a maximum absorbance peak at 415 nm in correspondence with the surface plasmon resonance of EWAgNPs (Fig. 1-A). A maximum absorbance peak around 430 nm is the distinctive property of silver nanoparticles [12].

The morphology and composition of EWAgNPs was carried out by FESEM and EDX analysis, respectively. Different sizes of spherical nanoparticles were observed which is because of aggregation of phyto-chemicals while preparing the samples for FESEM analysis. Fig. (1-B) shows the scanning electron micrograph of the EWAgNPs where the particle size was found to be on average 63 nm. While, primary composition for the presence of silver was examined with EDX determination (Fig. 1-C).

FT-IR is widely used for recognition as well as accurate and rapid identification of functional groups [32]. A limited portion of this range from 4000 to 625 cm^{-1} (2.5-16 μm) is useful for organic compounds [33]. FT-IR spectroscopic analysis was made in order to identify the functional groups or possible biomolecules involved in synthesis of the EWAgNPs from the EW extract. In FT-IR analysis, crude extract stretches were observed at

and the finger print region of polyphenols [34]. The bands appeared at 3271.0 cm^{-1} corresponding to -OH stretching vibrations, and at 2919.4 cm^{-1} for CH stretching of aromatic compounds of phenol groups. The peak at 1448.0 cm^{-1} corresponds to the O–H bend of polyphenols. The weaker band at 1205.1 cm^{-1} which corresponds to COC stretch was observed. In the EWAgNPs, peaks were changed from 2929.3 to 2919.4 cm^{-1}, 1698.5 to 1711.7 cm^{-1}, 1448.01 to 1452.6 cm^{-1}, 1205.1 to 1174.9 cm^{-1}, and 1032.8 to 1043.1 cm^{-1}. Shifting in the IR bands suggests the coordination of silver ions and the synthesis of nanoparticles with the extract [22, 35]. Shift in FT-IR peaks 1448.01 to 1452.6 cm^{-1} and 1032.8 to 1043.1 cm^{-1} with reduced band intensity suggesting the binding of silver ion to the alcohol groups and these shifting confirm the reduction of silver ions accompanied by the oxidation of these groups in the synthesis. While it can be inferred from the shifting of bands in EWAgNPs that carboxylate groups and hydroxyl were involved in the synthesis. Haq et al reported the presence of various phytochemicals like tannins, alkaloids, terpenoids, glycosides and flavonoids in the EW extract [15]. Ristig et al reported the band shifting from coordination compounds of Schiff ligand to individual Schiff ligand -from 1492 to 1490 cm^{-1} and 1456 to 1453 cm^{-1} respectively-indicating its potential to coordinate with the metals [36]. In FT-IR spectroscopy, nanoparticles show the absence as well as the shifting of peaks comparative to ligands. Shankar et al reported the reducing potential of terpenoids for metal ions by oxidation of aldehyde groups to carboxylic acids [37]. Aromatic compounds such as phenol compounds in *E. wallichii* extract may be involved in capping and stabilizing the EWAgNPs. Reducing potential of phenolic compounds for metal ions have been reported [38].

77. 46^0 are in agreement with the face centered cubic (fcc) structure of Ag (JCPDS Card No. -04-0783) and those are indexed to (111), (200), (220) and (311) crystallographic planes, respectively [39]. The well-defined intense and sharp peak in XRD pattern of synthesized silver nanoparticles confirms excellent crystalline nature of it. The peaks assigned as '*' indicates the crystallization of different bio-organic components present in the systems while preparing the silver nanoparticles [40, 41]. XRD pattern of EWAgNPs is as shown in fig. (3).

3.2. Determination of phenolic and flavonoids content

Bound phenolic compounds and bound flavonoids in nanoparticles and in the extract were also measured. The total phenolic content of synthesized EWAgNPs was found to be 46.7±2.41 µg GAE/mg as compared to the EW phenolic content of 149.5± 4.76 µg GAE/mg; while bound flavonoid content in nanoparticles was 11.71 µg QE/mg and 52.92 µg QE/mg was observed in EW (Fig. 4). Haq et al had reported 19.08 % phenolic and 0.26 % flavonoid content in EW [15]. Owing to the presence of aromatic rings, phenolic compounds and flavonoids are nucleophilic in nature, which is responsible for the chelating potential of these compounds [38]. It is also evident from the above results that other compounds which are neither phenolic nor flavonoids compounds are also involved actively in nanoparticle formation with metals, as indicated by the lower content of phenolic compounds and flavonoids measured in content determination.

2.3. Antioxidant activity assessment

Nanoparticles showed enhanced antioxidant activity in comparison to the extract alone in DPPH % scavenging, total antioxidant capacity and reducing power assays. The DPPH % scavenging results showed the effective free radical inhibition potential of

[15]. EWAgNPs and EW were assessed for reducing activity. This showed that EWAgNPs have higher reducing power comparative to the extract. Nanoparticles and crude extract showed reducing activity as 44.52±1.34 and 36.80±1.41 µg AAE/mg respectively (Fig. 5). Similar observations were made by Depankar and Murugan with silver nanoparticles using the extract of *Iresine herbstii* [44]. An increase in total antioxidant capacities were observed in nanoparticles as 60.48±2.2 and in EW as 38.59± 1.0 µg AAE/mg (Fig. 5).

2.4. Determination of toxic effects

We treated cancer cells with and without extract, nanoparticles and evaluated the cell viability/toxicity using MTT assay. The results showed that treatment with both samples studied affect the viability of hella cells by reducing their proliferation. Dose and time dependant effects of EW and EWAgNPs are shown in fig. (6). Dose (Fig. 6-A) and time (Fig. 6-B) dependant decreased proliferation indicates the anticancer potential of prepared nanoparticle.

Whether extract or nanoparticels induce geno-toxic effects, we assayed HeLa cells treated with extract or nanoarticles using the Tunnel assay. Assay detects apoptosis-induced DNA fragmentation. The presence of TUNEL-positive cells with fragmented DNA in their nuclei was indicated by a red fluorescence signal, exhibiting DNA strand breaks, and apoptosis induction (Fig. 7). The DAPI-stained nuclei appeared blue and merge image show overlaying of DAPI and Tunnel images (Fig. 7). In control cells, no nuclei of red-stained were detected due to the absence of apoptotic cells. However, the number of nuclei stained by the TUNEL reagents was higher in cells treated with nanoparticled treatment as compared to extract (Fig. 7). It is exhibiting the a greater level of apoptosis ultimately higher cytotoxic

assay was performed to evaluate the cytotoxicity of EWAgNPs and EW at the final concentration of 500, 250, 125, 62.5, 31.62 and 15.6 µg/ml. The LD_{50} values of the EWAgNPs and EW extract were found to be 66.83 and 134.94 µg/ml respectively (Table 1). The lower LD_{50} value of EWAgNPs suggesting the cytotoxic nature of nanoparticles when compared with EW extract. According to result the significant increase in the cytotoxicity of EWAgNPs reveals the involvement of toxic constituents of EW extract in silver nanoparticle synthesis. Enhanced dose dependent cytotoxicity may be correlated to another report in which higher concentrations of green synthesized nanoparticles of *Melia azedarach* had significant cytotoxic effects against cancer cell lines (Hela, HBL 100) [44]. The cytotoxic effect of nanoparticles on shrimps' larvae can be linked with anticancer potential and thus, nanoparticles could be an alternative source of anticancer drugs [45]. At present, much attention is being given to metallic nanoparticles and their anticancer activity. Various studies reported that cells experience different effects in *in vivo* and *in vitro* conditions. Cancer cells have different pathophysiology than normal cells and these are widely used for the toxicity testing of the nanoparticles. Silver nanoparticles are available in different coatings, each having different degrees of cytotoxicity. Furthermore, the toxicological results obtained from such experiment might be conflicting comparative to normal cells. Keeping in view of the distinct biokinetic features of silver nanoparticles, it is essential to address toxicity-related problems of these particles in suitable experimental *in vivo* models with respect to liver, kidney, lungs along with related effects on central nervous system disorders and endocrine functions [46].

3.5. Antimicrobial activity assessment

420 silver nanoparticles are used as antimicrobial agents [48]. The antimicrobial activities of
421 synthesized EWAgNPs and EW were investigated against various bacterial and fungal
422 species using the disc diffusion method. Zones of inhibition (mm) around each disc of EW,
423 EWAgNPs and control are represented in Table 2. Silver nanoparticles were observed to have
424 more bactericidal activity against gram positive comparative to gram negative bacterial
425 strains. These particles cause alteration in the cell permeability that led to cell death.
426 Moreover, shape and size determine the critical role in the pharmacological activity of the
427 nanoparticle. Usually, smaller sized nanoparticles are more active [49].

428 There are several reports for the enhanced antimicrobial activities of silver
429 nanoparticles and in present study synthesized nanoparticles showed enhanced antimicrobial
430 activity. The EWAgNPs were found to have the highest antibacterial activity against *S.*
431 *aureus* (14.5±0.5 mm, MIC 3.33 µg/ml) and highest antifungal activity against *A. fumigatus*
432 (11.5±1.3 mm, MIC 15 µg/disc). The detailed results are shown in Table 3. Awwad et al have
433 reported better antibacterial activity of nanoparticles of *S. tricobatum* extract against *Solanum*
434 *aureus* comparative to other bacterial strains [50]. These findings support our study.
435 Antifungal activities of silver nanoparticles are also reported [7]. The EWAgNPs were
436 proved to be more effective antimicrobial agents compared to EW. Nanoparticles are reported
437 to attach to the cell membranes of microorganisms for penetration. These particles attack
438 microbial cells by interfering with cell division and the respiratory chain, leading to cell death
439 [51]. Antimicrobial activity of silver nanoparticles is due to the release of silver ions in cells
440 [52] and attached bioactive constituents [22]. Liposolubility is the main factor that determines
441 the antifungal activity of the sample [53]. The lipid membrane around the cell controls

Conclusion

Natural products especially medicinal plants are potential sources of efficient and cost effective medicines by utilizing various techniques. In current study, cost-effective as well as fast biogreen method was used for the synthesis of silver nanoparticles of the methanolic extract of *E.wallichii* roots. Particles were varied in shape and size ranged with average 63 nm. The prepared nanoparticles were characterized by UV/vis spectroscopy, FT-IR spectroscopy and FESEM, EDX, XRD analysis and subjected to various antioxidant (DPPH scavenging, reducing power and total antioxidant assays), antimicrobial (Antibacterial and antifungal assays), cytotoxic assays (Brine shrimp lethality, MTT and TUNEL assays). Results suggest the significant and enhanced activities of nanoparticles as compared to crude extract, conferring these moieties as an important candidate for further potential application in many areas including chemoprevention, antimicrobial and cytotoxic field.

Ethics approval and consent to participate

Not applicable.

Human and animal rights

No Animals/Humans were used for studies that are base of this research.

Consent for publication

Not applicable.

Conflict of interest

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implications of nanoparticles. *Green Chem.,* **2006**, *8,* 417-432.

[2] Chandran, S.P.; Chaudhary, M.; Pasricha, R.; Ahmad, A.; Sastry. M. Synthesis of gold nanotriangles and silver nanoparticles using Aloevera plant extract. *Biotechnol. Prog.,* **2006,** *22*, 577-583.

[3] Joerger, R.; Klaus, T.; Granqvist, C. Biologically Produced Silver-Carbon Composite Materials for Optically Functional Thin-Film Coatings. *Adv. Mater.,* **2000,** *12*, 407-409.

[4] Gu, H.; Ho, P.; Tong, E.; Wang, L.; Xu, B. Presenting vancomycin on nanoparticles to enhance antimicrobial activities. *Nano Lett.,* **2003,** *3*, 1261-1263.

[5] Schabes-Retchkiman, P.; Canizal, G.; Herrera-Becerra, R.; Zorrilla, C.; Liu, H.; Ascencio, J. Biosynthesis and characterization of Ti/Ni bimetallic nanoparticles. *Opt. Mater.,* **2006,** *29*, 95-99.

[6] Gong, P.; Li, H.; He, X.; Wang, K.; Hu, J.; Tan, W.; Zhang, S.; Yang, X. Preparation and antibacterial activity of Fe3O4@ Ag nanoparticles. *Nanotechnology,* **2007,** *18*, 285604. http://dx.doi.org/10.1088/0957-4484/18/28/285604

[7] Vivek, M.; Kumar, P.S.; Steffi, S.; Sudha, S. Biogenic silver nanoparticles by *Gelidiella acerosa* extract and their antifungal effects. *Avicenna J. Med. Biotechnol.,* **2011,** *3*, 143-148.

[8] Kandile, N.G.; Zaky, H.T.; Mohamed, M.I.; Mohamed, H.M. Silver Nanoparticles Effect on Antimicrobial and Antifungal Activity of New Heterocycles. *Bull. Korean Chem. Soc.,* **2010,** *31*, 3530-3538.

152.

[10] Ahmad, N.; Bhatnagar, S.; Ali, S.S.; Dutta, R. Phytofabrication of bioinduced silver nanoparticles for biomedical applications. *Int. J. Nanomedicine,* **2015,** *10*, 7019-7030.

[11] Gardea-Torresdey, J.L.; Gomez, E.; Peralta-Videa, J.R.; Parsons, J.G.; Troiani, H.; Jose-Yacaman, M. Alfalfa sprouts: a natural source for the synthesis of silver nanoparticles. *Langmuir,* **2003,** *19*, 1357-1361.

[12] Vilchis-Nestor, A.R.; Sanchez-Mendieta, V.; Camacho-Lopez, M.A.; Gomez-Espinosa, R.M.; Camacho-Lopez, M.A.; Arenas-Alatorre, J.A. Solvent less synthesis and optical properties of Au and Ag nanoparticles using *Camellia sinensis* extract. *Mater. Lett.,* **2008,** *62*, 3103-3105.

[13] Kasthuri, J.; Veerapandian, S.; Rajendiran, N. Biological synthesis of silver and gold nanoparticles using apiin as reducing agent. *J. Colloids Surf. B Biointerfaces,* **2009,** *68*, 55-60.

[14] Verpoorte, R.; Kim, H.; Choi, Y. Plants as source for medicines: new perspectives. *R.J. Bogers, L.E. Craker and D. Lange editors. Med. Arom. Plant.,* **2006**; *17*, 261-273.

[15] Ul-Haq, I.; Ullah, N.; Bibi, G.; Kanwal, S.; Ahmad, M.S.; Mirza, B. Antioxidant and cytotoxic activities and phytochemical analysis of *Euphorbia wallichii* root extract and its fractions. *Iran. J. Pharm. Res*., **2012,** *11*, 241-249.

[16] Garipelli, N.; Runja, C.; Potnuri, N.; Pigili, R.K. Aanti-inflamatory and antioxidant activities of ethanolic extract of *Euphorbia thymifolia* Linn. whole plant. *Int. J. Pharm. Pharm. Sci.,* **2012,** *4(3)*, 516-519.

[18] Zahir, A.A.; Chauhan, I.S.; Bagavan. A.; Kamaraj, C.; Elango, G.; Shankar, J.; Arjaria, N.; Roopan, M.; Rahuman, A.A.; Singh, N. Synthesis of Nanoparticles Using *Euphorbia prostrata* Extract Reveals a Shift from Apoptosis to G0/G1 Arrest in Leishmania donovani. *J. Nanomed. Nanotechnol.,* **2014,** *5*, 1-12.

[19] Devi, G.D.; Murugan, K.; Selvam, C.P. Green synthesis of silver nanoparticles using *Euphorbia hirta* (*Euphorbiaceae*) leaf extract against crop pest of cotton bollworm, *Helicoverpa armigera* (*Lepidoptera: Noctuidae*). *J. Biopesticides,* **2014,** *7*, 54-66.

[20] Haq, I-u-.; Mirza, B.; Kondratyuk, T.P.; Park, E-J.; Burns, B.E.; Marler, L.E.; Pezzuto, J.M. Preliminary evaluation for cancer chemopreventive and cytotoxic potential of naturally growing ethnobotanically selected plants of Pakistan. *Pharm. Biol.,* **2013,** *51*, 316-328.

[21] Ali, I.; Naz, R.; Khan, W.N.; Gul, R.; Choudhary, M.I. Biological screening of different root extracts of Euphorbia wallichii. *Pak. J. Bot.,* **2009,** *41*, 1737-1741.

[22] Phull, A.R.; Abbas, Q.; Ali, A.; Raza, H.; kim, S.J.; Zia, M.; ul-Haq, I. Antioxidant, cytotoxic and antimicrobial activities of green synthesized silver nanoparticles from crude extract of *Bergenia ciliata*. *Future J. Pharm. Sci.,* **2016,** *2(1)*, 31-36.

[23] Zargar, M.; Hamid, A.A.; Bakar, F.A.; Shamsudin, M.N.; Shameli, K.; Jahanshiri, F.; Farahani, F. Green Synthesis and Antibacterial Effect of Silver Nanoparticles Using Vitex negundo L. *Molecules,* **2011,** *16*, 6667-6676.

[24] Clarke, G.; Ting, K.N.; Wiart. C.; Fry, J. High correlation of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing activity potential and total phenolics content indicates redundancy in use of all three assays to screen for

[25] Bibi, G.; Haq, I.; Ullah, N.; Muazzam, A.G.; Mannan, A.; Mirza, B. Phytochemical Evaluation of Naturally Growing Aster thomsonii Plant Species. *J. phrm. Harb. Form.,* **2012,** *2*, 33-39.

[26] Banerjee, J.; Narendhirakannan, R. Phytochemical analyses, antibacterial, *in vitro* antioxidant and cytotoxic activities of ethanolic extract of Syzygium cumini (L.) seed extract. *Int. J. Pharm. Res.,* **2011,** *2*, 1799-1806.

[28] Phull, A.R.; Eo, S.H.; Kim, S.J. Oleanolic acid (OA) regulates inflammation and cellular dedifferentiation of chondrocytes via MAPK signaling pathways. *Cell. Mol. Biol.* (*noisy le grand*), **2017,** *63(3)*, 12-17.

[29] Bibi, G.; Haq, I.; Ullah, N.; Mannan, A.; Mirza, B. Antitumor, cytotoxic and antioxidant potential of *Aster thomsonii* extracts. *Afr. J. Pharm. Pharmacol.,* **2011,** *5*, 252-258.

[30] Fatima, H.; Khan, K.; Zia, M.; Ur-Rehman, T.; Mirza, B.; Haq, I.U. Extraction optimization of medicinally important metabolites from Datura innoxia Mill.: an *in vitro* biological and phytochemical investigation. *BMC Complem. Altern. M.*, **2015,** *15(1)*, 1-18.

[31] Noginov, M.; Zhu, G.; Bahoura, M.; Adegoke, J.; Small, C.; Ritzo, B.; Drachev, V.; Shalaev, V. The effect of gain and absorption on surface plasmons in metal nanoparticles. *Appl. Phys.* B, **2007**; *86*, 455-460.

[32] Mallikarjuna, K.; Narasimha, G.; Dillip, G.; Praveen, B.; Shreedhar, B.; Lakshmi, C.S.; Reddy, B.; Raju, B.D.P. Green synthesis of silver nanoparticles using Ocimum leaf extract and their characterization. *Dig. J. Nanomater. Biostruct.*, **2011,** *6*, 181-

570 [34] Chen, C-X.; Mu, S-L. The electrochemical preparation of polyphenol with
571 conductibility. *Chin*. *J*. *Polym*. *Sci*., **2002,** *20*, 309-316.
572 [35] Mochochoko, T.; Oluwafemi, O.S.; Jumbam, D.N.; Songca, S.P. Green synthesis of
573 silver nanoparticles using cellulose extracted from an aquatic weed; water hyacinth.
574 *Carbohydr*. *Polym*., **2013,** *98*, 290-294.
575 [36] Ristig, S.; Kozlova, D.; Meyer-Zaika, W.; Epple, M. An easy synthesis of
576 autofluorescent alloyed silver-gold nanoparticles. *J*. *Mater*. *Chem*. B, **2014**; *2*, 8787-
577 8795.
578 [37] Shankar, S.S.; Rai, A.; Ahmad, A.; Sastry, M. Rapid synthesis of Au, Ag, and
579 bimetallic Au core-Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf
580 broth. *J*. *Colloid Interface Sci*., **2004,** *275*, 496-502.
581 [38] Sivaraman, S.K.; Elango, I.; Kumar, S.; Santhanam, V. A green protocol for room
582 temperature synthesis of silver nanoparticles in seconds. *Curr*. *Sci*., **2009**, *97*, 1055-
583 1059.
584 [39] N. Agasti, N. K. Kaushik, One Pot Synthesis of Crystalline Silver Nanoparticles,
585 American *J. Nanomaterials*, **2014,** *2*, 4-7.
586 [40] Suvith, V. S.; Philip, D. Catalytic degradation of methylene blue using biosynthesized
587 gold and silver nanoparticles. *Spectrochim. Acta A Mol. Biomol. Spectrosc*., **2014,**
588 *118*, 526–532.
589 [41] Philip, D. Biosynthesis of Au, Ag and Au–Ag nanoparticles using edible mushroom
590 extract, *Spectrochim. Acta A Mol. Biomol. Spectrosc*., **2009**, *73*, 374-381.
591 [42] Nie, Z.; Liu, K.J.; Zhong, C-J.; Wang, L-F.; Yang, Y.; Tian, Q.; Liu, Y. Enhanced

[43] Saikia, J.P.; Paul, S.; Konwar, B.K.; Samdarshi, S.K. Nickel oxide nanoparticles: A novel antioxidant. *J. Colloid Surf*. B, **2010**, *78*, 146-148.

[44] Dipankar, C.; Murugan, S. The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from Iresine herbstii leaf aqueous extracts. *J. Colloid Surf*. B, **2012**, *98*, 112-119.

[45] Ghareeb, M.A.;, Hussein, A.H.; AR, L. Antioxidant and cytotoxic activities of *Tectona grandis* Linn. Leaves. *Int. J. Pharmacol.*, **2014**, *5*, 143-157.

[46] Bahadar, H.; Maqbool, F.; Niaz, K.; Abdollahi, M. Toxicity of nanoparticles and an overview of current experimental models. *Iran. biomed. J.*, **2016**, *20 (1)*, 1-11.

[47] Seil, J.T.; Webster, T.J. Antimicrobial applications of nanotechnology: methods and literature. *Int. J. Nanomedicine*, **2012**, *7*, 2767-2781.

[48] Ravindra, S.; Murali Mohan, Y.; Narayana Reddy, N.; Mohana Raju, K. Fabrication of antibacterial cotton fibers loaded with silver nanoparticles via "Green Approach". *Colloids and Surfaces A: Physicochem Eng. Asp.*, **2010**, *367*, 31-40.

[49] Maity, D.; Pattanayak, S.; Mollick, M.M.R.; Rana, D.; Mondal, D.; Bhowmick, B.; Dash, S.K.; Chattopadhyay, S.; Das, B.; Roy, S.; Chakraborty, M. Green one step morphosynthesis of silver nanoparticles and their antibacterial and anticancerous activities. *New J. Chem.*, **2016**, *40(3)*, 2749-2762.

[50] Awwad, A.M.; Salem, N.M.; Abdeen, A.O. Green synthesis of silver nanoparticles using carob leaf extract and its antibacterial activity. *Int. J. Ind. Chem.*, **2013**, *4(1)*, 29-36.

[51] Rai, M.; Yadav, A.; Gade, A. Silver nanoparticles as a new generation of

2005, *16*, 2346-2353.

[53] Raman, N.; Muthuraj, V.; Ravichandran, S.; Kulandaisamy, A. Synthesis, characterisation and electrochemical behaviour of Cu (II), Co (II), Ni (II) and Zn (II) complexes derived from acetylacetone and p-anisidine and their antimicrobial activity. *J. Chem. Sci.*, **2003**, *115*, 161-167.

[54] Sadeek, S.A.; El-Shwiniy, W.H.; Zordok, W.A.; El-Didamony, A.M. Synthesis, spectroscopic, thermal and biological activity investigation of new Y(III) and Pd(II) norfloxacin complexes. *J. Argent. Chem. Soc.*, **2009**, *97*, 128-148.

Figures

Fig. (1). UV/visible spectra (A), FESAM micrograph (B), EDX spectrum (C), of silver nanoparticles (EWAgNPs) or extract of *E. wallichii* (EW) are depicted.

Fig. (2). The FT-IR spectra of synthesized silver nanoparticles (EWAgNPs) and extract of *E. wallichii* (EW) in the wavenumber frequency range of 4000-600cm^{-1}.

Fig. (3). XRD pattern of silver nanoparticles (EWAgNPs)

Fig. (4). Total phenolic (µg GAE/mg sample) and flavonoid content (µg QE/mg sample) determination in silver nanoparticles (EWAgNPs) and extract of *E. wallichii* (EW).Values are presented as mean ± Standard deviation from triplicate investigation. Asterisk symbol represents a significant difference between two groups (EW, EWAgNPs).

Fig. (5). Total antioxidant capacity (µg AAE/mg sample), Reducing power (µg AAE/mg sample) and % radical scavenging activity (DPPH) determination in Silver (EWAgNPs) and extract of *E. wallichii* (EW). Values are presented as mean ± Standard deviation from triplicate investigation. Asterisk symbol denotes a significant difference between two groups (EW, EWAgNPs).

Fig. (6). Effect of EW and EWAgNPs on the cell viability of breast cancer cells (Hella). To investigate the dose and time dependant effect cells were treated with (A) 0-5mg/ml for 24 h, or (B) 5 mg/ml for indicated time periods. Cellular toxicity was investigated through MTT assay. Untreated cells were used as control by considered 100% viability. Results are presented as mean of three experiments ±SD.

Tables

Table 1. Shrimps cytotoxicity assay of silver nanoparticles (EWAgNPs) and extract of *E. wallichii* (EW)

Table 2. Antibacterial activityof silver nanoparticles (EWAgNPs) and extract of *E. wallichii*(EW)

Table 3. Antifungal activity of silver nanoparticles (EWAgNPs) and extract of *E. wallichii*(EW)