Journal of Drug Delivery Science and Technology 45 (2018) 378–387

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Nanoparticles of *Combretum quadrangulare* leaf extract induce cytotoxicity, apoptosis, cell cycle arrest and anti-migration in lung cancer cells

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ARTICLE INFO

Keywords:
Combretum quadrangulare
Leaf
Apoptosis
Cell cycle arrest
Anti-igration
A549 cells

ABSTRACT

In the present study, the cytotoxic effect of *Combretum quadrangulare* leaf extract and nanoparticles against lung cancer cells was analyzed by MTT assay. The induction of apoptosis by the nanoparticles was investigated by a morphological study, Hoechst staining, Annexin V/Phycoerythrin assay, and cell cycle assay. The anti-migratory effect of the extract and nanoparticles was analyzed using a Transwell® assay. The results showed that the extract and nanoparticles were cytotoxic to A549 cells. The extract had IC_{50} values of 257.2 and 136.1 μg/mL at 24 and 48 h, respectively, while the nanoparticles had IC_{50} values of 545.2 and 350.4 μg/mL at 24 and 48 h, respectively. *Combretum quadrangulare* nanoparticles induced apoptosis in the A549 cell line in a dose- and time-dependent manner. At non-cytotoxic concentrations, the extract and nanoparticles inhibited the migration of A549 cells with concentration dependence. The nanoparticles increased the colloidal stability of the extract. The extract and nanoparticles contained phenolics, flavonoids, terpenes, and tannins. The results suggest that the nanoparticles of *Combretum quadrangulare* Kurz leaf extract may be a promising candidate for the development of drugs for lung cancer therapy.

1. Introduction

Lung cancer is the second most common cancer following prostate cancer and breast cancer in men and women, respectively. Currently, lung cancer remains the most common cancer-related cause of death. In 2012, approximately 1,590,000 lung cancer deaths occurred, outnumbering deaths from breast, prostate and colon cancers combined [1]. Lung cancer mainly occurs in the elderly. Approximately two out of three people diagnosed with lung cancer are 65 years old or older, whereas less than 2% are younger than 45 years old. The average age at the time of diagnosis is approximately 70 years old. Lung cancer therapy can be given by several methods, including surgery, radiation, chemotherapy, photodynamic therapy, laser therapy or a combination of these methods. The treatment selection depends on the type of lung cancer, the exact location of cancer, i.e., whether it is localized or metastasized, the tumor size, and the general health of the patient. Patients diagnosed with localized lung cancer may be treated by surgical resection or radiotherapy. For patients who have tumors that have already metastasized to other organs *via* the blood stream, chemotherapy and sometimes with radiotherapy are a typical treatment method. Chemotherapy can retard tumor growth and relieve symptoms in patients who cannot have surgery, but it may cause serious side effects. Although chemotherapy is the main method for both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) therapy, chemotherapy resistance limits the ability to effectively treat advanced lung cancer. The discovery and development of a promising lung cancer treatment strategy based on stimulating the apoptotic signal could be a promising strategy to overcome chemotherapy resistance in lung cancer. Natural substances, which may have fewer side effects than traditional chemotherapy, may be an alternative choice for the treatment.

Apoptotic cell death is one of the most widely studied subjects providing insights into the pathogenesis of a disease and how the disease can be treated. In cancer, there is a lack of balance between cell proliferation and cell death. When insufficient apoptosis occurs, the malignant cells do not die and cause tumor growth. Cancer research now focuses on the discovery and development of anti-cancer agents based on selectively killing cancer cells with minimal side effects on normal cells through inducing apoptotic cell death. The induction of apoptosis is one of the most important attributes of anti-cancer agents. The disruption of apoptotic programs can decrease cancer treatment sensitivity [2]. Some plant materials can induce apoptotic pathways in cancer cells [3]. Metastasis is the leading cause of the resultant mortality of patients with cancer. It derives from the invasion and migration

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https://doi.org/10.1016/j.jddst.2018.04.003
Received 23 December 2017; Received in revised form 17 March 2018; Accepted 2 April 2018
Available online 03 April 2018
1773-2247/

of tumor cells. Many potential anti-invasive and anti-metastatic compounds from natural compounds from plant materials have been discovered [4].

Combretum quadrangulare Kurz (*C. quadrangulare Kurz)* is an evergreen tree commonly found in Southeast Asia. According to Thai traditional medicine, its leaf has been used as an anti-helminthic, anti-dysenteric, and anti-pyretic agent [5]. There are several studies on the pharmacological activities of *C. quadrangulare Kurz* including antibacterial activity [6], antioxidant activity, anti-cancer activities [7], trypanocidal activity against epimastigotes of *Trypanosoma cruzi* [8], and *in vitro* HIV integrase inhibition [9]. In this study, the effect of the ethanol extract of *C. quadrangulare Kurz* leaf on the cytotoxicity, apoptosis induction, and migration of lung cancer cells was investigated. Nanoparticles (NPs) encapsulating the ethanol leaf extract of *C. quadrangulare Kurz* were fabricated for the first time to provide the suspendability and colloidal stability of the extract. The effects of NPs on the cytotoxicity and migration of A549 cells were initially investigated.

2. Materials and methods

2.1. Materials

The leaves of *C. quadrangulare Kurz* were collected from the Faculty of Pharmacy, Srinakharinwirot University botanical garden. The plant was identified by the Department of Pharmacognosy, Faculty of Pharmacy, Srinakharinwirot University. A voucher specimen of this plant (SWU10) was deposited at the Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand. A549 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco, MA, USA. 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amresco, OH, USA. Sodium carboxymethyl cellulose was purchased from Sigma, MO, USA. FITC Annexin V/Dead Cell Apoptosis Kit and Hoechst 33258 were purchased from Molecular Probes, CA, USA. Guava Nexin Reagent was purchased from Merck, NJ, USA. The 6.5-mm diameter chamber with an 8.0-μm pore polycarbonate membrane insert (Transwell®) was purchased from Corning, USA.

2.2. Preparation of C. quadrangulare Kurz leaf ethanol crude extract

C. quadrangulare Kurz leaves were dried in a hot air oven at 50 °C and ground to powder. The powdered plant material (100 g) was extracted in a Soxhlet apparatus for 7 h with 95% ethanol. The plant extract was concentrated and dried under reduced pressure using a rotary vacuum evaporator. The dried extract was weighed, and the yield was calculated to be 18.2% w/w.

2.3. Preparation and characterization of C. quadrangulare Kurz leaf extract NPs

NPs containing *C. quadrangulare Kurz* leaf extract were prepared by the solvent displacement method [10]. Sodium carboxymethyl cellulose (SCMC) (0.2% w/v) was used as a stabilizing agent. *C. quadrangulare Kurz* leaf extract (100 mg) was dissolved in 2 mL of 95% ethanol and infused into 15 mL of 0.2% SCMC at a rate of 10 mL/h under magnetic stirring (540 rpm). The NP suspension was dialyzed for 2 h to remove organic solvent and kept in water until characterization.

The size, polydispersity index (PDI) and zeta potential of *C. quadrangulare Kurz* leaf extract NPs were measured by a dynamic light scattering (DLS) technique. The NPs were dispersed in deionized water at a concentration of 6.7 mg/mL for size, PDI, and zeta potential measurements. The effective diameter and PDI were recorded at a 173° scattering angle under 25 °C (Zetasizer Nanoseries, Malvern instruments, Malvern, UK). The morphology of *C. quadrangulare Kurz* leaf extract NPs was observed using a transmission electron microscope (TEM) (Tecnai™ S/TEM Family, FEI Company, USA). A drop of nanoparticle suspension was deposited on a copper grid with carbon film and dried at room temperature. After another drop was repeatedly deposited on the grid, the samples were allowed to dry at room temperature before investigation.

2.4. Binding of C. quadrangulare Kurz leaf extract and NPs to A549 cells

A549 cells were used to determine the effect of *C. quadrangulare Kurz* leaf extract and NPs on cellular binding, cytotoxicity and apoptosis induction [11]. The binding of *C. quadrangulare Kurz* leaf extract and NPs to A549 cells was observed by optical microscopy. Cells were seeded into a 24-well cell culture chamber slide at a density of 3×10^4 cells/well and cultured for 24 h to approximately 80% confluence. The culture medium was removed, and *C. quadrangulare Kurz* leaf extract and NPs were added to the cells at 125, 250, and 500 mg/mL and incubated with the cells for 30 min at 37 °C and 5% CO_2. The cells were thereafter rinsed three times with phosphate buffer saline (PBS) of a pH of 7.4 to remove unbound samples and fixed with 4% paraformaldehyde. Micrographs were acquired using Nikon Eclipse TS100-F and NIS-Elements, version 4.0 software, Nikon Corporation, Tokyo, Japan.

2.5. In vitro cell viability study

A549 cells (8000 cells/well) were cultured in a DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin and maintained at 37 °C and 5% CO_2. The A549 cells were detached by trypsinization and transferred from the culture flask to a 96-well plate (8000 cells/well). The cells were allowed to grow in the 96-well plate until 80% confluence. *C. quadrangulare Kurz* leaf extract or NPs were added to the wells at defined concentrations (4–1000 μg/mL) and incubated for 30 min at 37 °C. The extract or NPs were removed, and cells were further incubated with a culture medium for 24 and 48 h. For the negative control experiment, the cells were not treated and incubated with the culture medium. The cells were washed three times with PBS and then incubated in the culture medium containing MTT (0.5 mg/mL) for 2 h at 37 °C. After incubation, the media were removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the water-insoluble formazan product which was a metabolite of a tetrazolium salt. The absorbance was measured at 550 nm. The absorbance of the extract and NPs at 550 nm was measured to subtract the absorbance value from the cells that might interfere with the extract. The percentage of cell viability was calculated as the ratio of the mean absorbance of triplicate readings with respect to the mean absorbance of the control wells. Dose-response curves were plotted to determine the half maximal inhibitory concentrations (IC_{50}) for *C. quadrangulare Kurz* leaf extract and NPs using GraphPad Prism 5 (GraphPad Software, CA, USA).

2.6. Apoptosis assessment using flow cytometry

The quantification of cell apoptosis was measured by Guava Nexin assay using Annexin V-Phycoerythrin (PE) to detect the phosphatidylserine on the external membrane of apoptotic cells and 7-amino-actinomycin D (7-AAD) as an indicator of cell membrane integrity. A549 cells (5×10^5 cells/mL) were treated with *C. quadrangulare Kurz* leaf extract NPs at concentrations of 125, 250, and 500 μg/mL for 30 min at 37 °C and 5% CO_2. Untreated cells served as the negative control. After incubation, the NPs were removed, and the cells were further incubated in a culture medium for 24 and 48 h. The cells in the 100 μL culture medium were suspended in a mixture of 100 μL Annexin V-PE and 7-AAD binding buffer. After incubation at room temperature for 20 min, the samples were analyzed by flow cytometry (Guava® easyCyte flow cytometer, Merckmillipore). The population was