ISSN 1991- 8690 الترقيم الدولي 8690 - 1991

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Inhibitory Effect of Ethanolic Extract Isolated from *Radix Pulasatillae* Seeds against Proliferation of A549 Human Lung Cancer Cells

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Abstract

The present study was evaluated the anticancer activity of *Radix Pulsatillae* ethanol extract at first by using high throughput screening HTS for herb fractions screening in concentration of 100μM to identify the active fraction and then we measured the cytotoxic effects by using morphology changes, and detected apoptosis by Hoechst 33258 staining. Our results showed that *Radix Pulsatillae* ethanol extract has a strong toxicity against A549 lung cancer cells and low toxicity with mice normal splenocytes and requires more investigation to identify the small natural molecule with new anticancer activity and further mechanistic for cancer pathways.

Keywords: Radix Pulsatillae, Lung cancer, Hoechst staining, Apoptosis, Splenocytes cells.

التأثير التثبيطي للمستخلص الايثانولي المعزول من بذور نبات رادكس بولساتيللا ضد تكاثر سرطان الرئة البشري نوع A549

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قسم الكيمياء- كلية التربية للعلوم الصرفة- جامعة البصرة

ملخص:

حضر المستخلص الايثانولي لنبات رادكس بولساتيللا باستخدام تقنية الحقن الانتاجي العالي الجريان. ولتحديد الفعالية السرطانية ضد خلايا سرطان الرئة تم قياس السمية عن طريق تجربة تأثير المستخلص قبل وبعد الاضافة وبتركيز 100 مايكرومولار على تغير شكل وطبيعة الخلايا السرطانية وكذلك تجربة كشف برنامج الخلايا المميت. دلت النتائج ان لمستخلص الايثانول لرادكس بولساتيللا فعالية قوية ضد سرطان الرئة واقل فعالية ضد خلايا طحال الفئران الطبيعية حيث يحتاج الى دراسة اعمق من اجل عزل وتشخيص المركب المسؤول عن هذه الفعالية المضادة لخلايا سرطان الرئة ودراسة الميكانيكية الفعالة لدورات الخلايا السرطانية

Introduction

Lung cancer is a disease starts in the lungs, in the cells lining and the lung airways and can break away and spread to other parts of the body in a process called metastasis. There are two main types of lung cancer and they are treated very differently (Small cell lung cancer (SCLC) and Non-small cell lung cancer (NSCLC)) (American Cancer Society,

2014), and the most common cause of lung cancer is long-term exposure to tobacco which causes 80–90% of lung cancers (Horn, et.al. 2012; Rahim,et.al., 2014). The chemotherapy of lung cancer is by using two drugs (cisplatin or carboplatin) (Murray and Turrisi, 2006), Combinations with carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan, and irinotecan are used and the side effects are still main reason to destroy human body immunity, therefore ; we are looking for active anticancer drugs which treat lung cancer cells with less side effects (Azim and Ganti, 2007; MacCallum and Gillenwater, 2006). The incidence of lung cancer is 1.37 million deaths in the worldwide, and Hungary is the first top country of highest death rate (male = 36.02 and female = 123.92, death rate per 100,000) (McNabola and Gill, 2009). In 2005-2008 in Basrah province-Iraq, the incidence of lung cancer is ranked in the fifth high ratio (Total >100) of cancer risk (male = 10.7 and female = 3.3) (Habib, et.al, 2010). Chinese Medicine herbs have a long history of diseases and cancer treatment and the screening of Chinese herbs will discover new herbs with new anticancer properties and identify the responsible small natural molecules for this cancer activity (Al Shawi, et.al., 2011; Khan, et.al., 2012). Chinese herbs have been used for lung cancer treatment and approximately 133 Chinese herbal medicines have been reported to possess anti-lung cancer effects such as Radix Rehmanniae, Radix Stemonae, Rhizoma Curcumae, Radix Ginseng, and Radix Adenophorae (Zhou, et.al., 2008; Guo, et.al., 2002), and some bioactive compounds isolated from Chinese medicinal herbs have been used to treat lung cancer such as Solamargine (SM) which is an alkaloid isolated from a herb, Solanum incanum, it was found to be a powerful cytotoxic agent in four human lung cancer cell lines (Liu, et.al., 2004). Radix Pulsatillae is a Chinese herb belong to Ranunculaceae family and native to East Asia, it is grown from east Siberia to Inner Mongolia, the northeast and north of China, it's bitter in flavor, cold in nature, it is related to the large intestine channel and it was used medicinally during ancient times as an external remedy for ulcers and eye inflammation, during the 19th century, European physicians noted that *Pulsatillae* used in the treatment of melancholy, swelling of the knees, and nervous system disorders (He, *et.al.* 2012; Du and Hu, 2004). Until now with depth literature survey, we could not find any published study shows the anticancer activity of *Radix Pulsatillae* against lung cancer and other cancers. In our study, we have used high throughput screening of *Radix Pulsatillae* ethanol extract fractions for first time to evaluate the anticancer activity against A549 small lung cancer cells by morphology changes and heochst33258 staining to detect apoptosis effect compared with mice normal splenocytes cells.

Materials and Methods

Chemical and reagents

Fetal bovine serum (FBS have a very low level of antibodies and containing more growth factors for many vitro applications) Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. DMEM culture medium, and Dimethyl sulfoxide (DMSO) were purchased from Sigma. Hoechst 5238 was purchased from Beyotime Institute of Biotechnology Jiangsu China.

Preparation of Radix Pulsatillae ethanol extracts fractions

Radix Pulsatillae herb was purchased from national institute for food and drug control and jilin xiancao medical herb limited company by the Key Research Laboratory of Cell Biology, Membrane Channels Research and Anti-Cancer Drug Discovery in the School of Life Science, Northeast Normal University, Changchun, Jilin Province China. Specimen was deposited in this Key Laboratory. Briefly, 1 kg of Radix Pulsatillae seeds were dried, pulverized and solubilized with 95% ethanol for 10 hours in a soxhlet extractor at (85-95)°C and more than 12 cycles to achieve maximum extraction of its ingredients. The ethanol extract was hemi-dried using rotary evaporator to get crude extract (40 g). To prepare ethanol extract fractions of radix pulsatillae by using HPLC preparative instrument, we dissolved the extract in 80% methanol (for HPLC preparative used) (Cannell, 1998). After centrifugation at 12000 rpm for 15 minutes (Centrifuge Force=26000 xg), the supernatant was separated and filtered with 0.18 μm filter paper (Buchner Funnel). Starting from the first peak to the end of the last peak, the extracted material was divided into 80 fractions on the basis of time (30 seconds per fraction) using HPLC. The fractions were dried and dissolved in dimethyl sulfoxide (DMSO) to obtain a 1 mg/mL stock solution. These fractions were subjected to screening for cytotoxicity against human A549 lung cancer cells; evaluate the

cancer toxicity by *using morphological changes (Rasul, et. al., 2012).*

Cell Culture

Human lung cell line (A549) was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 unit of Penicillin at 37℃ in a CO2 incubator with 5% CO2, 95% air and 100% humidity. Cells were plated in 10 cm culture dish and allowed to grow to approximately 60-80% confluence before experimentation (Freshney, (2001).

Analysis of toxicity on murine splenocytes

To observe the cytotoxic effect of Radix Pulsatillae ethanol extract on normal splenocytes cells, splenocytes was isolated from CD1 (cluster of differentiation 1 is a family of glycoproteins expressed on the surface of various human antigen-presenting cells) mouse. Briefly, mouse was euthanized by overdose of pentobarbital. Spleen was surgically removed and mashed using the plunger end of the syringe in cold phosphate buffered saline. Cell suspension was centrifuged at 1500 g for 5 minutes and pellet was re-suspended in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) medium. Red blood cells were lysed with lysis buffer (0.01 M $KHCO3^{-}$ and 0.15 M NH4Cl) for 40 s then 9 ml of medium was added and re-centrifuged. Supernatant was discarded again and the pellet was re-suspended in DMEM medium with 10% FBS. Cells were plated in 96 well plates at 20 cells/well and treated with (0 and 100) µM of Radix Pulsatillae ethanol extracts. After 24 h incubation, cells were stained with 0.4% trypan blue, observed and photographed under microscopy (Al Shawi, et.al., 2011).

Hoechst 33258 staining to detect apoptosis

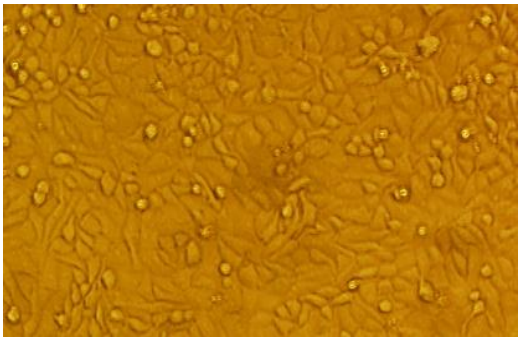
A549 lung cancer cells from exponentially growing cultures were harvested in 1×104 cells per well in 96-well plates. A549 cells were untreated or treated with (0 and 100) µM of Radix Pulsatillae ethanol extract for 24 h at 37◦C. The cells were then washed in ice-cold PBS and fixed in a solution of 3.7% paraformaldehyde for 15min at room temperature. To identify the apoptotic A549 cells, they were stained with Hoechst 33258 (5 µg/mL in PBS) for 10min at room temperature. The nuclei structure of the cells was examined by optical microscope with 1000 magnification (Lee, et.al., 2012; Zhang, 2000).

Results and Discussion

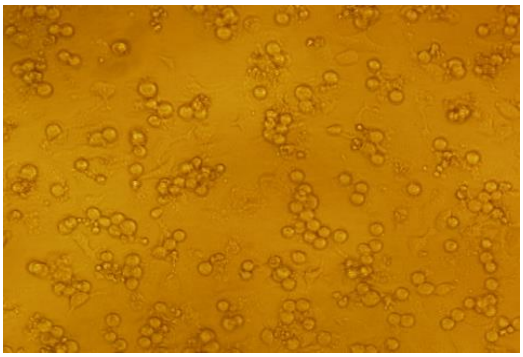
The previous studies of Radix pulsatillae constituents showed cytotoxic activity against pancreatic, leukemia cancers (Son, et. al. 2013; Li, et. al. 2013). In this study we identified several effective fractions which are (E2-E11), (F2-F4), and (F9-F11) through screening of Radix Pulsatillae ethanol extract fractions by using high throughput screening HTS with anti-lung activity and could use these fractions to identify the new small natural molecules which is responsible for the cancer activity as showing in table (1), the cells were treated with 0, 100 µM of Radix Pulsatillae ethanol extract fractions and showed clearly a strong high toxicity against A549 human lung cancer cells with low toxicity on normal splenocytes cells, perhaps due to the natural compounds mixture such as steroids, flavonoids, tannins, saponins, and carbohydrates (Mimaki, et. al. 2001; Goyal and Kumar, 2011) in Radix Pulsatillae ethanol extract which enhances the activity against lung cells, as showing in figure (1 and 2) and the small molecule in Radix Pulsatillae ethanol extract fractions which is responsible for anti-lung activity is still unknown and require further investigations by using HTS to identify it. Hoechst 33258 staining is a fluorescent stains for labeling DNA in fluorescence microscopy or flow cytometry, because these fluorescent stains label DNA, they are commonly used to visualize nuclei and mitochondria, because the Hoechst stains bind to DNA, they can disrupt DNA replication during cell division (apoptosis)(Elmore,2007). Consequently they are potentially mutagenic and carcinogenic so the cares are should be taken in their handling and disposal (Syed, et.al., 2013), and we used Hoechst 33258 staining to observe apoptosis affected by Radix Pulsatillae ethanol extract, the results showed an affective detection of apoptosis and could be supporting by flow cytometry analysis to determine apoptosis and necrosis cells ratio in the further studies as showing in figure 3. In conclusion, the results in vitro suggested that Chinese herb Radix Pulsatillae ethanol extracts have new small bioactive natural compounds with anti-lung cancer against A549 lung cancer cells and use the natural molecules for further cancer mechanistic researches.

Table 1: shows high throughput screening HTS procedure to detect different toxicity fractions of *Radix Pulsatillae* ethanol extract (negative control is untreated cells and positive control is cells treated with DMSO) (+= low toxicity; ++ = medium toxicity; +++ = high toxicity) against A549 lung cancer cells. (CS means cells suspension, cells growth to 70-80% and then digestion by trypsin and dilute it by DMEM+FBS to be single cells free of DMSO and herb extract as negative control); (A, B, C, ….H are lines mark for each well in the left side of 96 well/ plate to identify the fractions as A1, A2, A3, B1, B2,B3, C1,C2,C3,……H1 , H2, H3)

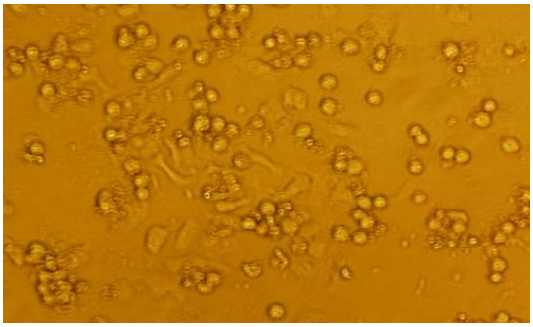
	Negative control	*Radix Pulsatillae* ethanol extracts fractions										Positive control
	1	2	3	4	5	6	7	8	9	10	11	12
A	CS											DMSO+CS
B	CS											DMSO+CS
C	CS											DMSO+CS
D	CS											DMSO+CS
E	CS	**E2** +++	**E3** +++	**E4** +++	**E5** +++	**E6** +++	**E7** +++	**E8** +++	**E9** +++	**E10** +++	**E11** +++	DMSO+CS
F	CS	**F2** +++	**F3** +++	**F4** +++					**F9** +++	**F10** +++	**F11** +++	DMSO+CS
G	CS											DMSO+CS
H	CS											DMSO+CS



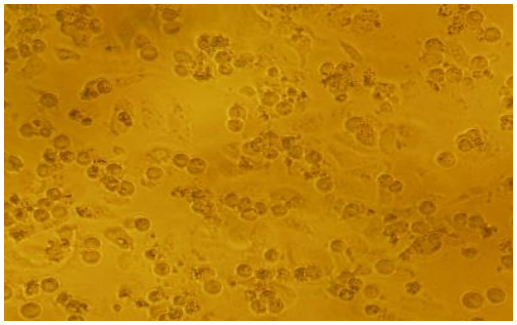
Control (0) µM



Fraction E5 (100) µM

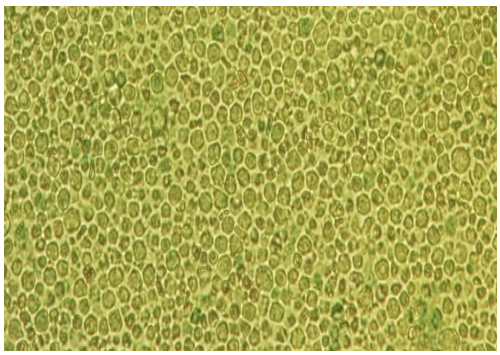


Fraction F3 (100) µM

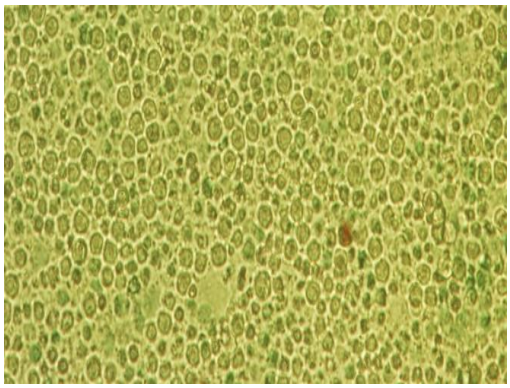


Fraction E3 (100) µM

Figure 1. Morphology changes of A549 lung cancer cells; Cells were treated with (0 and 100) µM of *Radix Pulsatillae* ethanol extracts fractions

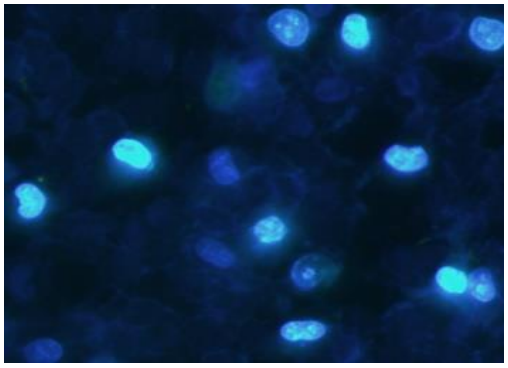


Control (0) µM

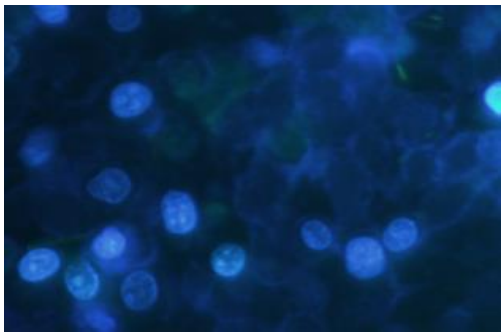


Treated with fraction E5-(100) µM

Figure 2. Normal fresh mouse splenocytes treated with (0 and 100) µM of *Radix Pulsatillae* ethanol extracts fraction E5



Control (0) µM



Treated with E5- (100) µM

Figure 3. Hoechst 33258 staining to detect apoptosis of A549 lung cancer cells after 24h of *Radix Pulsatillae* ethanol extract fraction E5 treated with (0 and 100) µM

Acknowledgments

The author is grateful to Northeast Normal University, institute of genetics and cytology, Membrane Channel Research Laboratory, Changchun city, Jilin province, China, for providing the materials, molecular techniques, and technical supports.

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