

## Original Research Article

# Biological efficacy of phyto-synthetic silver nanoparticles using ethanol extract of *Euphorbia wallichii* Hook Rhizome as bio-reductant and surfactant

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### Abstract

**Purpose:** To synthesize silver nanoparticles using rhizome extract (RE) of *E. wallichii* as a bio-reductant and surfactant.

**Method:** RE was used as a bio-reductant for the reduction of  $Ag^{+1}$  (aq.) and as a stabilizing agent for silver nanoparticles (AgNPs). The AgNPs were then characterized by UV-visible spectrophotometer, scanning electron microscopy (SEM), dynamic light scattering spectroscopy (DLS), x-ray diffraction (XRD), energy dispersive x-ray spectroscopy (EDS) and infrared (IR) spectroscopy. The antinociceptive potential of AgNPs was evaluated using Eddy's hot plate. Spasmolytic bioassay was measured in mice via GI charcoal movement test. Anti-leishmanial potency was examined against promastigotes of *Leishmania tropica*, while antioxidant bioassay was determined using DPPH reduction method.

**Results:** UV-Vis results showed characteristic surface plasmon resonance band from 440 – 460 nm, thus confirming the reducing ability of RE. SEM analysis showed spherical AgNPs with a typical face center cubic (fcc) structure as evidence by their x-ray diffraction (XRD) pattern. The role of RE as a stabilizing agent towards AgNPs was supported by IR and electronic absorption spectral data. Phytochemical investigation of RE confirmed the presence of alkaloids, flavonoids, phenols and glycosides etc. Mice treated with RE-AgNPs exhibited dose-dependent analgesic and spasmolytic effects. RE functionalized AgNPs exhibited promising antileishmanial and antioxidant effects ( $p < 0.05$ ).

**Conclusions:** RE-AgNPs exhibit substantial spasmolytic and antinociceptive activity, indicating central and peripheral analgesic effects. Thus, the formulation's antileishmanial and antioxidative effects, indicate that it might be useful for preventing infectious and oxidative stress disorders.

**Keywords:** *Euphorbia wallichii*, Silver nanoparticles, Antioxidant, Analgesic, Antispasmodic, Antileishmanial

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## INTRODUCTION

Nanobiotechnology is an interdisciplinary science with the application to the biological systems to manufacture new biochemical system. The transformed nanobiosystems exhibit completely new properties like morphology, density, diffusion rate, catalytic potential and size distribution [1]. Fabrication of nanoparticles via green route enhances physiological potential, much economical and ecofriendly compared to chemical and physical methods which requires high energy, use toxic and expensive chemicals [2].

The modulation of metals into their nanoscale structures by phytometabolites is of vital importance for the development of nanomedicine. Nanoparticles derived from metallic silver, made notable comeback as a potent antimicrobial, antimalarial and as antiangiogenic agent. Thus, AgNPs have diverse applications in modern health system like silver coated medicinal devices, dressings, nanogels and nano-lotions [3]. Plant extracts have the capability to reduce metals ions and to synthesize biogenic nanoparticles, improve their monodispersion and modulate their shapes and sizes. Different transition metals like Au, Mn, Zn, Ti, Cu and Ag have been used for the synthesis of nanoparticles but silver was proven to be most effective against bacteria, viruses and other pathogenic microbes [4]. Green and biogenic synthesis of nanoparticles is highly advantageous over both physical and chemical methods for its low cost and eco-friendly method as it avoids the use of high temperature, energy, pressure and toxic hazardous chemicals. Phytometabolites provide a better platform for biogenic nanoparticles as they are providing natural capping agents free from toxic chemicals [5].

*Euphorbia wallichii* is a himalayan herb, used in tibet as folkloric remedy for curing furuncle, exanthema, cutaneous anthrax and other skin disorders. Stem latex is using for healing external wounds while whole plant is highly laxative and causes severe diarrhea [6,7].

## EXPERIMENTAL

The plant specimen was collected from Abbottabad Pakistan and a voucher specimen (no. Bot.20078 PUP) was submitted to the herbarium of Department of Botany, University of Peshawar, Pakistan. The shade dried and powdered rhizome was subjected to successive extraction by 70 % ethanol and filtrate was evaporated with rotary evaporator at  $50 \pm 3$  °C.

Rhizome extract of *E. wallichii* (RE) was screened for different phytometabolites like reducing sugar, alkaloids, saponins, glycosides, proteins, phenols, flavonoids, phytosterols and tannins as per standard procedures [8].

### Biogenic synthesis of AgNPs

Aqueous solution of RE (200 µg/mL) was mixed with 1 mM AgNO<sub>3</sub> (Aqu) in different ratios (1:5, 1:10, 1:15, 1:20, 1:25, 1:30 and 1:35 v/v) on magnetic stirrer at  $35 \pm 3$  °C till the color of solution turned yellow brown. Optimization of AgNPs was brought by measuring UV-Visible spectrum against ethanol in deionized water as a blank with a resolution of 1 nm between the wavelength ranges of 300 to 800 nm. Dried powder of RE-AgNPs was mixed with KBr, pressed by hydraulic Pellet Press and subjected to FT-IR spectroscopy using "PerkinElmer spectrometer FT-IR Spectrum One". XRD pattern was analyzed using JEOL JDX 3532 X-ray diffractometer. The dried AgNPs was coated on a carbon tape, subjected to gold coating and analyzed for morphological features through SEM. Size distribution was analyzed through dynamic light scattering (DLS) technique using Malven Zetasizer. For EDX, powdered AgNPs mass was subjected to elemental mapping using the Oxford Inca 200 SEM instrument equipped with a Thermo EDX attachment.

### Antileishmanial bioassay

*In vitro* anti-leishmanial activity was performed against *Leishmania tropica* (KWH<sub>23</sub>) in 96 wells flat bottom plates. From the standardized promastigotes suspension ( $4 \times 10^6$  parasites/mL), 200 µL M-199 medium/well with  $1 \times 10^5$  promastigotes were transferred in 96-well plates, feed with 10 µg/mL, 50 µg/mL and 100 µg/mL of each of RE and RE-AgNPs, incubated at 26 °C for 48 hours and the number of promastigotes was counted with the help of Neubauer Hemocytometer in microscope.

### Assessment of analgesic activity

*In vivo* analgesic potency of RE and RE-AgNPs were determined using Swiss albino mice. The animals were divided into eight groups (n = 6), i.e. group I; received saline at 10 ml/kg bw (i.p); Group II; diclofenac sodium at 10 mg/kg bw (i.p); groups III, IV & V; received 50, 100 and 200 mg/kg bw of RE, respectively (i.p) and groups VI, VII and VIII received 50, 100 and 200 mg/kg bw of RE-AgNPs respectively (i.p).

The paw flicking and jumping response of animals at  $55 \pm 2$  °C was investigated using

eddy's analgesiometer, after 30, 60 and 90 min of drugs administration [9]. The experimental animals were dealt as per protocols of international guidelines for animal studies during the entire period of study [10].

### Evaluation of antispasmodic activity

The overnight fasted mice were divided into eight groups (six mice in each) i.e. group I; received saline at 10 ml/kg bw, (p.o), group II; received atropine sulphate at 10 mg/kg bw, (p.o), Group III, IV and V; respectively received doses of 50, 100 and 200 mg/kg bw of RE (p.o) and Group VI, VII & VIII received 50, 100 & 200 mg/kg bw of RE-AgNPs respectively (p.o). The experimental animals were dealt as per protocols of international guidelines for animal studies during the entire period of study [10]. 0.2 mL of activated charcoal was given to all animals after 15 min of doses administration. Animals were sacrificed by cervical dislocation after 30 min of charcoal treatment, dissected and the intestine was cut down from pyloric to caecum. The distance travelled by the charcoal relative to the total intestinal length was measured as percent propulsion using equation 1.

$$\text{Propulsion (\%)} = \text{CT/TL} \times 100 \dots\dots\dots (1)$$

where CT = charcoal meal traveled in small intestine and TL = Total intestinal (small) length.

### Determination of antioxidant potential

RE and RE-AgNPs were tested for free radical scavenging assay (antioxidant potential) using 2, 2-diphenyl- 1-picrylhydrazyl (DPPH). Different doses (10, 20, 40, 60, 80 and 100 µg/mL) of RE and RE-AgNPs were mixed with 0.1 mM DPPH. The reaction mixtures were tested for optical density (OD) at 517 nm after 30, 60 and 90 min of incubation. Untreated DPPH was run as control condition while ascorbic acid treated DPPH was run as a standard antioxidant drug. Antioxidant efficiency (E) was determined as in Eq 2.

$$E (\%) = (\text{Ab} - \text{At}/\text{Ab}) \times 100 \dots\dots\dots (2)$$

where Ab, At are the absorbance of blank and test, respectively samples.

### Evaluation of antibacterial activity

Sterile petri plates filled with Mueller–Hinton agar were autoclaved. Freshly prepared diluted culture of tested bacterial strains was swabbed on Mueller–Hinton agar plates. Wells of 6mm diameters were bored, filled with 2 mg/ml of each

streptomycin RE and RE-AgNPs, incubated at 30 °C for 24 h and evaluated for zone of inhibition (mm).

### Statistical analysis

Data obtained for characterization was plotted using Origin Pro.9.1. The effective concentration for antioxidant potential was determined using Probit analysis in Biostat plus. ANOVA was used for the comparison of mean effect followed by LSD as post-hoc test using SPSS. Antimicrobial, Analgesic and antispasmodic efficacy were expressed as mean ± SEM using GraphPad Prism 7.

## RESULTS

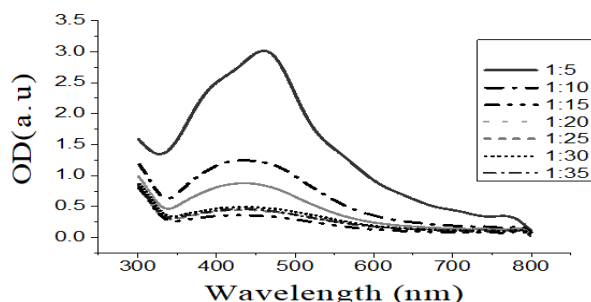
The phytochemical investigation of RE showed the presence of alkaloids, glycosides, reducing sugars, phenols, proteins, flavonoids, saponins, phytosterols and tannins (Table 1). Biogenic synthesis of RE-AgNPs was confirmed from color of reaction medium which turns from light pale (initial) to characteristic dark brown (final) color. This characteristic color, with a corresponding absorption at around 440 to 460 nm (Figure 1), arise from the collective oscillation of surface electron (palsmons) of AgNPs in resonance with the light wave. XRD pattern shows diffraction peaks at  $2\theta$  values of  $16.92^\circ$ ,  $38.20^\circ$ ,  $44.60^\circ$  and  $64.48^\circ$  correspond to Bragg's planes (1 1 1), (2 0 0), (2 2 0) and (3 1 1) of silver respectively, confirm crystalline phase of silver with face center cubic (fcc) dimension (Figure 2). The mean crystallite size was calculated using Scherrer's equation based on broadening of the (111) reflection was found to be 14 nm. Size distribution curve (Figure 3) shows a bimodal distribution pattern, representing polydispersity of AgNPs with average particle diameter of 30.57 nm. The electron micrograph of AgNPs at various magnification shows spherical to rod shaped morphology with uniform distribution where the average diameter is in the range of 20-58 nm. The larger particulate size is due to the agglomeration of individual particles (Figure 4). EDX spectrum shows the elemental composition of RE-AgNPs. One of the intense peak obtained for Ag at 3 KeV while other peaks for Mg, Al, K, Ca, S, Cl and Si were also observed (Figure 5). The characteristics X-ray emission peak of Ag (3 KeV) confirms the presence of metallic silver in RE-AgNPs. FT-IR spectrum shows vibration stretches at 1007, 1190, 1596 and 2926  $\text{cm}^{-1}$  (Figure 6). The stretch at 2926  $\text{cm}^{-1}$  assigned to O-H of carboxylic acid functional group, while 1007  $\text{cm}^{-1}$  stretch represent C-N bond may arise from aliphatic amines. 1190  $\text{cm}^{-1}$  vibrational mode representing C-O stretch of alcohols,

carboxylic acids, esters and ethers. Vibrational stretch of  $1596\text{ cm}^{-1}$  representing C-C bond correspond to aromatics and N-H bond to amines.

ANOVA results revealed significant ( $p < 0.05$ ) growth inhibition of all tested bacterial strains. RE-AgNPs was more potent in inhibiting the growth of all tested bacterial strains than RE alone (Figure 7). *E. coli* was found to be more susceptible to AgNPs than streptomycin, while *P. aeruginosa* was found more resistant to all treatments.

Both RE and RE-AgNPs were tested for their *in vitro* antileishmanial efficacy against *Leishmania tropica*. RE exhibited dose dependent mortality of 37, 53 and 62 % at 10, 50 and 100  $\mu\text{g/mL}$  doses respectively with  $\text{LD}_{50}$  of 34.73  $\mu\text{g/mL}$ . RE-AgNPs revealed pronounced anti-leishmanial property, exhibiting 28, 60 and 82 % lethality at 10, 50 and 100  $\mu\text{g/mL}$  respectively with  $\text{LD}_{50}$  of 27.30  $\mu\text{g/mL}$  (Figure 8).

Mice treated with RE at 50, 100 and 200 mg/kg bw, produced 30.51, 39.71 and 78.85 % spasmolytic responses compared to atropine sulphate. Experimental animals treated with RE-AgNPs, revealed dose dependent spasmolytic action, exhibiting 62.29, 77.54 and 83.21 % antispasmodic efficacy at 50, 100 and 200 mg/kg bw respectively compared to the atropine sulphate (Figure 9). In the paw flick test, pre-oral administration of RE and RE-AgNPs caused significant ( $p < 0.05$ ) dose related analgesia, though less analgesic than standard diclofenac sodium (Figure 10). RE-AgNPs were more potent than RE probably due to their rapid diffusion as they possess considerably small sizes (1 - 100 nm). Antioxidant potential of RE and RE-AgNPs was evaluated using DPPH as a source of free radical. The effective concentrations for 50 % DPPH inhibition ( $\text{EC}_{50}$ ) were 5.84, 5.45 and 3.46  $\mu\text{g/mL}$  for RE-AgNPs, RE and ascorbate respectively. RE at 30 and 60 min was generally more effective than AgNPs.

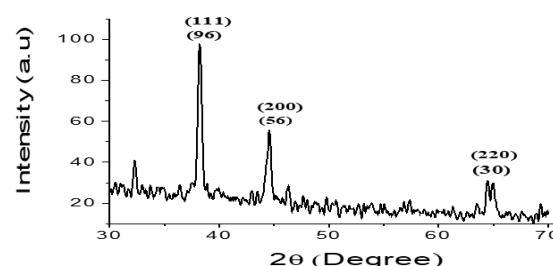


**Figure 1:** UV-Vis spectra of RE-AgNPs synthesized from different ratios of RE and  $\text{AgNO}_3$  showing the absorption spectra in the range of 400 – 500 nm

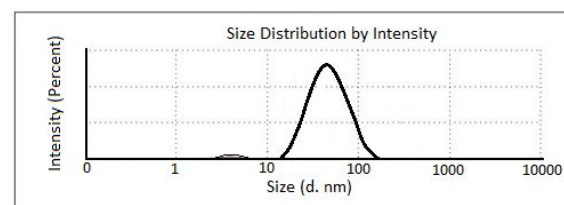
**Table 1:** The qualitative assessment of RE for its phytochemical profile

Phytometabolites	Result
Reducing sugars	++
Alkaloids	++
Flavonoids	+
Tannins	+
Phytosterols and triterpenes	+
Saponin	+
Glycosides	+
Proteins	+
Phenols	++

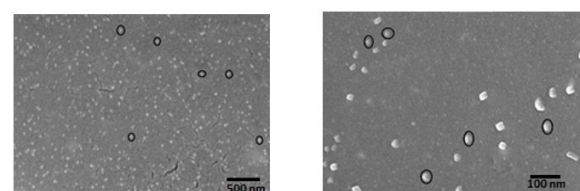
(+ +) high concentration, (+) Present, (-) Absent



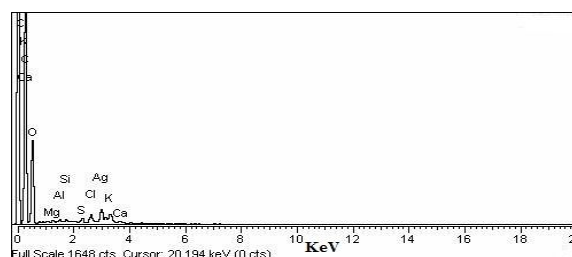
**Figure 2:** X-ray diffraction pattern of RE-AgNPs showing the crystalline phase



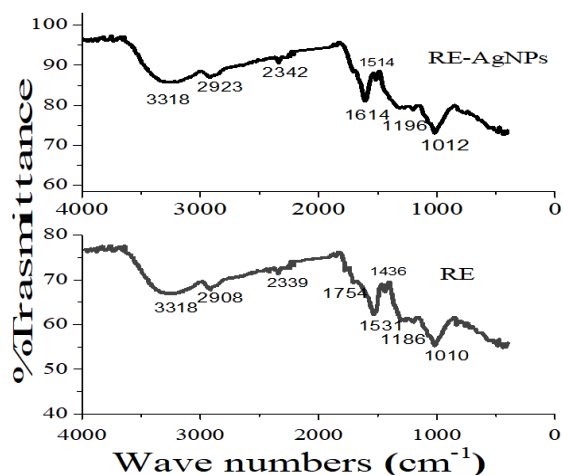
**Figure 3:** DLS spectrograph of RE-AgNPs showing bimodal size distribution



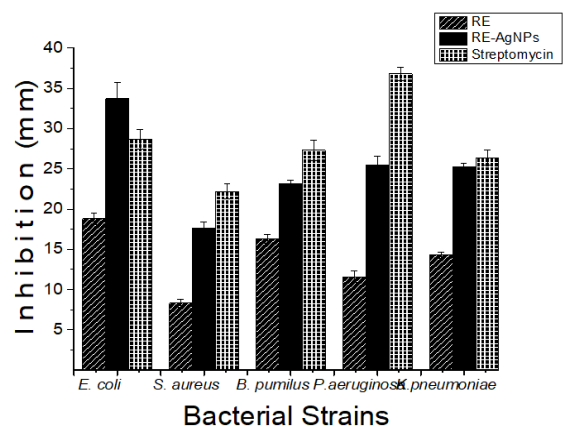
**Figure 4:** Scanning electron micrographs of RE-AgNPs showing spherical to rod-shaped morphology with mean diameter ranged between 20 - 58nm



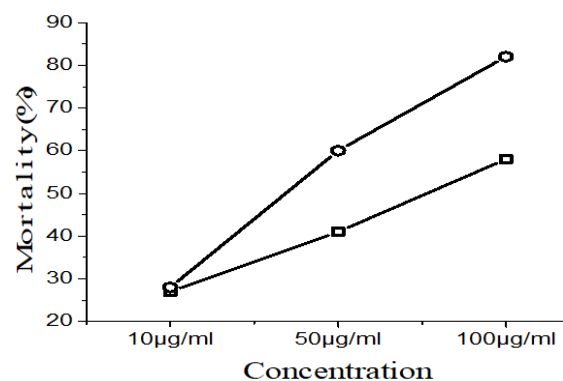
**Figure 5:** EDS of RE-AgNPs showing Ag signals at 3KeV representing metallic silver making the core of NPs



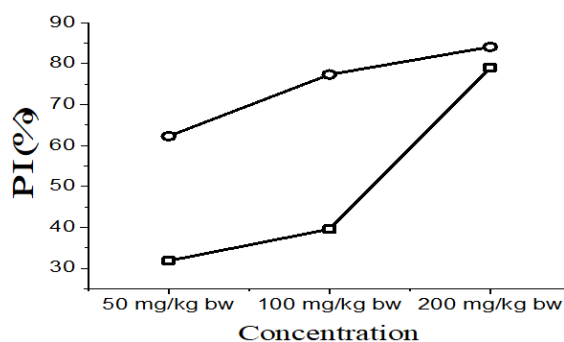
**Figure 6:** FT-IR spectra of RE and RE-AgNPs representing various stretched for different functional groups



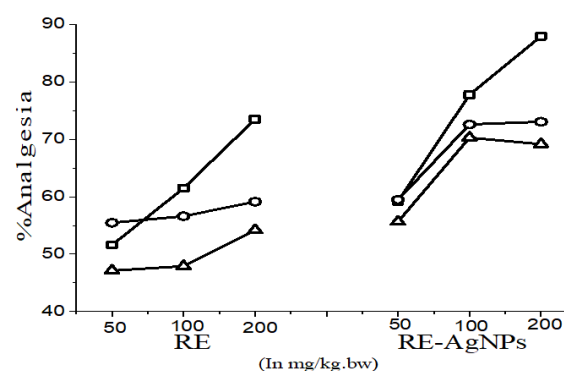
**Figure 7:** Antibacterial effect of RE and RE-AgNPs against different Gram +ve and Gram -ve bacterial strains in comparison to standard antibacterial agent (streptomycin). OW-ANOVA reveals statistical significant ( $p < 0.05$ ) inhibition (%) in linear growth of all tested strains



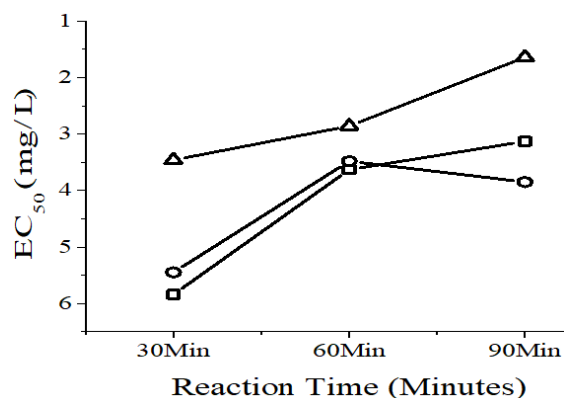
**Figure 8:** Antileishmanial effect of RE (-□-) and RE-AgNPs (-○-) against *L. tropica* showing a dose dependent leishmanicidal potency. Student t-Test reflects non-significant ( $p > 0.05$ ) effect at lower experimental dose but highly significant ( $p < 0.05$ ) effect at higher experimental dose



**Figure 9:** Spasmolytic response (propulsion inhibition "PI") of mice to RE (-□-) and RE-AgNPs (-○-) at various doses. Results revealed highly significant ( $p < 0.05$ ) differences between RE and AgNPs at 50, 100 and 200 mg/kg bw



**Figure 10:** Analgesic response of mice produced after 30 (-□-), 60 (-○-) and 90 minutes (-Δ-) of administration of RE and RE-AgNPs at different time intervals of dose administration.



**Figure 11:** Effective concentration ( $EC_{50}$ ) for DPPH scavenging by RE (-□-), and RE-AgNPs (-○-) at different interval of reaction mixture. Ascorbic acid (-Δ-) produced highly significant ( $p < 0.01$ ) scavenging effect against DPPH at all reaction times, RE-AgNPs exhibited significant ( $p < 0.05$ ) antioxidant potential at 90 minutes of reaction time

## DISCUSSION

Phytochemical screening validates the presence of various primary and secondary metabolites. Available literature reveals that aqueous extract

of *E. wallichii* rhizome shows the presence of saponins, steroids, and triterpenoids, while alkaloid was absent. Similarly, ethanol extract of *E. thymifolia* showed the presence of alkaloids, flavonoids, phenols and phytosterols [11]. Synthesis of AgNPs was primarily confirmed by change in colour of reaction mixture to dark brown which is due to excitation of electron on the surface (SPR) of the newly synthesized nanoparticles [12].

Synthesis of AgNPs accomplished by the reduction of  $Ag^+$  to  $Ag^0$  by phytometabolites, form oligomeric clusters due to agglomeration which lead to the synthesis of colloidal AgNPs. AgNPs shows typical absorption band in visible region due to its characteristic SPR [13]. DLS of RE-AgNPs showed bimodal size distribution with mean particle size of 30.57 nm which is different from size distribution revealed by SEM of RE-AgNPs (20 – 58 nm). This difference is due to the fact that electron microscopy considers only the metallic core while DLS includes the ligand shell and determines the hydrodynamic size [14]. EDX spectral analysis of RE-AgNPs shows intense optical absorption approximately at 3 KeV, whereas other less intense peaks for other elements are related to the binding energies of bio-compounds capped the AgNPs [13].

Metallic nanoparticles target multiple cellular components including cell wall, cell membrane, cytoplasmic organelles and DNA, hence there is least chance for microbe to develop resistance [15]. The antibacterial mechanisms of these nanoparticles include binding of positively charged silver to negatively charged DNA and proteins, thus disrupting cellular metabolic pathways [16,17]. AgNPs also shown attachments to the bacterial plasma membrane proteins thus disturbing its permeability and respiration, hence making AgNPs, a more potent antimicrobial agents [18].

Accumulation of nanoparticles at targeted site has seems to be in high concentration than normal drugs which often leads to reduced systemic toxicity. AgNPs has the tendency to impair with glycoprotein 63 and lipophosphoglycan found on the leishmanial parasite surface leads to generate oxidative stresses and inhibit the growth of leishmanial parasite [19]. The GI tract motility is mainly initiated by the local reflexes and is myogenic. The extrinsic nerves of GI tract have limited role in modulating the peristalsis. Opioid delays gastric emptying through acting on GI sphincters hence decrease intestinal transit. The smooth muscles showing mark contraction when exposed to neurotransmitter where acetylcholine

released from acetylcholine receptors (parasympathetic nerves) [20].

Antispasmodics like atropine and dicyclomine are antagonist of muscarinic acetylcholine receptors and RE-AgNPs exhibited promising antispasmodic effect by antagonizing muscarinic acetylcholine receptors. The spasmolytic effect of RE-AgNPs was more evident than RE due to the provision of large surface area for antagonism. The current results suggest the involvement of  $\mu$ -opioid receptors mediated by RE and RE-AgNPs resulting in the generation of analgesic response through central system. Non-thermal nociceptive tests are sensitive to  $\kappa$ -agonists opioid while thermal analgesic tests are more sensitive to  $\mu$ -agonists opioids [21]. The analgesic activity of *E. wallichii* is attributable to the presence of potent secondary metabolites i.e. alkaloids, flavonoids and saponins known for triggering prostaglandins responsible for peripheral nociceptive perception [22]. The ability of phytometabolites to bleaching DPPH purple color indicating their electron donation tendency [23].

Plants with adequate phenolic contents have been observed with potent antioxidant properties. The antioxidant potential is correlated to the phenolic contents of the plants which has the redox potential where they act as proton donor [24]. Other phytometabolites like reducing sugars, tannins and proteins present in plant extracts may also be responsible for antioxidant activity [25]. The promising antioxidant potential of *E. wallichii* is attributed to the presence of reducing biomolecules like phenols, flavonoids, reducing sugars etc. as evident from biochemical tests. RE delivered elevated DPPH scavenging properties than AgNPs, due to the affinity of both DPPH and  $Ag^+$  ions for the reducing agents.

## CONCLUSION

The findings of this study demonstrate that RE has tremendous reducing potential for silver ions and functionalizes the synthesized AgNPs, Biomimetic RE-AgNPs significantly inhibits the growth of leishmanial parasite and pathogenic bacterial strains. Although RE produces significant analgesia and spasmolytic effects in mice, RE-AgNPs alleviates antispasmodic and analgesic phenomena and hence has clinical potentials for use in humans as such.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

## Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rehman Ullah as a principal author designed the study, the synthesized and characterized RE-AgNPs. Sumaira Shah and Saiqa Afriq Jan collectively performed biological screening. Zahir Muhammad statistically analyzed the data and plot the graphs of the manuscript. Siraj ud Din supervised the whole study and wrote the manuscript. We all authors approved the manuscript for publication.

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