Antitumor Sterol Isolated from the Fruiting Body of *Pleurotus eryngii*

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Activity-guided fractionations led to the isolation of antitumor compound, ergosterol peroxide (5α, 8α-epideoxy-24(R)-methylcholesta-6, 22-dien-3β-ol) from the fruiting body of *Pleurotus eryngii* that was cultivated artificially. This sterol structure was established by using spectroscopic methods (^{1}H and ^{13}C nuclear magnetic resonance and high resolution mass spectra). The purified compound showed a molecular formular of $C_{28}H_{44}O_3$, displaying characteristic features of epidioxy sterols. The 50% inhibitory concentrations (IC_{50}) of ergosterol peroxide against human lung cancer cell line (A549) and human ovarian cell line (SK-OV3) were 7 μM and 14 μM, respectively. In the DNA fragmentation assay, the compound showed the programmed cell death causing the chromosomal DNA fragmentation. It reveals that ergosterol peroxide arrests G1 phase of the cell division cycle.

Key words – *Pleurotus eryngii*, ergosterol peroxide, 50% inhibitory concentration, A549, SK-OV3

Introduction

Mushrooms have been used for the diet in many countries due to their good taste and nutritive value. Bioactive molecules have been isolated from mushrooms including lectin[3,7,33], nucleases[23,29,32], proteases[2,9-11,18,19,22, 24-26,34], ribosome inactivating proteins[11,13,30,31], ubiquitin-like protein[8], polysaccharides[17,25] and polysaccharide-peptide and polysaccharide -protein complexes [4,6,15,26]. Some of these compounds have exploitable potential or have been proven to have clinical efficacy[17,27].

One of these, ergosterol peroxide (5,8-epidioxyergosta 6,22-dien-3-ol) is a natural steroid that has been found in a variety of fungi[5] and yeast[20,21,23]. In fungi, ergosterol peroxide[1], synthesized by the conversion of ergosterol to its epideoxide[22] plays a pivotal role in the detoxication of reactive oxygen species and has shown tumoricidal properties[28]. Extracts of seven mushrooms, *Pleurotus ostreatus, Agaricus balzei, Poria cocos, Cordyceps militaris, Flammulina velutips, Phellinus linteus* and *Pleurotus eringii*, were investigated to have the antitumor activities.

The objective of the present investigation is to isolate bioactive compounds from the mushroom *P. eryngii* which is now commonly cultivated in South Korea. We investigated the biochemical properties of a bioactive compound from *P. eryngii* and apoptotic effects against lung cancer and ovarian cell lines, A549 and SK-OV3, respectively.

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Materials and Methods

Cytotoxicity of seven mushrooms

Seven mushrooms, *P. ostreatus, A. balzei, P. cocos, C. militaris, F. velutips, P. linteus* and *P. eringii* were purchased from local farmers and supermarkets. Human lung cancer cell line (A549) and human ovarian cell line (SK-OV3) were purchased from Korean cell line bank (Seoul, Korea). All sample are dried at 30℃ for 3 days and grinded in the grinder and then added in sodium phosphate buffer (pH 6.7) in the ratio of 50% (w/v). The solution were boiled at 80℃ for 3 days to extract biofunctional materials and then centrifuged and filtered with micro syringe filter (0.2 ㎛). The extract was added to the cell lines for cytotoxicity.

Extraction of biofunctional material from *P. eryngii.*

One hundred kilograms of *P. eryngii* were teared with fingers and dried at 30℃ for 7 days. The dried mushrooms were grinded at local miller. Ten kilograms of mushroom powder were extracted with MeOH as shown in Fig. 2A and then step by step followed as shown in Fig. 2B.

Cytotoxicity of the purified compounds

The cytotoxicity assay was conducted with MTT method[16].

Identification of the purified PEC-4 compound

Melting points were measured on a Thomas Scientific capillary melting point apparatus. IR spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (KBr), and UV spectra were measured on a Beckman DU650 spectrophotometer. ^{1}H and ^{13}C NMR along with 2D-NMR data were obtained on a Bruker AM 500 (^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz) spectrometer in $CDCl_3$ and CD_3OD; EIMS and HREIMS data were collected on a JEOL JMS-700 spectrometer.

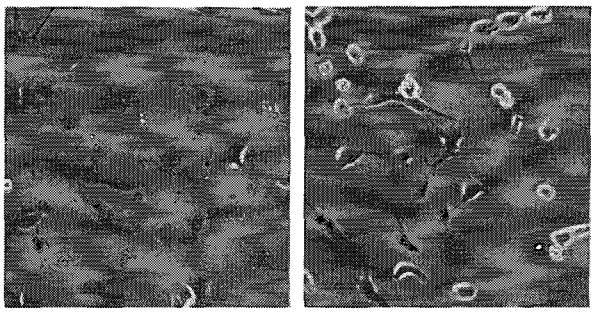
DNA fragmentation assay

Cells were grown in 60×15 mm^3 cell culture plate and treated for 6 hrs with PEC-4 compound before being transferred into 1.5 ㎖ micro-centrifuge tubes. The cells were centrifuged at 16,100×g for 5 min. The pellet was resuspended in 0.5 ㎖ TTE [ethylenediamine tetracetic acid (Tris-EDTA)] followed by vigorous vortexing. To the 0.5 ㎖ solution being already in the tube, 0.1 ㎖ ice-cold 5 M NaCl and 0.7 ㎖ ice-cold isopropanol were added. The tubes were vigorously vortexed and placed at -20℃ overnight to precipitate the DNA. Samples were washed once with 0.5 ㎖ 70% ice-cold ethanol and centrifuged at 16,100×g for 10 min at 4℃. The supernatant was removed and the tube was dried at 37℃. The pellets were dissolved in 45 ㎕ Tris-EDTA containing 5 ㎕ loading buffer. The samples were analysed by electrophoresis on 1.8% agarose gels stained with ethidium bromide (0.5 ㎍/㎖).

Table 1. Anticancer activities of seven mushrooms used in this study

	SK-OV3	A549	HL-60
P. eryngii	+++	++++	+++
P. ostreatus	++++	+++	+++
A. balzei	-	+	-
P. cocos	-	-	-
C. militaris	+	++	+
F. velutipes	-	-	-
P. linteus	+	+	-

≧+++ : high, ++ : middle, + : low



0 ㎍/ml 20 ㎍/ml

Fig. 1 Cytotoxic activity of *P. eryngii* against the lung cancer cell line (A549).

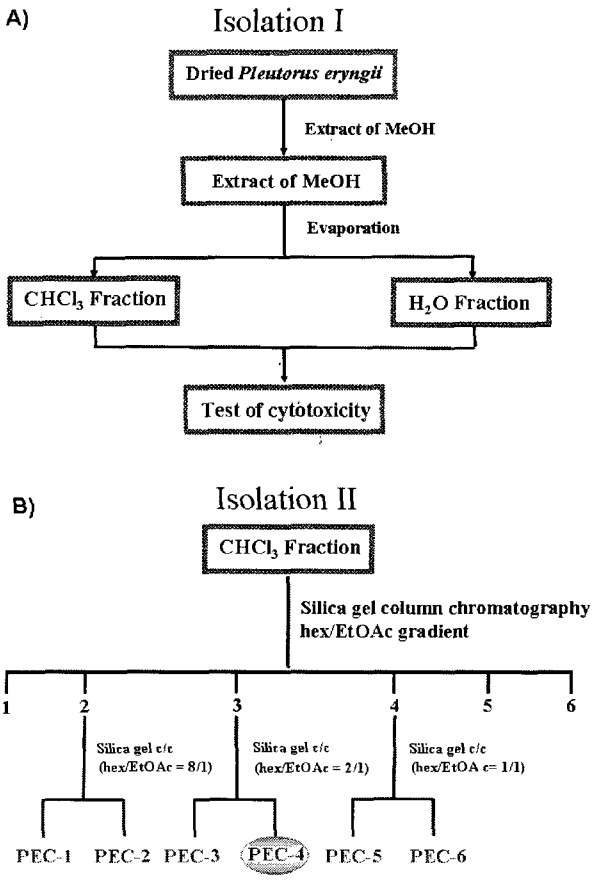


Fig. 2. Purification procedure of 6 compounds from *P. eryngii*.

Flow cytometry analysis

1×10^6 lung cancer cells treated with PEC-4 were resuspended in PBS, fixed by paraformaldehyde (final concentration 2% paraformaldehyde) for 1 h at RT, and twice washed. The cells were resuspended in 100 ㎕ permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and treated for 2 min on ice. After washing, the cells were incubated with TUNEL reaction mixture (Roche Molecular Biochemicals, Mannheim, Germany) for 1 h at

37℃ in a humidified atmosphere in the dark. The re-suspended cells were analyzed by flow cytometry with a FAC sort FCM machine (Beckman Coulter, UK)[14].

Results and Discussion

Cytotoxicity of seven mushrooms

Mushrooms form part of the diet in many countries due to their good taste and nutritive value. A host of bioactive molecules have been isolated from mushrooms including lectin[1-3], nucleases[6]. Seven mushrooms, *P. eringii, P. ostreatus, A. balzei, P. cocos, C. militaris, F. velutips, P. linteus* which are commonly cultivated in South Korea were detected antitumor activities against three cancer cell lines, SK-OV3, A549 and HL-60. As shown in Table 1, *Pleurotus* genera have strong cytotoxic effect to the cancer cell lines (Fig. 1).

Extraction of biofunctional material from *P. eryngii*

One gram of dried *P. eryngii* was extracted with chloroform and the extract was examined by TLC. Under UV light, followed by spraying with a solution of vanillin in sulphuric acid, six components (PEC-1, PEC-2, PEC-3, PEC-4, PEC-5 and PEC-6) were detected as shown in Fig. 2 Activity guided fractionation led to isolate PEC-4 which has the strongest antitumor activities against lung and ovarian cancer cell lines.

Identification of the purified PEC-4 compound

Compound PEC-4 was obtained as white powder having the molecular formular, $C_{28}H_{44}O_3$, and seven degrees of unsaturations, as deduced from its HREIMS data. IR spectrum of PEC-4 showed absorption bands on 3519 cm^{-1} (OH), 3203 cm^{-1} (=C-H), and 2950 cm^{-1} (aliphatic C-H). ^{1}H-NMR spectrum showed signals due to two tertiary methyl groups [H 0.82 (*s*, ^{3}H), 0.88 (*s*, ^{3}H)], four secondary methyl group [H 0.91 (*d*, ^{3}H, 1.0 (*d*, ^{3}H), 2.0 (*d*, ^{6}H)), one oxygenated methine protons [H 3.9 (*m*, ^{1}H)] and four sp^2 methylene group [H 5.13 (*dd*, ^{1}H), 5.23 (*dd*, ^{1}H), 6.23 (*d*, ^{1}H), 6.49 (*d*, ^{1}H)]. The ^{1}H and ^{13}C-NMR data with DEPT experiment showed the presence of twenty eight carbon atoms with seven sp^3 methylenes, eleven methins, six methyls, and four quaternary carbons. The ^{13}C-NMR spectral data enabled four double bonds to be characterized, and these account for two of the total seven degrees of unsaturations. The five extra degrees of unsaturations were presumed to be due to five rings. All spetral data (Fig. 3, Fig. 4, Fig. 5, Fig. 6 and Fig. 7) of compound PEC-4 were in good agreement with those of ergosterol peroxide.

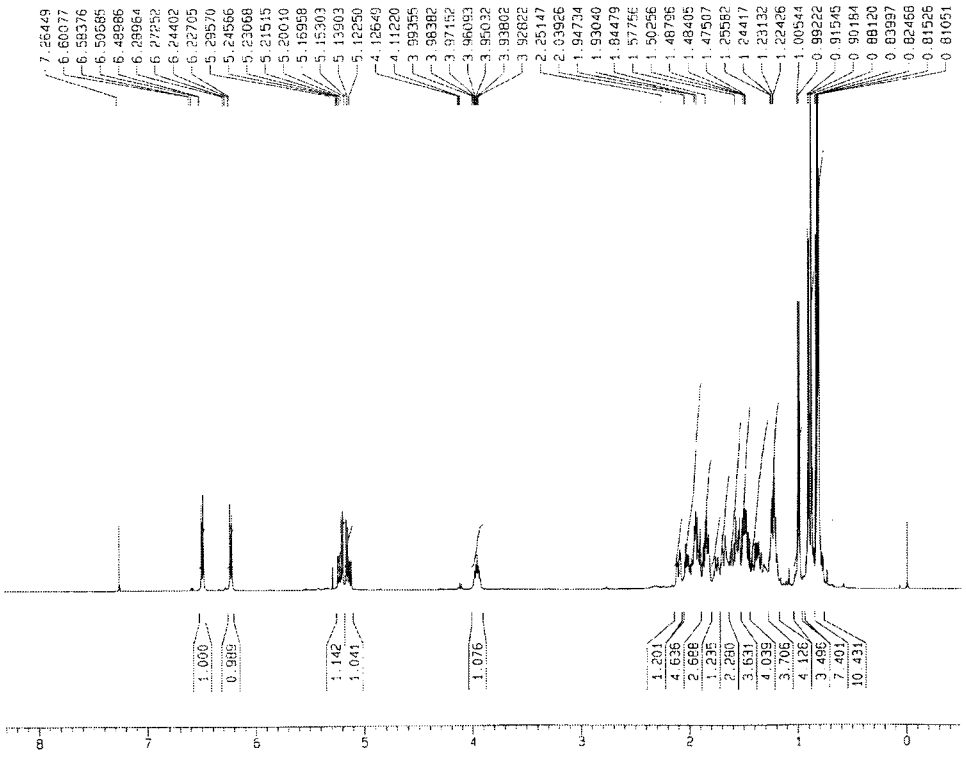


Fig. 3. ^{1}H NMR spectrum of PEC-4.

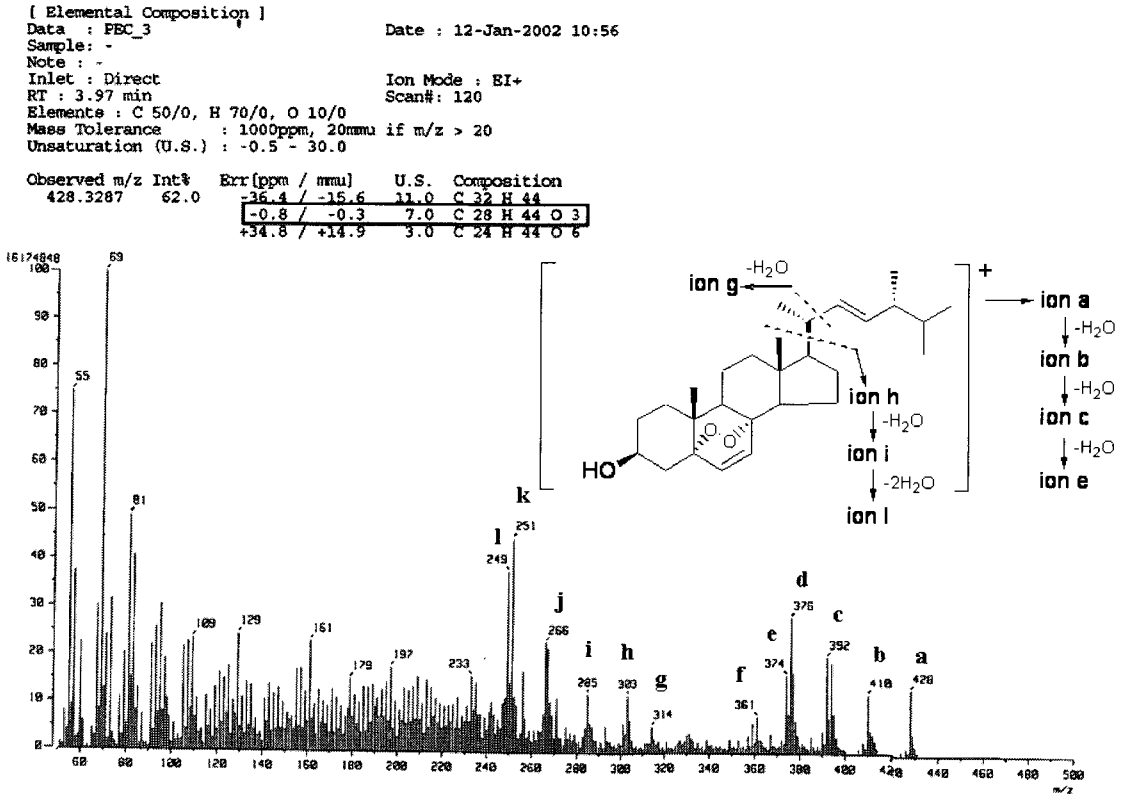


Fig. 7. Mass spectrum of PEC-4.

As shown in Table 2, ergosterol peroxide is stable compound with 182℃ of melting point and white color. Even though it was already known compound, ergosterol peroxide has strong cytotoxic effect against lung cancer (A549)(Fig. 8) and ovarian cancer cell line (SK-OV3)(Fig. 9).

DNA fragmentation assay

To gain more information of the mechanism of ergosterol peroxide-associated cytotoxicity in lung cancer cells, we determined whether ergosterol peroxide causes DNA fragmentation, a hallmark of apoptosis. As shown in Fig. 10, DNA fragmentation occurred upon exposure of lung cancer cells to ergosterol peroxide in a dose-dependent manner.

Table 2. Physico-chemical properties of ergosterol peroxide

	Ergosterol peroxide	Reference
Mol. Formula	$C_{28}H_{44}O_3$	
HRMS	428.3287	
mp	181-182℃	181.5-183℃
$[\alpha]_D^{20}$	-28(*c* 1.5, $CHCl^3$)	-29(*c* 0.8, $CHCl_3$)
Physical	White powder	
descrlption	Enough stable	

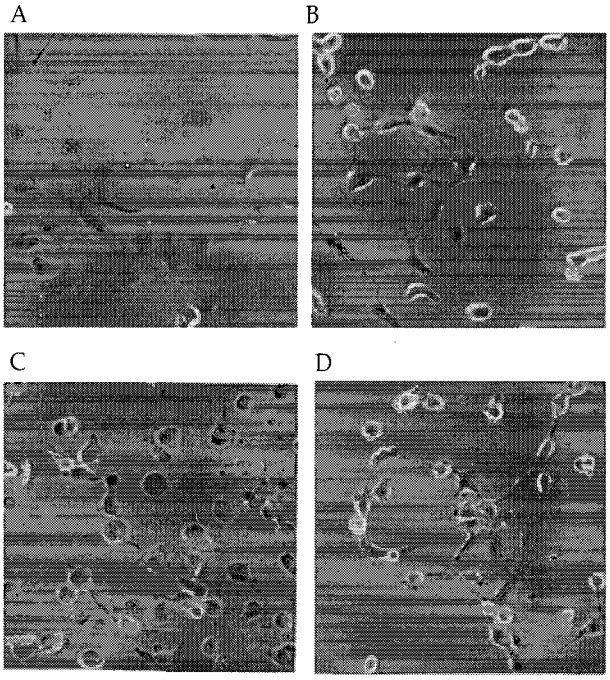


Fig. 8. Anticancer activity of the ergosterol peroxide against the lung cancer cell line (A549).
A : A549 without ergosterol peroxide,
B and C : A549 treated with ergosterol peroxide, 5 μg /ml and 10 μg/ml, respectively,
D : A549 treated with 5 μg/ml of taxol.

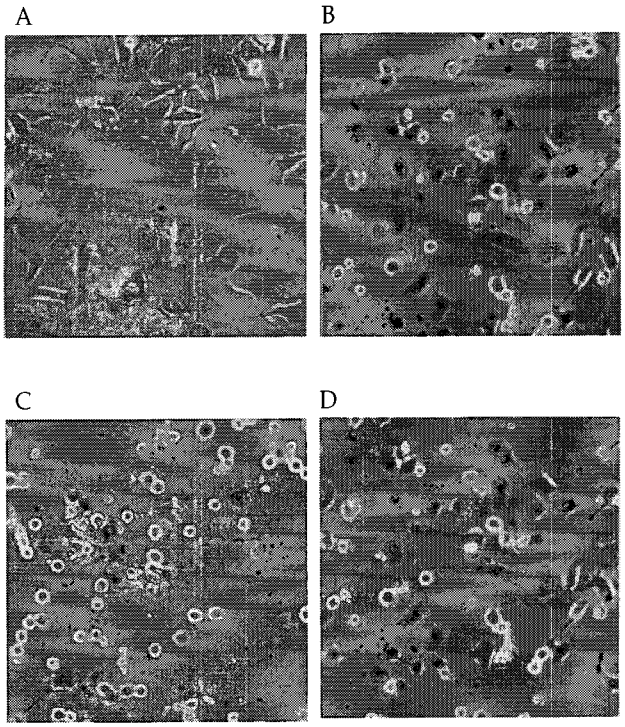


Fig. 9. Anticancer activity of the ergosterol peroxide against the ovarian cancer cell line (SK-OV3).
A : SK-OV3 without ergosterol peroxide,
B and C : SK-OV3 treated with ergosterol peroxide, 5 μg/ml and 10 μg/ml, respectively,
D : SK-OV3 treated with 5 μg/ml of taxol.

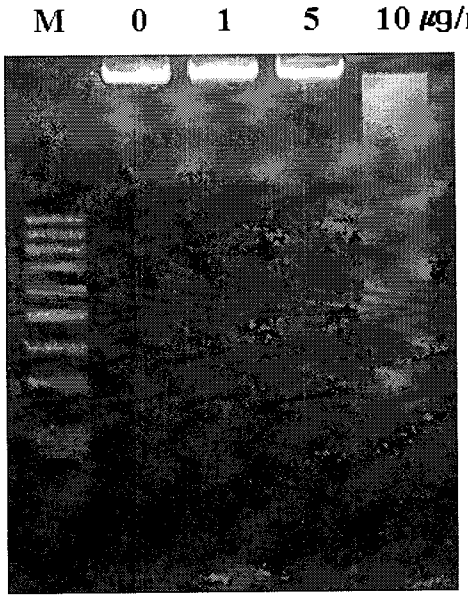


Fig. 10. Analysis of DNA fragmentation by agarose gel. Cell cultures were treated with ethanol or various concentrations of ergosterol peroxide for 48 hours and floating cells were harvested and the fragmented DNA was isolated and analyzed in a dose-dependent manner on agarose gel.

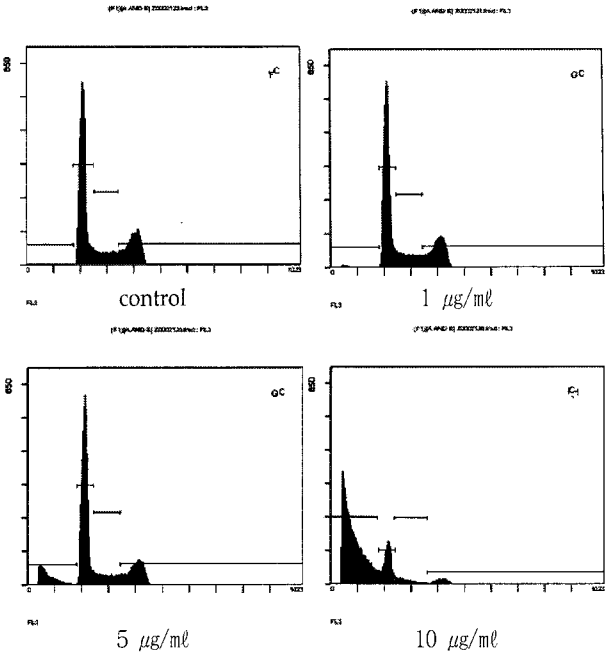


Fig. 11. Flow cytometry analysis of ergosterol peroxide in lung cancer cell line (A549).

Flow cytometry analysis

Through the flow cytometry analysis ergosterol peroxide arrested the G1 phase of the cell cycle(Fig. 11). We concluded that ergosterol peroxide is a strong candidater for anti-cancer agent against lung cancer.

Acknowledgments

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초록 : *Pleurotus eryngii* 로부터 항암물질의 분리

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새송이버섯으로부터 활성추적법으로 항암활성이 있는 물질인 에르고스테롤 프록사이드를 분리하였다. 이 스테롤의 구조는 분광법과 NMR법으로 확인하였으며 분자식은 $C_{28}H_{44}O_3$이었다. 폐암과 난소암에 IC_{50}값은 각각 7 μM과 14 μM이었다. DNA단편화 실험에서 이 화합물은 암세포의 chromosimal DNA 를 사닥다리모양으로 분해하였고, 세포 분열주기의 억제실험에서 G1단계를 억제함을 관찰하였다.