



## Original article

Anticarcinogenic effect of zinc oxide nanoparticles synthesized from *Rhizoma paridis saponins* on Molt-4 leukemia cellsZheng Xu<sup>a</sup>, Yuhui Wu<sup>a</sup>, Ling Song<sup>a</sup>, Arunachalam Chinnathambi<sup>b</sup>, Sulaiman Ali Alharbi<sup>b</sup>, Liwei Fang<sup>c,\*</sup><sup>a</sup> Department of Hematology, Xinyang Central Hospital in Henan Province, Xinyang City, Henan Province 464000, China<sup>b</sup> Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia<sup>c</sup> MDS and MPN Centre, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin City 300020, China

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## ABSTRACT

Leukemia is considered as a multistep progression by progressively genetic modifications that make the conversion of normal human hematopoietic progenitor/stem cells into leukemic cells. In this current investigation, we optimized the reaction of parameters to control the nanoparticle size that was examined via SEM. A various characterization techniques, for example UV–visible spectroscopy, FTIR and EDX were carried out for the synthesized ZnONPs from *Rhizoma paridis saponins* plant extract. The RPS-ZnONPs were evaluated to inspect their cytotoxic activity against Molt-4 cells via MTT test. Apoptosis was determined through the ROS, MMP and AO/EtBr fluorescent staining techniques. Moreover, pro and anti-apoptotic signaling protein expression was inspected through RT-PCR method. We observed the fabricated RPS-ZnONPs have a crystalline nature, spherical form and various biomolecules were exist. As well, we detected the cytotoxicity effect of synthesized RPS-ZnONPs at a range of 23.5 µg followed to 24hrs treatment. Apoptosis was triggered via the RPS-ZnONPs along with augmented ROS, decreased MMP and altered dual staining. Moreover, the apoptosis were identified through significant up-regulation of Bax, caspase-9 and caspase-3, but the anti-apoptotic protein expression i.e., Bcl-2 was down regulated. Though, this study, we investigated the synthesis, characterization and anticancer activity of ZnONPs of *Rhizoma paridis saponins* plant extract (RPS-ZnONPs) were useful for treatments of leukemia.

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## 1. Introduction

Nanotechnology is a fundamental branch in the main fields of chemistry, biology, material sciences and physics. The NPs have a broad range of functions in the various fields like electronics, medicine and therapeutics (Khan et al., 2017). The nanomaterials can be produced by various techniques containing biological, physical and chemical. The improvement of physical or chemical methods has revealed in the environmental contaminations, which produced a huge amount of dangerous byproducts (Bhumi and Savithramma, 2014). Therefore, there is a demand of green synthe-

sis methods that contains an eco-friendly, safe, clean and nontoxic. Furthermore, in this process, there are none need to utilize more energy, temperature, pressure and toxic chemicals (Savithramma et al., 2011). Metal oxide nanoparticles have been widely used for medicinal functions in the last decades. Zinc oxide nanoparticles (ZnONPs) are a hopeful kind of nanostructures with broad ranges of health related applications (Datta et al., 2017; Raliya and Tarafdar, 2013).

Deregulation of apoptosis disturbs the delicate and complex balance between cell survival, cell proliferation and cell death, which takes a central function on the growth of ailments like cancer, mainly leukemia (Hanahan and Weinberg, 2000). Leukemia is considered as a multistep progression by progressive genetic modifications that make the conversion of normal human hematopoietic progenitor/stem cells into leukemic cells (Plummer et al., 2016). Leukemia initiates from a single cell that has undergone malignant conversion by regular genetic mutations. These actions are clonally selection of mutated cells that exhibits progressively more aggressive actions. There is an obvious indication demonstrating that the molecular and cellular actions resulting

\* Corresponding author.

E-mail address: lwf052987@sina.com (L. Fang).

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to leukemia are organized by leukemic stem cells (Cassier et al., 2017; McBride et al., 2019).

Medicinal plants are utilized conventionally all over the globe for treatments of numerous diseases containing cancer. The WHO reported, 80% of the world's people are reliant on remedial plants for the cure of various diseases (Javed et al., 2017). *Rhizoma paridis saponins* (RPS) as important segments of *P. polyphylla* Smith var. *yunnanensis* was utilized as anticancer agents in conventional Chinese medicine (Man et al., 2013). Moreover, earlier studies demonstrated that the RPS exhibits antioxidant, anti-lung cancer, anti-hepatocarcinoma and anti-inflammatory properties (Man et al., 2014; Man et al., 2015; Wang et al., 2018). The RPS also possessed the inhibitory effects against liver fibrosis and cirrhosis, suppressed the growth numerous types of cancers in both *in vitro* and *in vivo* like lung, ovarian, liver and cervical cancer (Man et al., 2014; Shuli et al., 2011; Yan et al., 2009). For the finest of our understanding, biological methods of using plant extract of RPS the first time as a reducing agent plus surface stabilizing material for the ZnONPs synthesis. A lot of work had been studied on anticancer activities, but synthesis of nanoparticles mainly ZnONPs are insufficient. Therefore, in the current research, we have investigated the biological synthesis of ZnONPs by using the leaves of RPS and distinguished these NPs by FTIR, EDX, UV-vis spectroscopy and SEM. Additionally, the synthesized RPS-ZnONPs were assessed for the anticancer activity against Molt-4 cells.

## 2. Materials and methods

### 2.1. Chemicals

DMEM, PBS, MTT, FBS, EtBr and DMSO was bought from Himedia-Lab Ltd., Mumbai, India. Zinc acetate is obtained in Sigma-Aldrich (St. Louis, MO, USA). Entire additional chemicals were utilized as of diagnostic range.

### 2.2. Cell culture

Molt-4 cells were procured and cultured using DMEM with FBS (10%) and antibiotic antimycotic solution (1%). They were maintained at sterilized condition in a CO<sub>2</sub> chamber (5%) at 37 °C. Then cells get 80% of confluence were allowed to sub cultured using trypsin solution and used for further research.

### 2.3. Preparation of RPS leaf extract

The 30 g of the prepared powder form of RPS leaves were dissolved in distilled water (100 ml) and located in a water bath at 60 °C for 1hrs. After that, the solution and powder layer was divided through the Whatman-No.1 sift paper and funnel. Then filtrated sample was gathered and utilized for the synthesis of RPS-ZnONPs.

### 2.4. Synthesis of RPS-ZnONPs

The 20 ml of collecting RPS filtrate was mixed with 80 ml of zinc acetate (1 mM). Then, the mixtures were located in a water bath at 60 °C for 3 h. After the suspension completed the fabrication of RPS-ZnONPs, sample was centrifuged at 3000 rpm for 20 min. Centrifugation was done thrice alongwith the addition of deionized water for synthesis of RPS-ZnONPs. After centrifugation, the upper aqueous phase was eliminated and the pellets were kept in a furnace at 60 °C for overnight to get a powdered RPS-ZnONPs.

### 2.5. Characterization of RPS-ZnONPs

Synthesized RPS-ZnONPs were examined via UV-visible spectroscopy to confirm the bio-reduction of NPs. Bio-fabricated RPS-ZnONPs was amalgamated with KBr to form pellets then measured through FTIR spectroscopy (Shimadzu, Japan). The SEM and HR-TEM were utilized to measure the morphology of the synthesized RPS-ZnONPs (FEI Company, Hillsboro, OR, USA). For the EDX investigations, the drop of RPS-ZnONPs were dehydrated on covered with carbon film and employed in the Hitachi-S-3400 N SEM machine equipped with thermo-EDX attachments.

### 2.6. Anticancer activity of RPS-ZnONPs

#### 2.6.1. Cell viability by MTT assay

Cytotoxic effectual of RPS-ZnONPs on Molt-4 cells were determined by MTT analyze. The cells ( $1 \times 10^4$ ) were cultured on 96-well plates then added dissimilar volumes (1, 5, 10, 15, 20 and 25 µg/ml) of RPS-ZnONPs for 24 hrs. Then, the MTT solution(15 µl) was placed to every well then kept at 37 °C for 2hrs, every well was then discarded and add DMSO (100 µl) to all well to liquefy formazan crystals. The results were employed to measure the cell viability.

#### 2.6.2. Measurement of intracellular reactive oxygen species (ROS)

The Molt-4 cells were treated with various quantities of RPS-ZnONPs (15 and 20 µg/ml). Then, cells were treated with DCFH-DA (Hi-media) (10 µM) at 37 °C for 30 min. Later than incubation, cells were cleaned twice with PBS and ROS formation was measured using flow cytometry.

#### 2.6.3. Measurement of mitochondrial membrane potential (MMP)

MMP was studied through JC-1 MMP test kit (Sigma-Aldrich, USA) via adopting the manufacturer's protocol. Cells were supplemented with various dosages (15 and 20 µg/ml) of RPS-ZnONPs. Then cells were gathered and stained with JC-1 stain for 30 min at 37 °C. The cells were washed and measured by a flow cytometer equipped with Cell-Quest software.

#### 2.6.4. Dual staining study (AO/EtBr)

Dual stained (AO and EtBr) cells were clearly exhibits differentiation among live and dead cells. Initially, cells were supplemented with RPS-ZnONPs (15 and 20 µg/ml) then 0.1% TritonX-100 was added then stained with 10 µl of AO and EtBr (Sigma-Aldrich, USA). Then stained cells were kept for 15 min in a dark stipulation and finally measured beneath the fluorescent microscope. The green fluorescence exhibiting cells were pointed out as viable and red fluorescent meant the dead cells.

### 2.7. RT-PCR analysis

The RNA were extracted from control as well as RPS-ZnONPs(15 and 20 µg/ml) supplemented Molt-4 cells via utilizing the Trizol-reagent (Sigma-Aldrich, USA). After extraction, the RNA was subjected to spectrophotometric investigation through absorbance taken at 260 nm. Cleansed RNA sample were then reverse transcribed to construct the single-strand cDNA. The cDNA was amplified with the primers (Sigma-Aldrich, USA) of Bax, caspase-3, Bcl-2 and caspase-9.

### 2.8. Statistical analysis

The statistical investigation were done with the aid of SPSS tool (ver.17) (SPSSInc., Chicago, IL, USA). The one-way ANOVA was employed for expressing significance of the current study. Statistical significance was established at a level of  $p < 0.05$ .

### 3. Results

#### 3.1. Characterization of RPS-ZnONPs

##### 3.1.1. UV-visible spectroscopy study of RPS-ZnONPs

The biosynthesized RPS-ZnONPs were examined via UV-visible spectroscopy at the 200–700 nm range. From the experimental findings, it was noted that the maximum absorbance of the synthesized RPS-ZnONPs was observed on the 289 nm range, which reveals the changes of the initial material to end product. It confirms the bio-reduction of ZnONPs in the test solution and it was evidently demonstrated in Fig. 1.

##### 3.1.2. SEM and TEM analysis of RPS-ZnONPs

The biosynthesized RPS-ZnONPs were inspected via SEM and TEM analyses to detect the exact magnitude and shapes. From the experimental findings of electron microscopical investigations, it was indicated that the fabricated RPS-ZnONPs possessed a spherical-shaped nano particles and irregular formations. The electron microscopical images of synthesized RPS-ZnONPs were illustrated on Fig. 2.

##### 3.1.3. EDX pattern analysis of RPS-ZnONPs

The synthesized RPS-ZnONPs were subjected to the EDX spectral investigations for measuring the combinations of oxides and metallic nature of the synthesized RPS-ZnONPs in a test solution. The spectrum of EDX evidently demonstrated that the 84% of zinc (Zn) were present on the surface area of the synthesized RPS-ZnONPs. The spectrum of EDX analysis was portrayed in the Fig. 2.

##### 3.1.4. FTIR spectroscopic study of RPS-ZnONPs

FTIR examination of ZnONPs from RPS leaves extract demonstrates in Fig. 3. The interaction of nanoparticles with biomolecules of RPS-ZnONPs illustrated intensive peaks at 3199.85, 1736.67, 1564.72, 1408.82, 1241.41, 1082.98, 1006.96, 845.56, 765.93, 677.98, 632.21 and 530.09. The peaks of FTIR was possessed the bonds that is imperative for the existence of alkynes ( $3199.85\text{ cm}^{-1}$ ), aldehyde ( $1736.67\text{ cm}^{-1}$ ), alkanes ( $1408.82\text{ cm}^{-1}$ ), alkyl ketone ( $1241.41\text{ cm}^{-1}$ ), alkyl amine ( $1082.98\text{ cm}^{-1}$ ), esters ( $1006.96\text{ cm}^{-1}$ ), aromatic ( $765.93\text{ cm}^{-1}$ ) and halogen compound ( $677.98, 632.21$  and  $530.09\text{ cm}^{-1}$ ).

#### 3.2. Anticancer activity of RPS-ZnONPs

##### 3.2.1. Effect of RPS-ZnONPs on MTT assay

Fig. 4 illustrating a cytotoxic effectual of RPS-ZnONPs on Molt-4 cells by MTT analyze. The Molt-4 cells were supplemented with various doses of 1, 5, 10, 15, 20 and 25  $\mu\text{g/ml}$  for 24 h with RPS-ZnONPs. Cell viability was determined to be decreased with

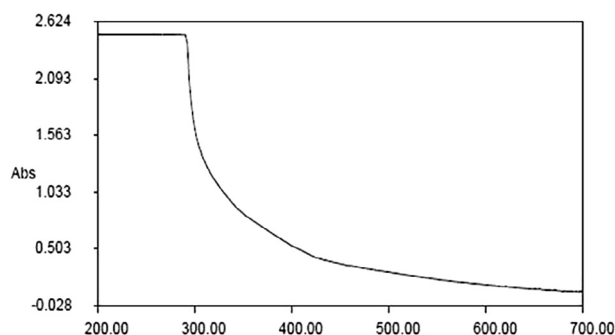


Fig. 1. Illustrating the UV-visible spectral pattern of the synthesized ZnONPs from *Rhizoma paridis saponins*.

increasing levels of the biosynthesized RPS-ZnONPs. The inhibitory concentration ( $\text{IC}_{50}$ ) level of the Molt-4 cells was found as 15  $\mu\text{g/ml}$  and we have chosen for 15 and 20  $\mu\text{g}$  concentrations of RPS-ZnONPs to the additional study.

##### 3.2.2. Effect of RPS-ZnONPs in generation of ROS

Fig. 5 displaying the efficiency of RPS-ZnONPs on the production of intracellular ROS level in the Molt-4 cells. The Molt-4 cells were administrated with two concentrations of RPS-ZnONPs (15 and 20  $\mu\text{g/ml}$ ) and ROS statuses were examined. The result was revealed the considerable augmentation in the intracellular ROS status on Molt-4 cells. It was observed that the RPS-ZnONPs treated Molt-4 cells elevated ROS when comparing it to normal cells.

##### 3.2.3. Effect of RPS-ZnONPs on the status of MMP

Fig. 6 evidently showed the potency of RPS-ZnONPs in the status of MMP of Molt-4 cells. The Molt-4 cells were supplemented with different dosages of RPS-ZnONPs (15 and 20  $\mu\text{g/ml}$ ) and then the MMP statuses were inspected. The result was evidently exhibited that the considerable diminution of the MMP status was identified in the RPS-ZnONPs supplemented Molt-4 cells which is contrasting to the control cells.

##### 3.2.4. Effect of RPS-ZnONPs on status of AO/EtBr

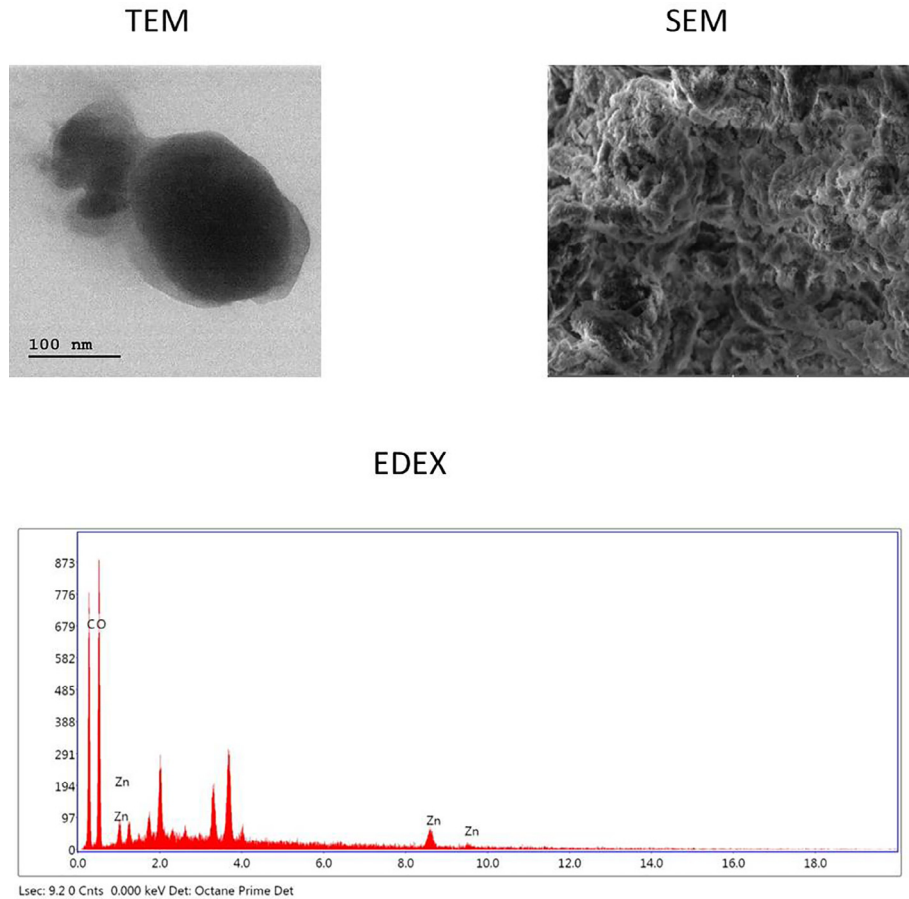
Fig. 7 demonstrates the effectual of RPS-ZnONPs in apoptotic morphological modifications were evaluated via AO/EtBr staining. In current results showed that control cells had a deep green fluorescence nucleus, which point out for live cells. Conversely, different volumes of RPS-ZnONPs (15 and 20  $\mu\text{g/ml}$ ) added Molt-4 cells exhibited orange (early apoptosis) and red stained (late apoptosis) apoptotic cells.

#### 3.3. Effect of RPS-ZnONPs on apoptotic protein expressions

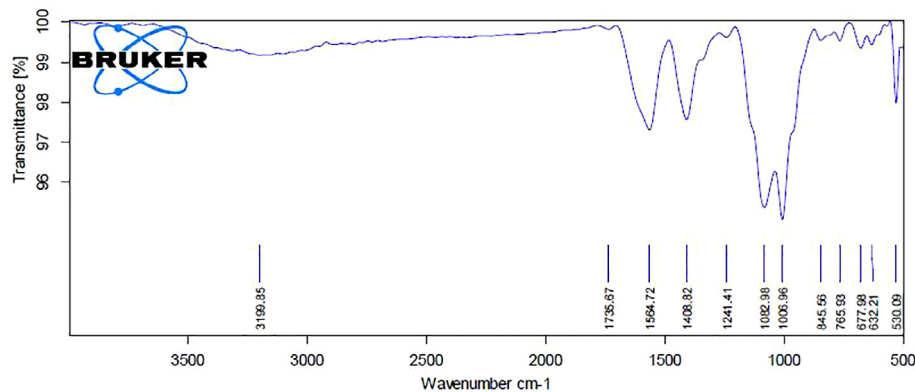
Fig. 8 illustrating the expression patterns of apoptotic genes in normal as well as RPS-ZnONPs added Molt-4 cells. The up-lifted expression of Bcl-2 and lowered expression of caspase-9, Bax and caspase-3 were noted in the normal cells. In opposition, RPS-ZnONPs (15 and 20  $\mu\text{g/ml}$ ) added cells exhibited decreased expression of the Bcl-2 and enhanced the caspase-3, Bax and caspase-9 expressions were observed.

### 4. Discussion

Synthesis of nanoparticles with particular morphologies and properties is one of the mainly significant features of nanoscience, which studies materials whose size lies within the nanometer range (Montasser et al., 2017). The chemical synthetic methods can lead to the production of poisonous chemical by-products or need high temperatures and/or pressure, whereas biosynthesis of nanoparticles using plant extracts offered a simplistic and green approach (Bala et al., 2015). The ZnONPs are presently under exploration owing to its consumption on sarcoma management and diagnostic purposes (Salam et al., 2014). Since the curing of cancer via chemotherapy was inadequate as a result of the difficult outcome of tumor repressing drugs and drug resisting of cancer cells, herbal plant derivative drug investigates have attention on surmounting those limits. The ZnO nanopowder taken upon optimization was distinguished using different analytical methods to determine their size, shape and functionalization (Jamdagni et al., 2018). The present study, we have been finding that the changing of colour of the fabricated nanoparticles with maximum absorbance, size and shape by SEM, qualitative elemental investigation through EDX (Fig. 2) and existence of various functional groups via testing the FTIR method (Fig. 3) of fabricated RPS-ZnONPs.



**Fig. 2.** Showing the images of SEM, TEM and EDX analyses of the synthesized ZnONPs from the *Rhizoma paridis saponins*.

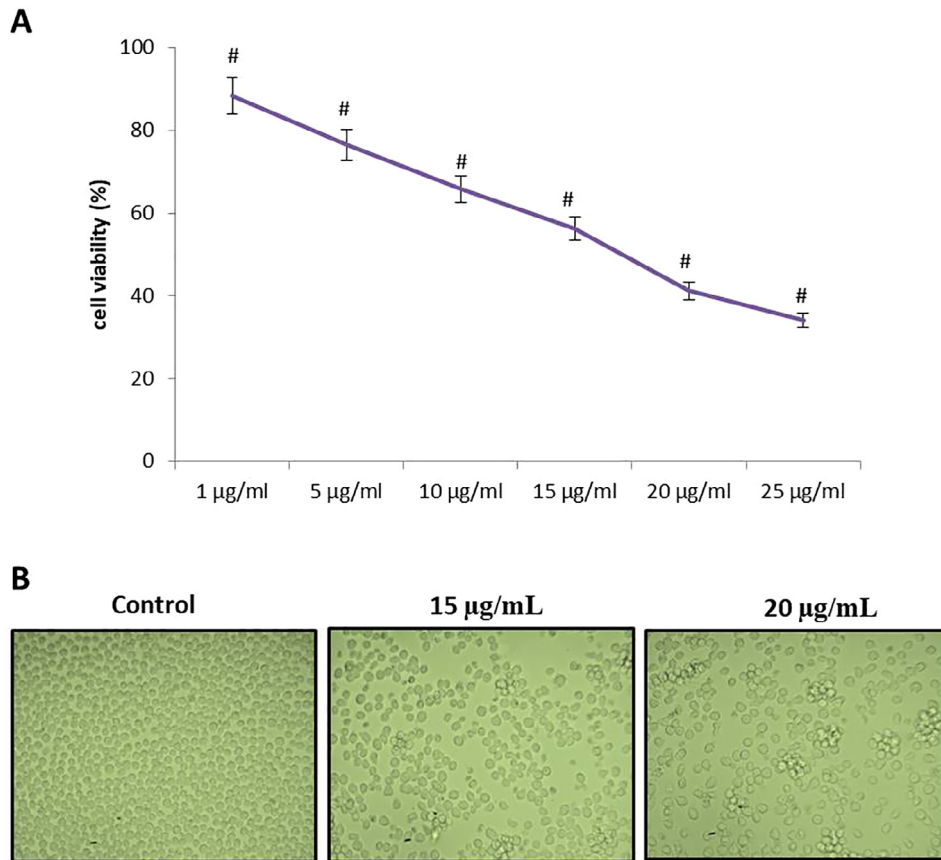


**Fig. 3.** Showing the FT-IR spectral analysis of the synthesized ZnONPs from *Rhizoma paridis saponins*.

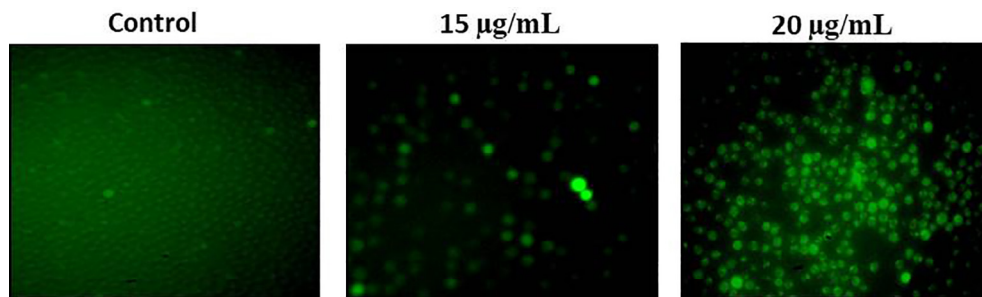
Mitochondria are one of the vital organelles that control the cell necrosis as well as pointing the apoptosis. Functional modifications of mitochondria was revealed to took a imperative task on cell apoptosis (Rajeshkumar et al., 2015). ROS mediated intracellular signaling cascades could activate redox signaling pathways, containing cell cycle arrest, apoptosis and oxidative stress (Gupta et al., 2012). According to this latest study, several anticancer agents able to enhance the production of ROS and activated the apoptosis in cancer cells through the mitochondrial-dependent signaling-pathway (Chen et al., 2018). Additionally, the allocation of the MMP is one of the apoptotic developments enthused via anticancer drugs (Liu et al., 2017). The present study shows that

the RPS-ZnONPs has caused cell necrosis via destructing the mitochondria (Fig. 6) and rising intracellular ROS generation in Molt-4 cells (Fig. 5). This supports an earlier work that demonstrated the cytotoxic potential of ParisSaponin-I, a functional constituent of *Rhizoma paridis* in SKOV3 cells (Xiao et al., 2009). The cells undergoing apoptosis reveal following characteristic features such as membrane blebbing, condensation of chromatin, mitochondrial depolarization, shrinkage of the nucleus and DNA degradation by endonucleases into fragments (Jamunakumari and Sakthisekaran, 2014). In this investigation, we found that the RPS-ZnONPs supplementation, considerably modified the morphological alterations linked with Molt-4 cells directing to induction of apoptosis.

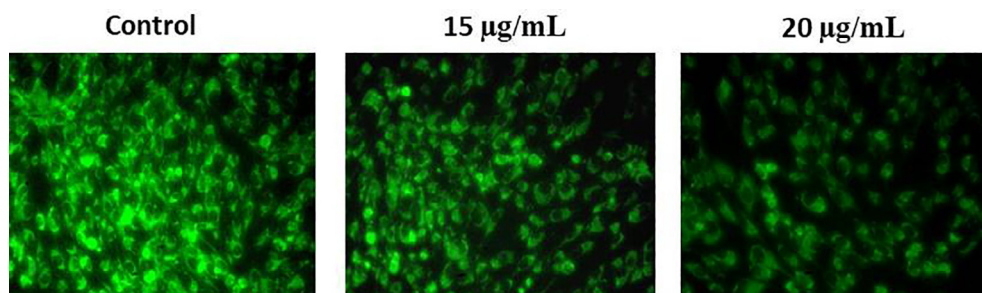




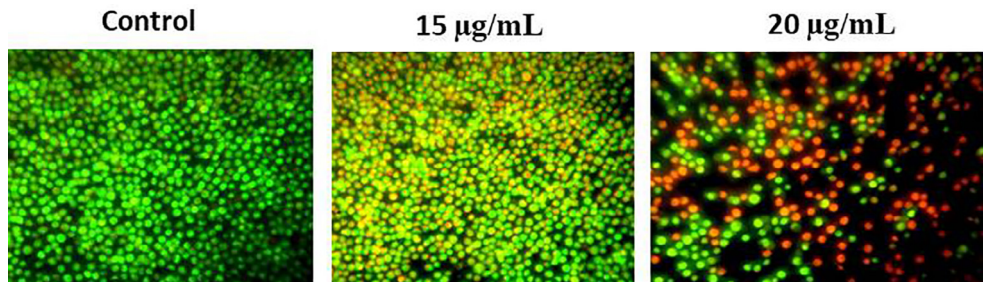
**Fig. 4.** RPS-ZnONPs inhibit the viability of Molt-4 cells by MTT assay. The statistical analysis was carried out using one way ANOVA. Data represent mean  $\pm$  SD of triplicate, \* $P < 0.05$  as compared with the control group. a) MTT assay b) Cell morphological analysis.



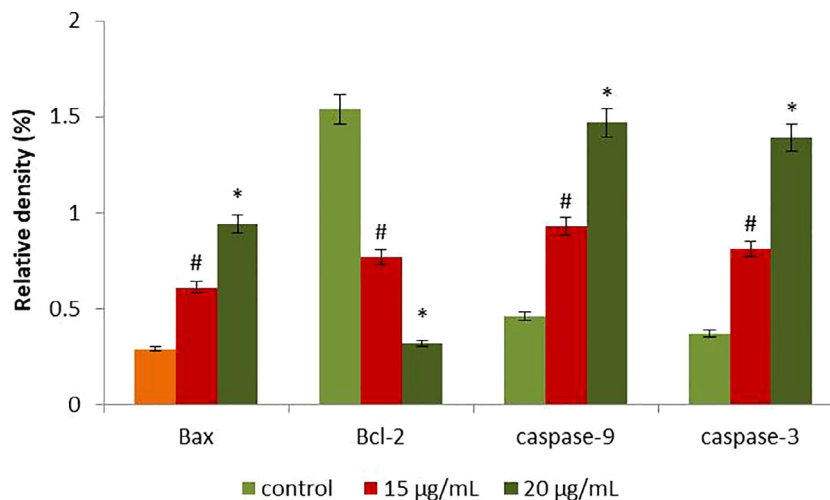
**Fig. 5.** RPS-ZnONPs induce intracellular reactive oxygen species generation in Molt-4 leukemia cells. Data represent mean  $\pm$  SD of triplicate, \* $P < 0.05$  as compared with the control group.



**Fig. 6.** RPS-ZnONPs decreased the mitochondrial membrane potential in Molt-4 leukemia cells. Data represent mean  $\pm$  SD of triplicate, \* $P < 0.05$  as compared with the control group.



**Fig. 7.** RPS-ZnONPs activate apoptosis through activation of a double staining method by AO/EtBr in Molt-4 leukemia cells. Data represent mean  $\pm$  SD of triplicate, \* $P < 0.05$  as compared with the control group.



**Fig. 8.** The anticancer effect of RPS-ZnONPs on apoptosis signaling pathway gene expressions in Molt-4 cells were analysed by RT-PCR. The cells were treated with RPS-ZnONPs (15 and 20 µg/ml) for 24 hrs and the gene expressions of Bax, caspase-3, Bcl-2 and caspase-9 were determined. Each bar represents mean  $\pm$  SD of three independent observations. # $p > 0.01$ , \* $p > 0.05$ . The GAPDH was used as an internal control.

Apoptosis occurs mainly during the extrinsic and intrinsic pathways (Li et al., 2012). In extrinsic pathway was activated via caspase-8, while caspase-9 was implicated in the intrinsic pathway (Shimizu et al., 2015; Alabsi et al., 2016). The Bcl-2 protein was an key controllers of cytochrome *c* release from mitochondria and the diminution of Bcl-2 expressions triggers the releasing of cytochrome *c*, which leads to the induction of cell necrosis (Hata et al., 2015). The reduced Bcl-2 and augmented Bax escorts to the deliverance of cytochrome *c* and consequently activate caspase-9 and caspase-3 (Czabotar et al., 2014). In the present research, RPS-ZnONPs treatments promoted the expression of Bax, caspase-3 and caspase-9 and Bcl-2 was considerably reduced in Molt-4 cells (Fig. 8). Previous study also informed that the *Rhizoma paridis saponins* induce apoptosis in A549 cells, which as recommended in this current research (Zhang et al., 2015).

## 5. Conclusion

In conclusion, we found that the fabricated ZnONPs from RPS was characterized by various techniques. It was principally identified with the changes of color solution and UV absorption spectra also confirmed the maximum absorbance peak. The SEM and EDX images illustrated the size and morphological structures of nanoparticles. The FTIR results showed synthesized RPS-ZnONPs having different functional groups were observed. Furthermore, RPS-ZnONPs induced cytotoxicity at a concentration range 15 µg/ml and also triggered apoptosis through augmented ROS forma-

tion, decreased MMP, altered AO/EtBr staining and induced proapoptotic and suppressed anti-apoptotic protein were observed.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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