دانشگاه علوم پزشکی و خدمات بهداشتی،درمانی کرمانشاه
Kermanshah University Of Medical Sciences

Microbial Cell Factories jurnal

Evaluation of the anticarcinogenic potential of the endophyte, *Streptomyces* sp. LRE541 isolated from *Lilium davidii* var. *Unicolor* (Hoog) Cotton

Presenter: Forough kazemzadeh

Supervisor : Dr. Yadollah Bahrami

Faculty of medicine, class No.3 [dr. Nowroznezhad]

12:30 p.m

Department of Medical Biotechnology
Kermanshah University of Medical Sciences

Ma *et al. Microbial Cell Factories* (2021) 20:217
https://doi.org/10.1186/s12934-021-01706-z

Microbial Cell Factories

RESEARCH Open Access

Evaluation of the anticarcinogenic potential of the endophyte, *Streptomyces* sp. LRE541 isolated from *Lilium davidii* var. *unicolor* (Hoog) Cotton

Aiai Ma[1,2], Kan Jiang[5], Bin Chen[5], Shasha Chen[3], Xinge Qi[1], Huining Lu[4], Junlin Liu[4], Xuan Zhou[1,2], Tan Gao[1,2], Jinhui Li[1] and Changming Zhao[1,2*]

Introduction

•Although major progress has been achieved in cancer therapy for the past few decades, cancer remains a serious public health threat.

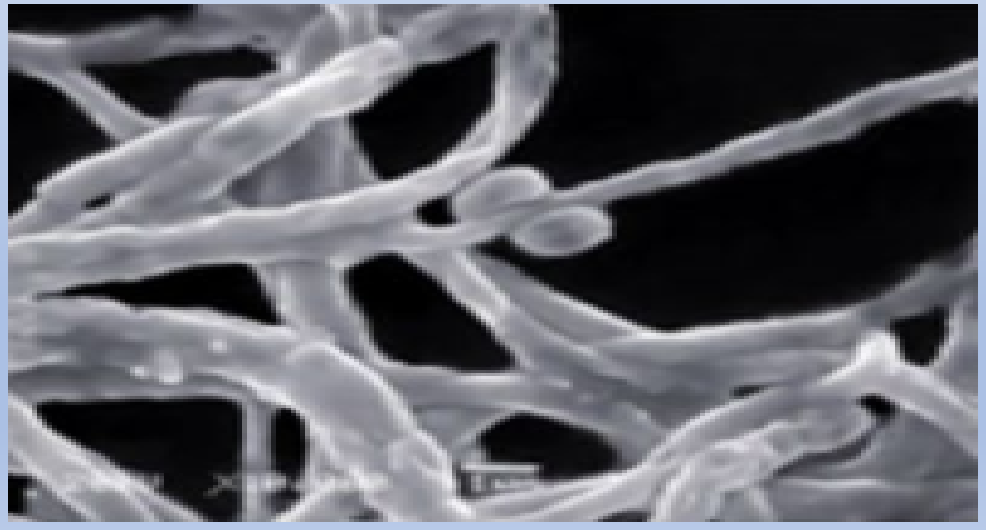
•Chemotherapy is one of the common therapeutic approaches for controlling cancers. Unfortunately, most patients eventually relapse and develop drug resistance.

•On this account, a continuous supply of novel drugs with high effectiveness and safety is urgently needed.

•extensive and intensive studies on the underlying antitumor mechanisms of the drugs are also required

Introduction

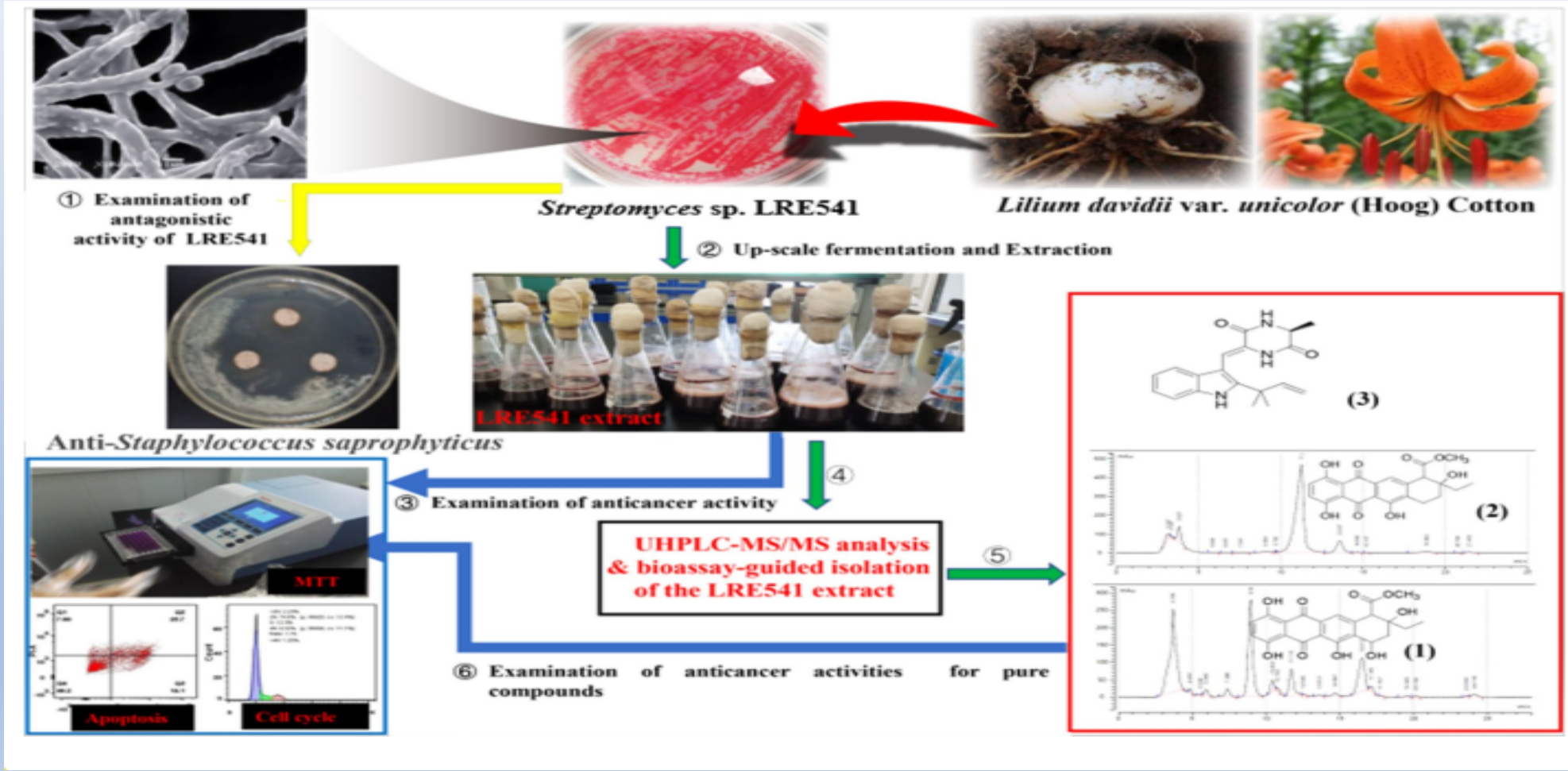
- The genus *Streptomyces*, with its vast distribution and innate capability of producing diverse **bioactive secondary metabolites**, has served as an important source of novel antibiotic candidates for decades.



Introduction

- ***Lilium davidii var***. *unicolor* (Hoog) Cotton (commonly called Lanzhou lily), a famous healthcare edible medicinal plant rich in amino acid, vitamins, glycosides, alkaloids, and polysaccharides, possesses **antioxidant activities**

Graphical Abstract



Materials and Methods

- **Sample collection and actinomycetes isolation：**

Thirty healthy roots L. davidii var. Monochrome cotton (Hogg) was randomly selected. The roots of the plant were carefully dug to ensure its integrity, then stored in aseptic plastic bags at 4 ° C and processed within 24 hours after collection.

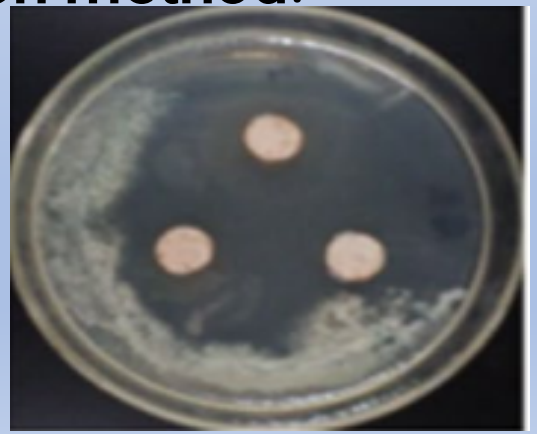
Materials and Methods

- **Primary screening of endophytic isolates for antimicrobial activity:**
- All 15 isolates were cultured on agar
- The spore suspension was prepared and inoculated on the culture medium for 7 days at 23 degrees
- Then it was covered with 5 ml of soft nutrient agar 0.6% (weight by volume)
- Seed was cultured with 500 µl of cultured marker microorganisms
- Then, the coated plates were incubated at 28 ° C for 24 h, the apparent inhibition zone around each isolate was recorded for positive antimicrobial activity.

Materials and Methods

- **Antimicrobial assay of LRE541 isolate:**

The actinomycete-like isolate, which exhibits an apparent inhibitory zone against all pathogenic microorganisms tested, was further evaluated by **disk diffusion method**.



Materials and Methods

Morphological and physiological characteristics of LRE541 isolate

- Pure culture of LRE541 daily in ISP medium
- Micromorphology and sporulation of culture by light microscopy
- Aerial mycelium and spores after 14 days of growth in culture medium with electron microscopy
- Physiological characteristics such as extracellular enzyme activity, carbon / nitrogen source utilization, and temperature / pH tolerance

Materials and Methods

- **16S rRNA sequencing and phylogenetic analysis:**
- Genomic DNA (**gDNA**) of LRE541 isolate was extracted.
- The sequence of the 16S rRNA gene isolated from LRE541 was matched with the closest gene sequence of **Streptomyces spp**.

- The 16RE rRNA gene sequence isolated from LRE541 was sent to GenBank nucleotide sequence databases under registration number **MK138546**.

Materials and Methods

- **Fermentation and extraction of secondary metabolites from LRE541:**
- LRE541 isolate was inoculated on mile agar medium
- the biomass was centrifuged and the supernatant was extracted three times with an equal volume of ethyl acetate.
- The ethyl acetate fractions were then concentrated at 40 ℃ C in a rotary vacuum distillation appa

Materials and Methods

- **In vitro cytotoxic assay of LRE541 extract:**

➤Cell culture: Antitumor activity of LRE541 extract against a wide range of cell lines, including nine human cancer cell lines

➤Cytotoxicity test: Cell survival rate using **MTT method**

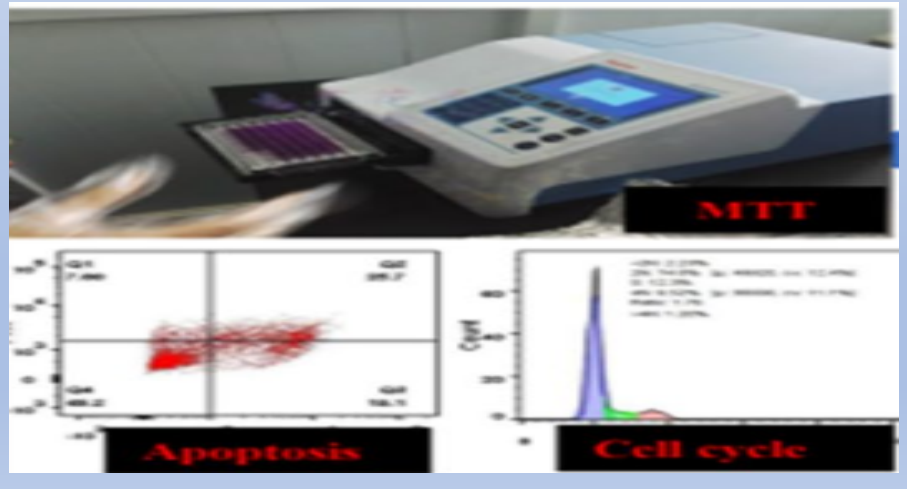
➤Analysis of cell apoptosis: Data were obtained and analyzed using **flow cytometer**.

➤Cell cycle analysis: Cell cycle analysis was also performed by **flow cytometry**

Materials and Methods

- Analysis of cell apoptosis
- Cell cycle analysis

flow cytometer



Materials and Methods

- **Purification and identification of bioactive metabolites from LRE541 extract:**
- The ethyl acetate extract of LRE541 was separated and purified on a HP-20 macro-porous **column** and washed with a mixture of H2O-EtOH gradient (70:30, 50:50, 20:80) to give three fractions.

- Structural identification of purified metabolites in spectroscopy using **spectroscopy** techniques

Materials and Methods

- **statistical analysis:**

SPSS software was used for statistical analysis

Results

- **Isolation of endophytic actinomycetes and screening for antimicrobial activities**

Table 1 Antimicrobial activities of isolate LRE541 against various pathogenic microorganisms

Test microorganisms	Inhibition zone (mm diameter)
Gram-positive bacteria	
Staphylococcus aureus (MRSA) ATCC25923	16.67 ± 2.31
Diplococcus pneumoniae (clinical isolate)	16 ± 2.65
Enterococcus faecalis (clinical isolate)	12.33 ± 0.58
Staphylococcus saprophyticus (clinical isolate)	21.33 ± 1.53
Gram-negative bacteria	
Escherichia coli ATCC25922	12.33 ± 1.53
Pseudomonas aeruginosa ATCC27853	12 ± 1.00
The yeast-like fungus	
Candida albicans ATCC66415	7.67 ± 0.78

Results

- **Phenotypic characteristics of isolate LRE541**

Table 2 Cultural characteristics of *Streptomyces* sp. LRE541 on various media

Media	Growth	Color of colony mycelia		Diffusible pigment
		Aerial	Substrate	
ISP2	Good	Light pink to red	Orange-yellow to red	–
ISP3	Good	Bright red	Red	–
ISP4	Good	Pinky white	Pinky white	–
ISP5	Good	Red in white	Red in white	–
ISP6	Good	Transparent to pale violet red	Pale violet red	–
ISP7	Good	Brick red	Vivid red	–
Gause's No. 1	Good	Vivid pink	Red	Claret-colored pigment

Results

- Phenotypic characteristics of isolate LRE541

Table 3 Physiological properties of *Streptomyces* sp. LRE541

Tests	Results	Tests	Results
Cellulose utilization	+	**Nitrogen sources utilization**	
MR test	−	Urea	+
H_2S production	−	Glycine	+
Extracellular enzyme activity		Peptone	+
Urease	+	Maizena	−
Catalase	+	Tyrosine	+
Starch hydrolysis	+	Aspartic acid	−
Gelatin hydrolysis	+	Soybean meal	+
Degradation of		Ammonium sulfate	+
Tween 20	−	L-Proline	+
Tween 40	+	L-Arginine	+
Tween 80	+	L-α-Alanine	−
Carbon sources utilization		**Growth at pH**	
Xylose	++	pH 2	−
Starch	+	pH 4	+
Glucose	++	pH 6	+
Maltose	+++	pH 7	+++
Lactose	+++	pH 8	++
Sucrose	++	pH 10	++
Fructose	+	pH 12	+++
Mannose	++	**Growth at Temp**	
Trehalose	−	4–16 °C	−
Raffinose	−	18–20 °C	+
Arabinose	+	23 °C	+++
Rhamnose	+	28 °C	++
Growth at NaCl (w/v)		37 °C	+
0–6%	++	**Gram staining**	+

Results

- Phenotypic characteristics of isolate LRE541

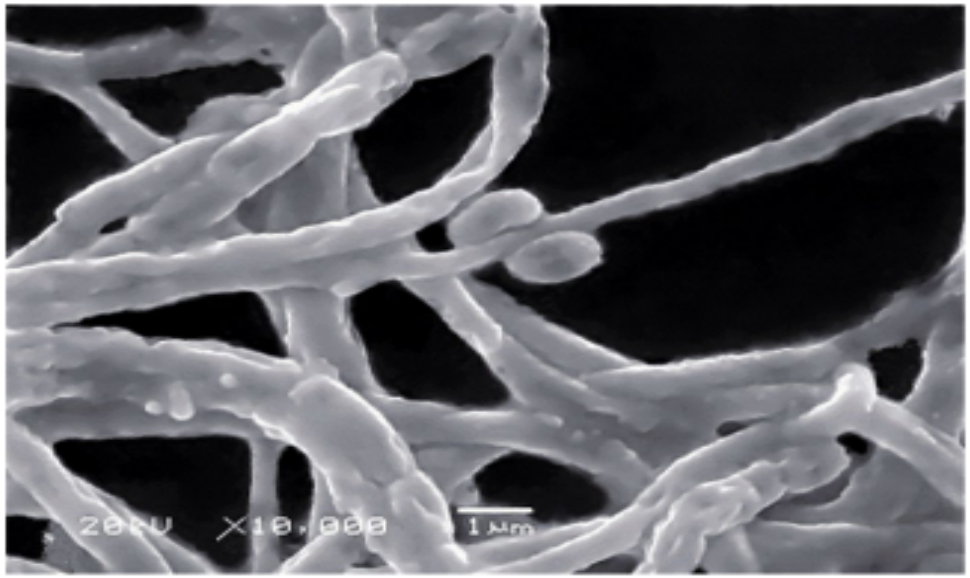


Fig. 1 The scanning electron micrograph of *Streptomyces* sp. LRE541 cultured on the Gauze's No. 1 medium for 2 weeks showing aerial mycelia and spores

Results

- 16S rRNA gene-based phylogenetic analysis

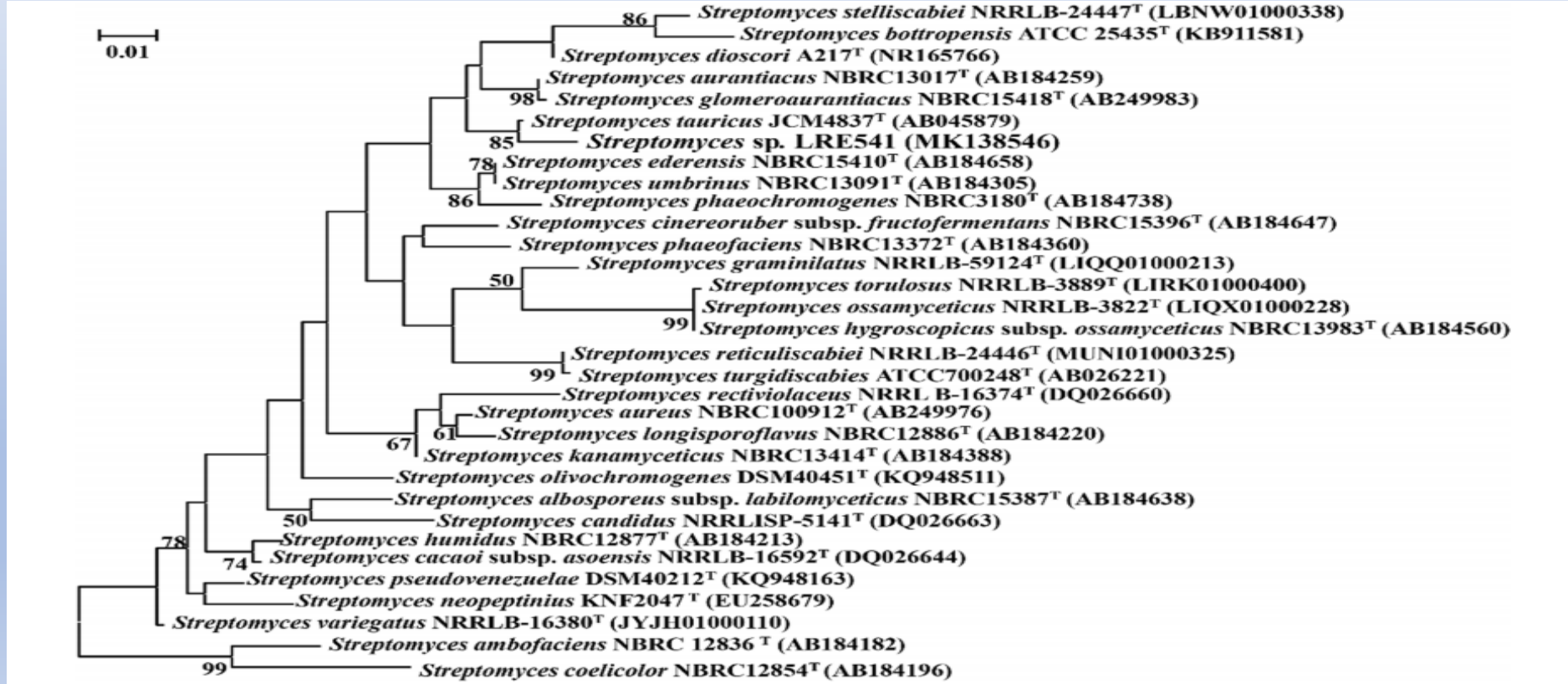


Fig. 2 Maximum Likelihood tree exhibiting phylogenetic relationship between isolate LRE541 and the closely related representatives of *Streptomyces* spp. Only bootstrap values above 50% are present at the tree nodes. The scale bar denotes 0.01 substitutions per site

Results

- Cytotoxicity of the LRE541 extract towards various cell lines

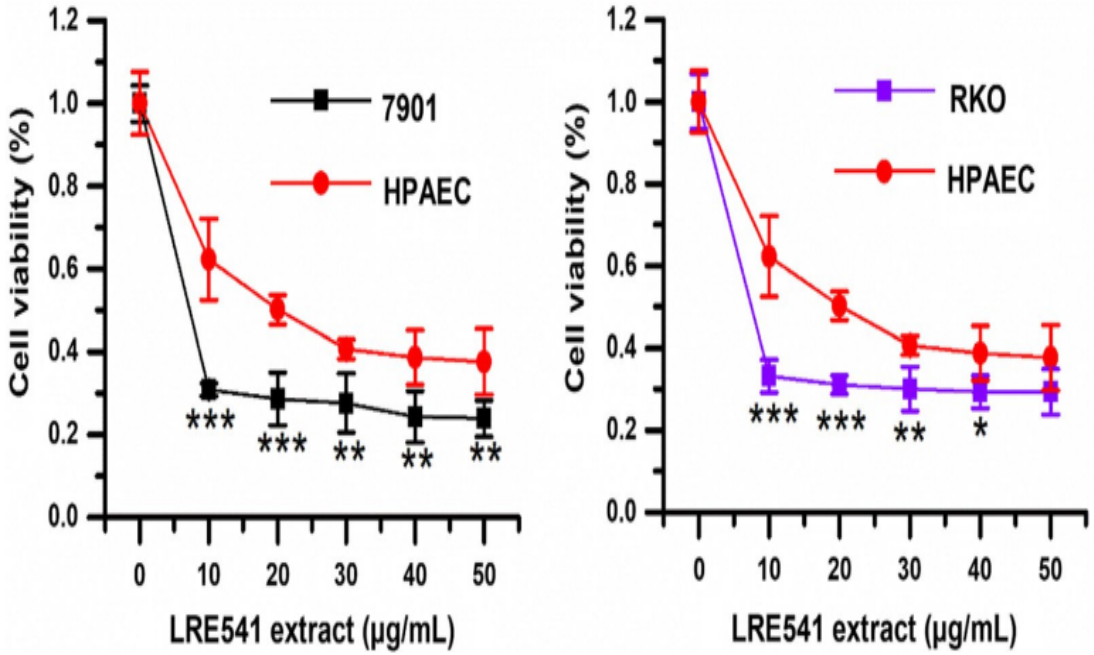


Fig. 3 Sensitivity of various types of cell lines (7901, RKO and HPAEC) to the LRE541 extract. The three cell lines were incubated with increasing concentrations of the LRE541 extract for 48 h, and their viabilities were determined by the MTT method. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. the HPAEC cell line

Table 4 IC_{50} values of the LRE541 extract against various cell lines (μg/mL)

Cell types	IC_{50}
Human colon cell RKO	0.02127
Human gastric adenocarcinoma 7901	0.2904
Human liver carcinoma cell HepG2	1.484
Human tongue cancer cell CAL-27	4.861
Human breast carcinoma cell MCF-7	6.986
Human chronic promyelocytic leukemia cell K562	8.106
Human cervical cancer cell Hela	10.87
Human pancreatic cancer cell SW1190	12.98
Human non-small cell lung cancer A549	16.94
Human pulmonary artery endothelial cell HPAEC (human normal cell)	20.14

Results

- Induction of apoptosis in 7901 and RKO cell lines

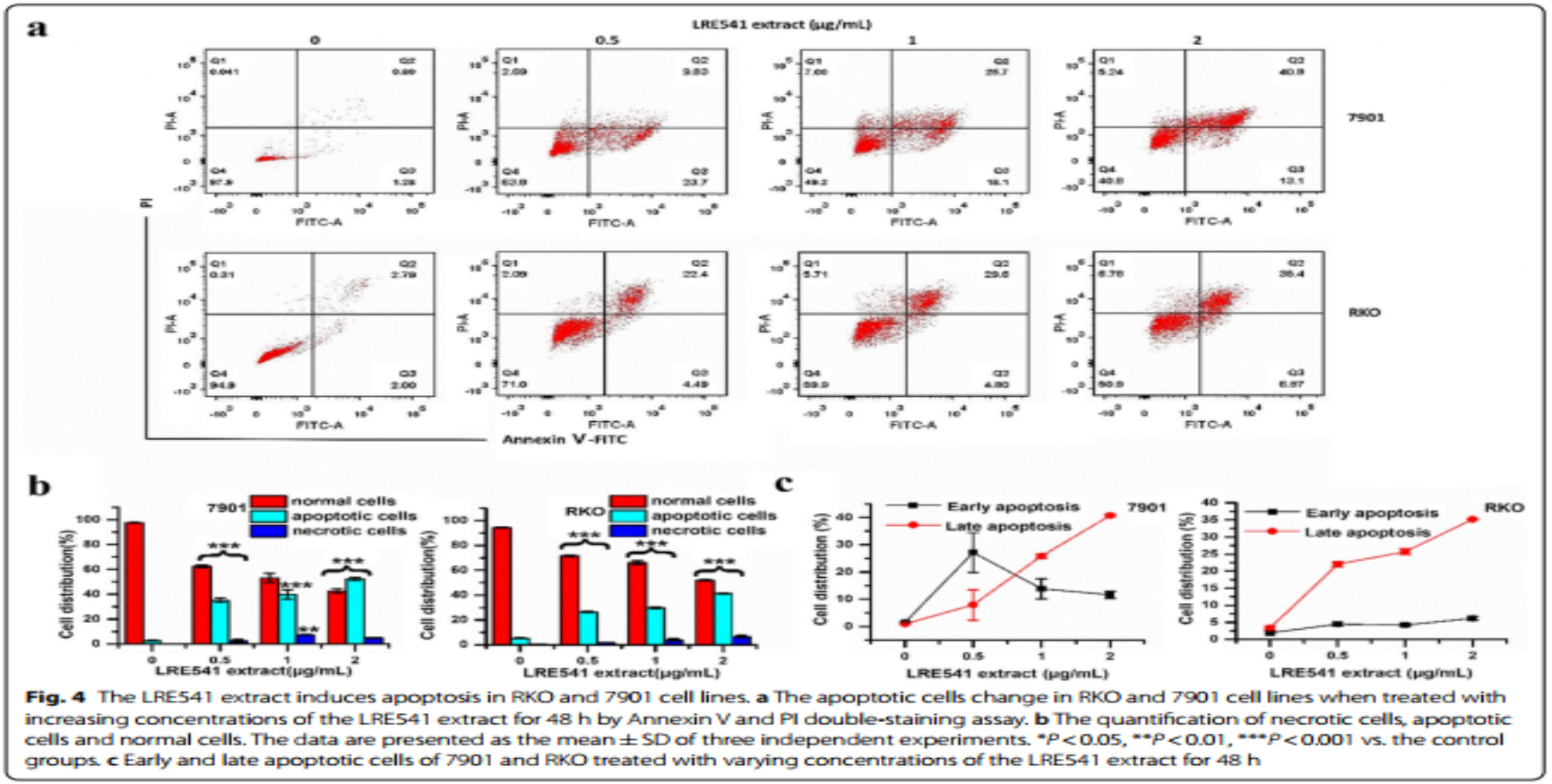


Fig. 4 The LRE541 extract induces apoptosis in RKO and 7901 cell lines. **a** The apoptotic cells change in RKO and 7901 cell lines when treated with increasing concentrations of the LRE541 extract for 48 h by Annexin V and PI double-staining assay. **b** The quantification of necrotic cells, apoptotic cells and normal cells. The data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control groups. **c** Early and late apoptotic cells of 7901 and RKO treated with varying concentrations of the LRE541 extract for 48 h

Results

- The LRE541 extract inhibits the cell cycles of 7901 and RKO cell lines

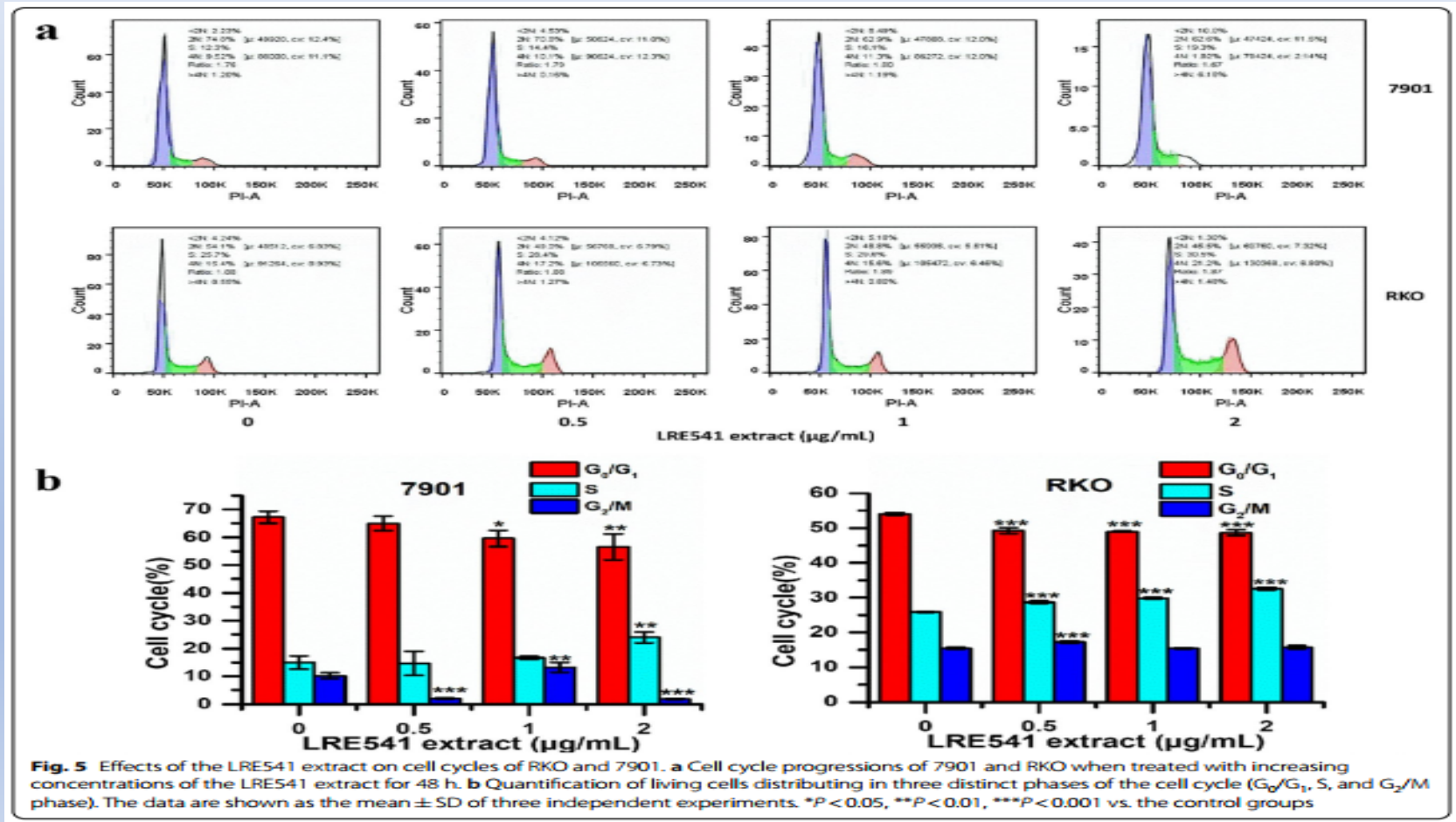


Fig. 5 Effects of the LRE541 extract on cell cycles of RKO and 7901. **a** Cell cycle progressions of 7901 and RKO when treated with increasing concentrations of the LRE541 extract for 48 h. **b** Quantification of living cells distributing in three distinct phases of the cell cycle (G_0/G_1, S, and G_2/M phase). The data are shown as the mean ± SD of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the control groups

Results

- **Chemical profiling of the LRE541 extract using UHPLC-MS/MS analysis**
  To investigate the compounds that may be responsible for its anti-neoplastic properties, LRE541 extract was subjected to ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS / MS).
- There are about 700 compounds in LRE541 extract. More than one-seventh of the compounds have been recorded to show various biological activities, including thirty-nine antitumor compounds, ten antioxidant compounds and sixteen antimicrobial compounds.

Results

- Structure elucidation and cytotoxicity of compounds from LRE541 extract

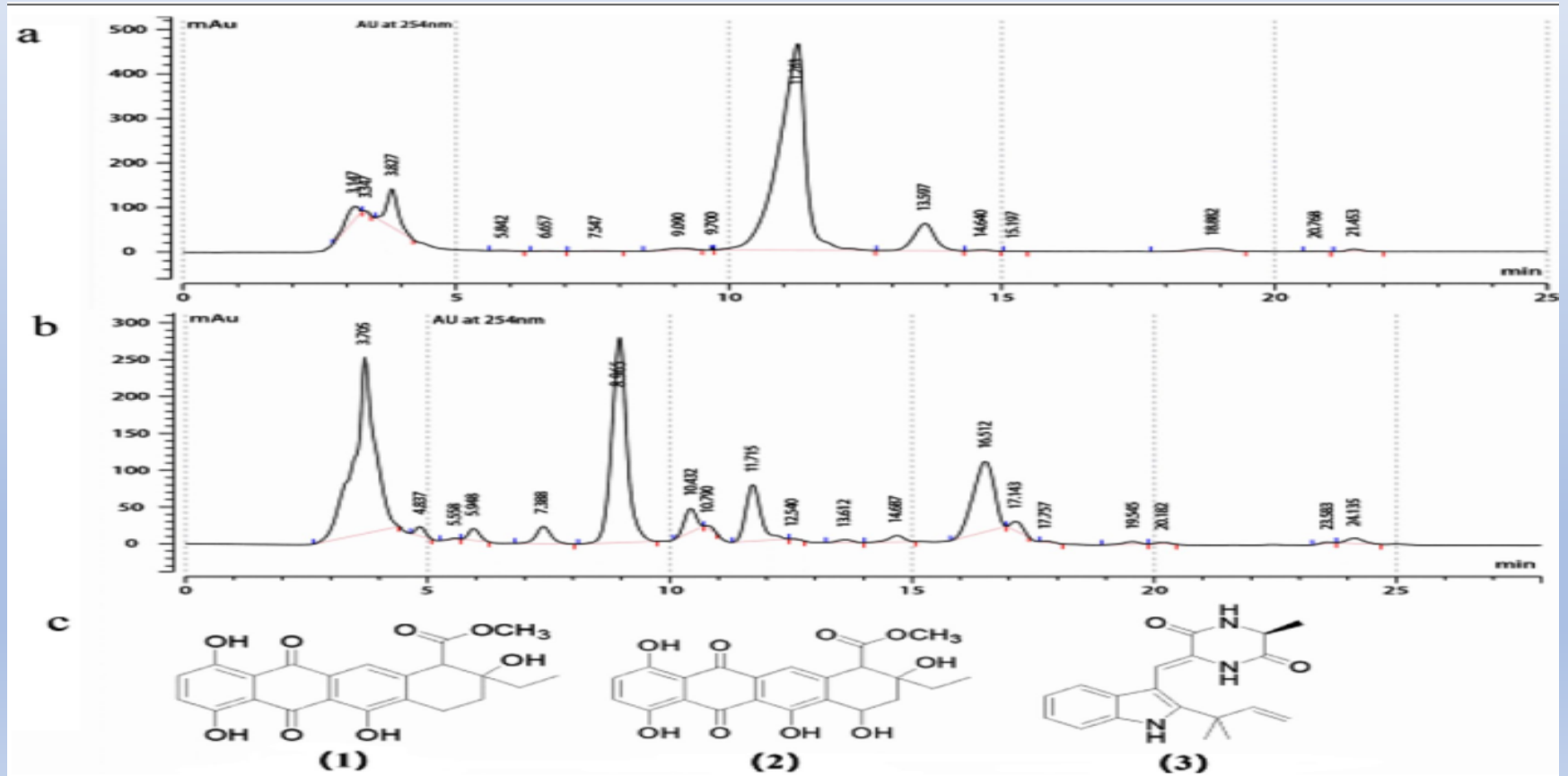


Fig. 6 **a, b** Isolation and purification of 4-deoxy-ε-pyrromycinone at 11.263 min and epsilon-pyrromycinone at 8.965 min, respectively, by semi-HPLC; **c** chemical structures of the three pure compounds

Results

- Structure elucidation and cytotoxicity of compounds from LRE541 extract

Table 6 IC_{50} values of the compounds 4-deoxy-ε-pyrromycinone (1), epsilon-pyrromycinone (2), and cisplatin (DDP) against various cancer cell lines (μg/mL). The data are shown as the mean ± SD of three independent experiments

Compounds	A549	HepG2	SW1990	RKO
(1)	19.55 ± 5.2	20.42 ± 4.24	17.87 ± 2.73	14.96 ± 2.6
(2)	16.8 ± 0.75	18.6 ± 3.03	19.3 ± 4.32	12.9 ± 2.13
DDP	12.8 ± 0.37	13.3 ± 1.2	17.1 ± 2.8	16.72 ± 3.5

Table 5 ^{13}C NMR spectroscopic data of compounds (1)–(3) [400 MHz,δ (ppm)] purified from the LRE541extract

(1) ($CDCl_3$)		(2) ($CDCl_3$)		(3) ($(CD_3)_2CO$)	
Position	δ_C	Position	δ_C	Position	δ_C
1	159	1	158.6	2	144.8
2	130	2	130.3	3	104.4
3	129.7	3	129.8	3a	126.6
4	157.9	4	158	4	119.7
5	191.2	5	191.1	5	120.7
6	161.8	6	161.2	6	127.3
7	20.3	7	62.6	7	110.7
8	28.8	8	32	7a	136.2
9	71.8	9	70.1	8	112.4
10	57.3	10	57.6	9	122.2
11	121	11	120.8	10	160.3
12	186.5	12	185.3	12	52.2
13	171.7	13	170.3	13	166.9
14	53.4	14	53	15	40.1
15	32.6	15	34.7	15a × 2	27.9
16	7	16	6.3	16	146
4a	113	4a	112.5	17	112.3
5a	131.3	5a	132.2		
6a	134.5	6a	132.8		
10a	142.1	10a	142.6		
11a	114	11a	114.9		
12a	112.9	12a	112.3		

Conclusion:

- ✔LRE541 isolated from root tissue of L. davidii var. Cotton monochrome (Hoog) and study of **cytotoxic activity** of isolates secondary to a panel of human malignant cell lines
- ✔Further detection of **cell apoptosis** and cessation of the RKO and 7901 cell cycle by flow cytometry revealed a basic mechanism underlying biological action.
- ✔Secondary metabolites and may elucidate the potential application of metabolites in the **treatment** of RKO and 7901 cell lines.

Conclusion:

- ✔The chemical profile of LRE541 extract identified by UHPLC-MS / MS analysis showed the presence of **antitumor** and **antimicrobial** compounds in the extract.
- ✔Further chemical analysis of Streptomyces sp. LRE541 led to the discovery of a prenylated indole dictapopyrazine (**DKP**) alkaloid, identified as neoquinoline A, an antitumor agent. The two anthraquinones, **4-deoxy-ε-pyromycinone** and **epsilon-pyromycinone**, both have anti-cancer activity.

Thank you for your attention