SUPPLEMENTARY MATERIAL

Anticancer and antibacterial potential of *Rhu spunjabensis* and CuO nanoparticles

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Abstract

The present study reports ecofriendly synthesis of CuO nanoparticles (NPs) using an extract of *Rhus punjabensis* as a reducing agent. NPs structural and composition analysis are evaluated by X-rays diffraction (XRD), Fourier transform infrared, Energy dispersive spectroscopy, Scanning electron microscopy, Transmission electron microscopy, and Thermal analysis. The NPs have pure single phase monoclinic geometry with spherical structure and high stability toward heat and with average particle size of about 36.6 and 31.27 nm calculated by XRD and SEM, respectively. NPs are tested for antibacterial, protein kinase (PK) inhibition, SRB cytotoxic, and NF-κB activities. Antibacterial activity is observed against *B. subtilis* and *E. coli*. Significant PK and SRB cytotoxic activity is observed with some NF-κB inhibition. NPs IC_{50} values against HL-60 and PC-3 prostate cancer cells are 1.82 ± 1.22 and 19.25 ± 1.55 μg/ml. The results encourage further studies for antibacterial and anticancer drug development of NPs using animal models.

Key words: SRB cytotoxic; antibacterial; nanoparticles; anticancer; green synthesis

Experimental Part

Plant Collection and Extract Preparation

Leaves of *R. punjabensis* were collected from the local vicinity of Karak, KPK Pakistan and identified by Prof. Dr. Mir Ajab, Taxonomist, Biological Sciences, Quaid-i- Azam University, Islamabad. A voucher specimen HMP 491 was deposited in the Herbarium of Medicinal Plants, Quaid-i-Azam University Islamabad, Pakistan. Leaves were washed, air dried, grinded and used for extract preparation.

To prepare the leaf extract of *R. punjabensis* plant, leaves powder was suspended in distilled water and placed in shaking incubator at 37°C at 200 rpm for 5 hr. The resulting liquid extract was passed through a muslin cloth to remove solid particles and then again filtered through a Whatman filter paper. The filtrate was stored at 4°C till further experiments.

Synthesis of CuO NPs

All chemicals were purchased from Merck and Sigma Aldrich and used without further purification. Aqueous leave extract was warmed up for 10 min before the addition of copper (II) acetate monohydrate. Then, the reaction mixture was placed on a hot plate at 70°C under continuous stirring for 3 hr. Color change from greenish brown to dark brown was observed, which indicated synthesis of NPs. Isolation of NPs was done by centrifugation at 10,000 rpm for 6 min and washed thrice with distilled water. NPs were oven dried at 60°C for 6 hr and calcination was done at 450°C for 2 hr. After calcination, these NPs were finely crushed by pestle and mortar, and collected for characterization and biological applications.

Characterization of CuO NPs

The nature of synthesized CuO NPs was evaluated via using X'Pert³ Powder (PANalytical), working on 40 kV voltage and 30 mA current at room temperature. Nickel monochromator had an angle of diffraction (2θ) in the range of 20° to 80° with radiation source of Cu Kα with 1.5406 Å wavelength. Particle size was calculated with the help of Scherrer's equation ($D=0.9\lambda/\beta \cos\theta$) where D is the average crystalline size, λ wavelength of X-rays (1.5406 Å), β the angular full

width at half maximum, and θ is the angle of diffraction. For elucidation of involvement of biological molecules in the CuO NPs synthesis, FTIR spectroscopy was done by using SHIMADZU FTIR (Kyoto, Japan) with wavelength in the range of 400–4,000 cm-1 **(Fig. S2.)**. The morphological study and size evaluation of CuO NPs were completed by using JEOL-JSM-6490LA SEM (JEOL, Tokyo, Japan), operating at 20 kV with 2,838 counts per second field-emission transmission electron microscope (FE-TEM; JEOL JEM-2100F, Japan), working at 200 kV using LaB6 electron gun and 0.18 nm point-to-point resolution. Elemental analysis of synthesized CuO NPs was done by Energy Dispersive X-Ray Analysis (EDS) JEOL-JSM-6490LA SEM (JEOL, Tokyo, Japan), working at the energy range of 0-20 keV, voltage of 20.0 kV with probe current of 1.00000 nA and counter current of 3411 counts per second. The thermal stability study of CuO NPs was performed by using thermogravimetric and differential thermal analyzer having N_2 gas flowing at a rate of 20 ml/min, heating at 20°C/min in a temperature range of 25 – 850 °C **(Fig. S3.)**.

Sample Preparation for Biological Evaluation of CuO NPs

For biological applications of CuO NPs, 4 mg of NPS were crushed, dissolved in 1 ml of distilled water and sonicated for 20 min to get homogeneous suspension.

Antibacterial assay of CuO NPs

The antibacterial properties of the plant extract and green synthesized CuO NPs (4 mg/ml) were evaluated by using the disc diffusion assay against *S. aureus, P. aeruginosa, E. coli* and *Bacillus subitilis* bacterial strains (Zia et al., 2016). Rothrimycine was used as a positive control for this assay.

Protein kinase inhibition assay of green synthesized CuO NPs

The liquid TSB and ISP4 media was used for mycelium and spores production of *Streptomyces*. Refreshed *Streptomyces* culture was inoculated homogenously on autoclaved ISP4 media in Petri plate. Samples of 5 µl were loaded on sterile disc of 6 mm separately for plant extracts and CuO NPs (4 mg/ml). Zone of inhibition was measured after incubation of plate for 24 h at 37°C. Surfictinis was used as a positive standard.

Sulforhodamine B (SRB) cytotoxicity assay

The following cell lines were used in SRB cytotoxic assay; MDA-MB-231 (estrogen receptor negative breast cancer cells: ATCC® HTB-26™), PC-3 (prostate cancer: ATCC® CRL-1435™), DU-145 (prostate cancer: ATCC® HTB-81™), HL-60 (human promyelocytic leukemia cells*:* ATCC® CCL-240™), HT-29 (human colon carcinoma cell line: ATCC® HTB-38™). The HeLa (cervical cancer ATCC® CCL-2™) cell lines used for NF-□B TNF-α activated nuclear factor-kappa B (NF-κB) assay.

The SBR cytotoxic assay of CuO NPs was determined by following a previous method based on colorimetric principle (Ngamrojanavanich*, et al.*, 2007). DMEM media was used for all other cell lines except for HL-60 cell lines where RPMI-1640 medium was used. Basic medium contained5% FBS and 1% antibody solution. Cells were placed in a CO_2 incubator (5%) at 37°C to reach required confluence level. Hemocytometer was used for cell count and their density was maintain at 5×10^4 cells/mL.

Test sample (10 µL) was gently mixed with cultured cells (190 µL) in a 96 well plate and incubated at 37°C for 72 h. Then for cell fixation, TCA 20 % w/v (100 µL) was added and incubated at 4°C for 30 min. Cells were washed, air dried and re-incubated with 0.4 % w/v SRB (100 µl) at room temperature for 30 min. After the wells were washed, air dried and subjected to 10 mMTris base (200 µL) for 5 min with agitation. Absorbance was taken at 492 nm. The test samples exhibiting over 50% inhibition at 20 ug/ml were also tested at four concentration levels (0.16 µg/ml – 20 µg/ml), to obtain inhibitory concentration (IC_{50}). Taxol was used as positive control and medium without any growth was taken as negative control.

NF-κB Assay

The NF-κB assay was carried out following a previously published protocol (Kim et al, 2010). In brief, HeLa cells were treated with different concentration levels of test samples. After treatment with test samples, cells were treated with TNF-α (10 ng/mL) and the nuclear extract from the cells extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA). Protein quantitation was completed using the Bradford protein assay kit from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA).The specific binding ability of activated NF-κB p65 to the biotinylated-consensus sequence under the presence of test samples was assessed using the Pierce™ NF-kB p65 Transcription Factor Assay kit from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA). The binding activity was measured by detecting the chemiluminescent signal in a Fluostar Optima plate reader (BMG Labtech Inc., Durham, NC). The test samples exhibiting over 50%

inhibition at 50 ug/ml were also tested at four concentration levels (0.05 µg/ml – 50 µg/ml), to obtain inhibitory concentration (IC_{50}). Rocaglamide was used as a positive control and untreated cells as negative control.

Statistical analysis

All the experiments were done in triplicate. The data were presented as mean ± standard deviation (SD). SPSS Ver. 21 software was used for "Post Hoc" Multiple Comparison test in two Way ANOVA and IC_{50} was determined by using TableCurve software 2D Ver. 4System Software Inc., San Jose, CA, USA)

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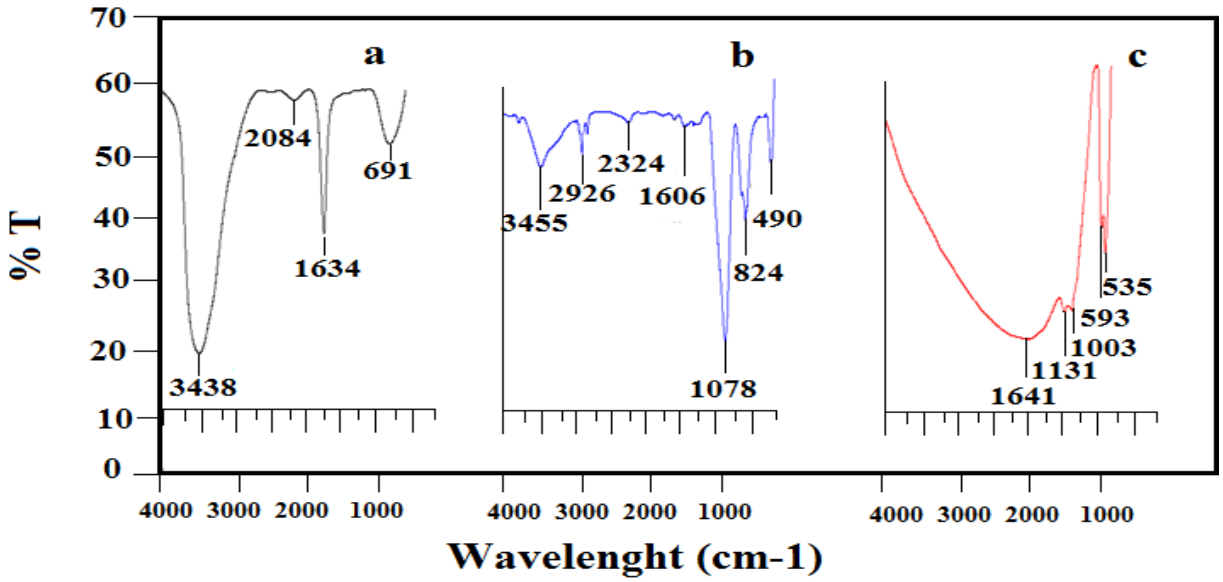

Fig. S2. FTIR of green synthesized CuO NPs. **(a)** Leave Extract **(b)** Reaction Mixture **(c)** CuO NPs

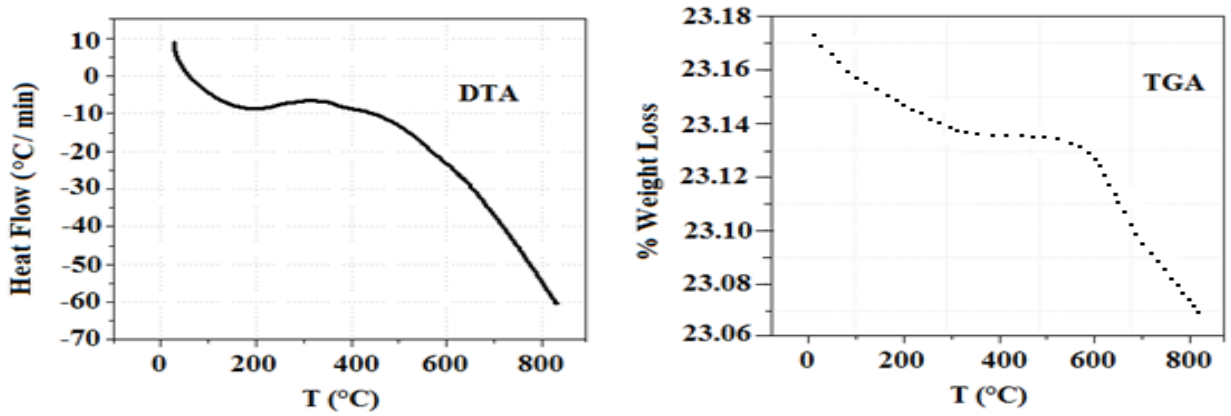

Fig S3. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) of CuO NPs.