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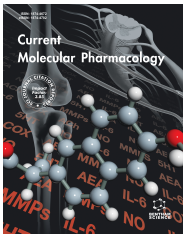

RESEARCH ARTICLE

Sanguinarine Attenuates Lung Cancer Progression *via* Oxidative Stress-induced Cell Apoptosis

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Abstract:

Background:

Lung cancer (LC) incidence is rising globally and is reflected as a leading cause of cancer-associated deaths. Lung cancer leads to multistage carcinogenesis with gradually increasing genetic and epigenetic changes.

Aims:

Sanguinarine (sang) mediated the anticancer effect in LCC lines by involving the stimulation of reactive oxygen species (ROS), impeding Bcl2, and enhancing Bax and other apoptosis-associated protein Caspase-3, -9, and -PARP, subsequently inhibiting the LC invasion and migration.

Objective:

This study was conducted to investigate the apoptotic rate and mechanism of Sang in human LC cells (LCC) H522 and H1299.

Methods:

MTT assay to determine the IC_{50}, cell morphology, and colony formation assay were carried out to show the sanguinarine effect on the LC cell line. Moreover, scratch assay and transwell assay were performed to check the migration. Western blotting and qPCR were done to show its effects on targeted proteins and genes. ELISA was performed to show the VEGF effect after Sanguinarine treatment. Immunofluorescence was done to check the interlocution of the targeted protein.

Results:

Sang significantly inhibited the growth of LCC lines in both time- and dose-dependent fashions. Flow cytometry examination and Annexin-V labeling determined that Sang increased the apoptotic cell percentage. H522 and H1299 LCC lines treated with Sang showed distinctive characteristics of apoptosis, including morphological changes and DNA fragmentation.

Conclusion:

Sang exhibited anticancer potential in LCC lines and could induce apoptosis and impede the invasion and migration of LCC, emerging as a promising anticancer natural agent in lung cancer management.

Keywords: Lung cancer, Sanguinarine, Reactive oxygen species, Apoptosis, Vegf, Flow, Cytometry.

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1. INTRODUCTION

Lung cancer (LC) is the foremost trigger of death in the world. According to 2020, worldwide data about 2.2 million cases were recorded, and 1.8 million deaths occurred; like in other cancers, LC also presents molecular and epigenetic abnormalities [1, 2]. The first case of non-small cell lung cancer (NSCLC) death was reported in Western countries, accounting for about 85% of LC cases [3]. Combinational chemotherapy is the most common treatment option in NSCLC patients; nevertheless, the complexity of such a therapeutic strategy remains associated with organ failure and low patient survival due to higher cytotoxicity, poor patient selection/stratification, and the diversity of patients' phenotypes and genotypes [4]. The need to find a new therapeutic agent for better efficacy against LC remains an urgent global health challenge.

Apoptosis is highly investigated due to its prime role in cancer progression; it is recognized as the most important process of programmed cell death and development. In 1972, for the first time, the term 'apoptosis' was used to illustrate a distinctive cell death morphology [5]. Apoptosis is controlled by extracellular and intracellular signaling mediators, which induce cell morphological alterations containing fragmentation and nuclear condensation, mitochondrial permeabilization, cell shrinking and apoptotic development and membrane blebbing [6]. Different studies explored the essential role of apoptosis in homeostasis and normal development [7]. Besides, apoptosis also shows a protective role in immune responses or eradicating impaired cells, helping as a superiority regulator for homeostasis [8]. Apoptosis is a hallmark of cell death after chemotherapy or radiotherapy in sensitive organs, including the tongue, testis, gastrointestinal system, and bone marrow [9]. The perilous role of apoptosis is to confiscate precancerous cells and inhibit the malignancy progression [10]. The increased frequency of apoptosis in normal tissue after chemotherapy or radiotherapy leads to unembellished responses, which may limit the therapeutic proportion of modalities [11]. Apoptosis in the intestinal stem cell and parotid gland is responsible for the origination of mucositis and xerostomia, which is common in patients who undergo radiotherapy or chemotherapy for abdomen or head and neck cancers [10]. In tumor cells, apoptosis is the major type of cell death that plays a main role in cancer treatment [12]. Apoptosis inflection in both tumor cells and normal tissue is a fascinating approach for refining the therapeutic window and reducing toxicity [13]. Impeding of apoptosis in the gastrointestinal system and bone marrow has enhanced toxicity from radiation therapy in such organs [14]. Apoptosis induction in the tumor by the inflection of different signaling pathways has created fascinating outcomes [15].

The cell death process, apoptosis, is facilitated by mitochondrial permeabilization, which affects apoptosome creation, stimulation of caspase 9, and subsequent stimulation of caspase effector. Intrinsic apoptosis is due to intracellular stress and growth factor withdrawal, whereas transmembrane death receptor is responsible for extrinsic apoptosis. The instigation and execution of this process are controlled by BCL2 and the caspase proteins family. Stimulation of BCL2 proteins, Bak and Bax, is due to mitochondrial permeabilization and pro-apoptotic protein release. Due to the activation pathway, the CASPASE-9 is stimulated, which is directly responsible for the activation of clave and activated caspase-3 and caspase-7 [16]. For morphological mitochondrial alterations, Caspase-9 and ROS generation are required by cleaving and stimulating Bid into tBid. After stimulation by caspase 9, caspase 3 impedes ROS generation and is required for effective apoptosis induction, whereas effector caspase-7 triggers cell detachment during apoptosis.

ROS contains exceedingly reactive molecules like hydrogen peroxide (H2O2), hydroxyl radical (●OH), singlet oxygen (1O2), and superoxide anion radical (●O2−) [17]. Generally, ROS is the derivative of oxygen metabolism, which explains that mitochondria are the prime cause of ROS. Basal intensities of ROS help to control normal cell differentiation and proliferation; however, enhanced levels of ROS can cause severe impairments to protein and DNA, which leads to cell apoptosis [18, 19]. Moreover, extreme oxidative stress particularly targets mitochondria, which may lose mitochondria membrane and activate mitochondria, attenuating apoptosis [18, 20, 21].

Recent studies have reported that numerous natural compounds have incredible anti-tumor actions or can improve the effectiveness of chemotherapy [22]. Natural molecules are safe and stable due to pollution-free, toxin-free, residue-free, and non-hazardous properties, and thus, provide cancer management opportunities [23]. For instance, polyphenols (*e.g.*, flavonoids) and natural agents (*e.g.*, metformin and melatonin) are less toxic in normal tissue and enhance cell death in tumours [11, 24, 25].

Sanguinarine (C20H14NO4) is a naturally occurring benzophenanthridine alkaloid found in the papaveraceae root of the plant Sanguinaria canadenisis L. and the seeds of Argemone mexicana L [26, 27]. Sanguinarine elicits anti-inflammatory and anti-bacterial activity [28 - 30]. Although recent data determined that Sanguinarine impedes hypoxia-induced tumor in breast and liver cancers [31, 32], resists hypoxia-induced stimulation of EphB4 [33], inhibits tumor metastasis, regulates epithelial to mesenchymal transition [34], and impairs lysosomal function by enhancing ROS dependent mitophagy in liver cancer [35]. Sang exhibits prominent inhibitory and anti-proliferation effects with significant inducing capability in LCC. So, inclusive outcomes of our investigation emphasized the pre-clinical significance of Sang in the *in-vitro* model. Further studies in the *in-vivo* model will be required to create pharmacokinetics and other effects of Sang so that it can be a probable applicant for future LCC therapy.

2. MATERIALS AND METHODS

2.1. Reagents

Sang (C20H14NO4, HSO26156198, 98% purity) and other

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high-grade reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). MTT, phosphate-buffered saline (PBS), dimethylsulphoxide (DMSO), trypsin, and RPMI-1640 were also obtained from Sigma Aldrich (St. Louis, MO, USA). FBS (Fetal Bovine Serum) was obtained from Excell Bio (Shanghai, China). The antibiotics penicillin and streptomycin were bought from Harbin Pharmaceutical (Shijiazhuang, China). Antibodies against the promoter of apoptosis Bax, the inhibitor of apoptosis Bcl2, Caspase-3 (ca-3, effector), Caspase-7 (ca-7, effector), and Caspase-9 (Ca-9, initiator) were procured from Protein Technology Group (Chicago, IL, USA). ECL and BCA test kits were bought from Pierce Biotech (Rockford, USA), and buffer RIPA lysis was obtained from Applygen Tech. (Beijing, China). Crystal violet was bought from Beijing Chemical Co. Ltd. (Beijing, China).

2.2. Cell Lines and Cell Culture

Human LCC lines A549, H1299, and H522 were used in this study and were all obtained from the Chinese Academy of Sciences (Beijing, China). A549 cells were grown in the F-12 medium, while RPMI-1640 medium was used for H522 and H1299 cells. All the mediums were used by adding 10% FBS and 1% penicillin (100 units/mL) and streptomycin (100 mg/mL). The cell lines were grown in an incubator at 37°C under 5% CO2. The respective cell culture medium was changed after every 24 to 48 hours of culture.

2.3. Cytotoxicity Assay

For cell viability, an MTT assay was carried out using H1299, A549, and H522 LC cell lines [36]. Cells were grown for 24 h in 96-well plates and were then incubated with several concentrations of Sang and further incubated for the next 48 h. Moreover, 5 mg/mL (100 µL) of MTT solution was added to each well and kept for 4-6 h in the incubator. Subsequently, the MTT solution was aspirated, 150 µL of DMSO was added to 96 plates, and the plate was kept for 15 minutes on a shaker. Optical density (OD) was observed spectrophotometrically at 490 nm in a microplate reader.

2.4. TCGA Database

The Cancer Genome Atlas (TCGA) (http://cancergenome .nih.gov/), publicly available, was used to determine the related gene expression of LC patients. We analyzed the mRNA expression of 59 non-tumor, 533 adenocarcinomas in LUAD, 49 non-tumor, and 502 squamous cell carcinoma LUSC data. A significant alteration was noticed after using a t-test GraphPad Prism (Prism 6.0, Graph Pad, La Jolla, CA, USA).

2.5. RT-PCR

Total RNA from LCC was isolated (Vazyme, Nanjing, China). Reverse transcription was performed as described [37]. The primer sequences for bcl2, bex, and β-actin are listed in Table **S1**. Relative expression of mRNA was normalized by β-actin.

2.6. Western Blotting

LCC lines were grown, and after 24 h, they were treated with Sanguinarine at different concentrations. The concentration of protein was measured by BSA protein assay (bio-rad, Hercules, Calif, USA) according to instructions of the manufacturer [34]. For immunoblotting, the same concentrations of protein were inserted into the SDS-polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (Schleicher and Schuell, Keene, N.H., USA). The blot was probed with targeted primary antibody overnight and incubated with secondary antibody for 1 h. The bands were visualized by enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL, USA). Tanon5200 imaging systems (Tanon, Shanghai, China) were used to take images. GAPDH was employed as a control protein.

2.7. Scratch Assay

Scratch assay was performed to determine the migration of LCC [38]. In 12-well plates, 3 × 105 cells/well were cultured until reaching 80% to 90% confluency. With the help of a 200 µL pipette, a monolayer was scratched, the medium was removed and rinsed with PBS to eliminate debris/residues, treated with Sanguinarine by adding the complete cultural medium and cultured for 48 hours. Cell migrations were monitored, and images were taken at a designated time under the inverted fluorescence microscope.

2.8. Transwell Migration Assay

Transwell chambers with 8 µm aperture (Transwell, Costar, UK) were used for migration assay [38]. The LCC lines were cultured for 48 h after treatment with Sanguinarine at different concentrations. The chamber medium was altered with the empty medium, and 30% FBS-containing medium was added for chemo-attractant. After 24 h of incubation, the cells sited on the chamber's upper surface (non-migrated) were distant with cotton swabs. The lower surface of the chamber, which contained the migrant cells and 4% paraformaldehyde, was used to fix the cells and crystal violet for about 15 min to stain it. Migrated cell images were taken by an inverted fluorescence microscope.

2.9. Flow Cytometry for Cell Cycle and Apoptosis Analysis

Cells were cultivated for 24 h and were treated with numerous concentrations of Sang for the next 24 h [17]. The apoptosis was examined using the FITC Annexin-V/DAPI apoptosis Kit, corresponding to the instruction of the manufacturer (BD BioSciences, San Jose, CA, USA).

Cells were cultured in 6-well plates and treated with the indicated concentrations of Sanguinarine for 48 h after serum starvation for 24 h. Then, the cells were harvested and fixed in 70% ice-cold ethanol at −20 °C overnight. After fixation, the cells were incubated with 1 mL RNase (50 µg/mL) for 30 min and stained with 1 mL PI (60 µg/ mL). The cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The obtained data were analyzed with Modfit LT software.

2.10. Colony Formation Assay

A total of 10 cells were seeded into each 12-well plate and incubated for 24 to 48 hours [39]. After that, the cells were treated at various dilutions and incubated for 5 to 7 days, but

the treated medium was changed after 48 h. The colonies were fixed with 4% paraformaldehyde and treated with 0.1% crystal violet solution for staining. The images were taken under an inverted fluorescence microscope (DM505, Nikon Co., Ltd., Otawara, Tochigi, Japan).

2.11. Hoechst Staining

The nuclear damage of Sang was observed by Hoechst staining [40]. Cells were plated in 96-well plates, followed by Sang treatment for 24 h and subsequently washed and stained with Hoechst 33258 (1 μg/mL) for 15 min and rinsed with 1X PBS. Blue fluorescent signals were imaged under the inverted fluorescence microscope in the dark.

2.12. ELISA

Cells in 24-well plates were seeded for 24 h [41]. Subsequently, the cells were exposed to Sang treatment and cultured for a further 24 h. Subsequently, the cells were analyzed for VEGF (Sino Biological, Beijing, China) by utilizing commercially accessible ELISA kits.

2.13. Immunofluorescence

Cells in 96-well plates were seeded for 24 h 96-well plate, followed by Sang treatment for the next 24 h. Then, the cells were aspirated and carefully washed twice with 1x PBS and subsequently fixed with paraformaldehyde solution (4%) for 15 min [42], followed by 30 minutes of incubation with 10% BSA to block the unspecific sites (epitopes). The primary antibody (Bcl2 and Bax diluted at 1:200) was incubated with the cells at 37°C for 4 h. Thereafter, the secondary corallite conjugated (diluted at 1:50) was incubated with the cells at 37°C for 1 hour. The nucleus was stained with DAPI (reference, see reagents section). Pictures were taken by applying an inverted fluorescence microscope.

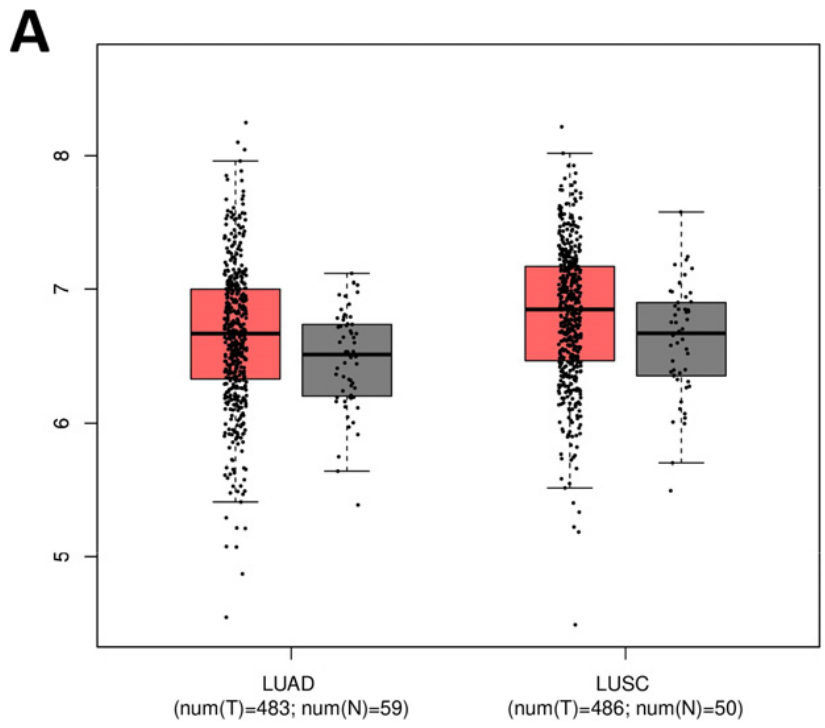
2.14. Statistical Analysis

Data are articulated as a mean ± standard deviation (SD). The statistical difference was analyzed by one-way ANOVA and unpaired student's t-test.

3. RESULTS

3.1. Apoptosis-associated Mediators Influence LCC Progression

The overexpression of Bax/Bcl2 correlated with poor prognosis in LC [43]. To check the progression of LCC, we analysed data from The Cancer Genome Atlas (TCGA) and found that Bax was overexpressed in LC tumor samples compared with adjuvant non-tumour (Fig. **1A** and **B**). High Bax, cas-3, cas-9, and PARP expressions were linked to unfavourable survival consequences in LCC. Besides, as shown in Fig. (**1C** and **D**), the different stages of cancer showed increased expression of Bax in LCC (Fig. **1E** and **F**). Significant changes were observed between Bax and Bcl-2 in transcripts per million (TPM) in both lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (Fig. **1G** and **H**). The correlation between Bax and Bcl2 is shown in Fig. (**1I**).



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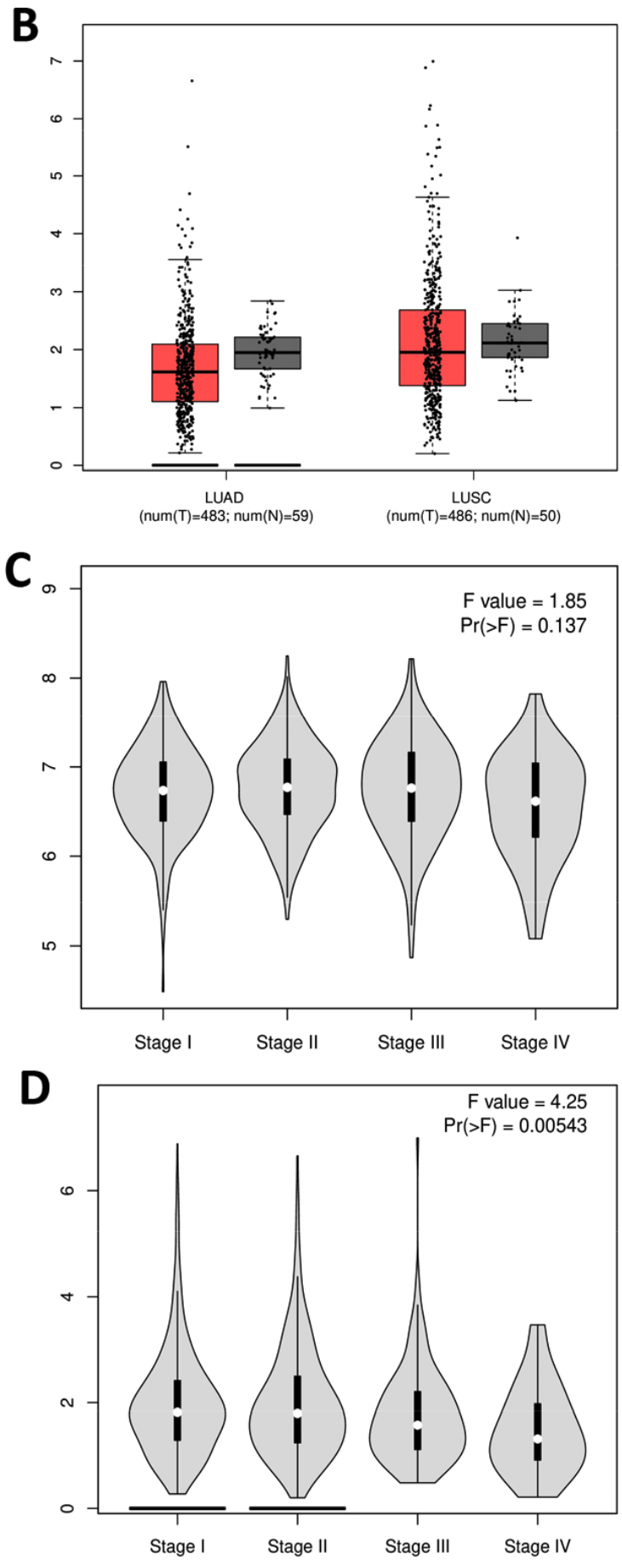
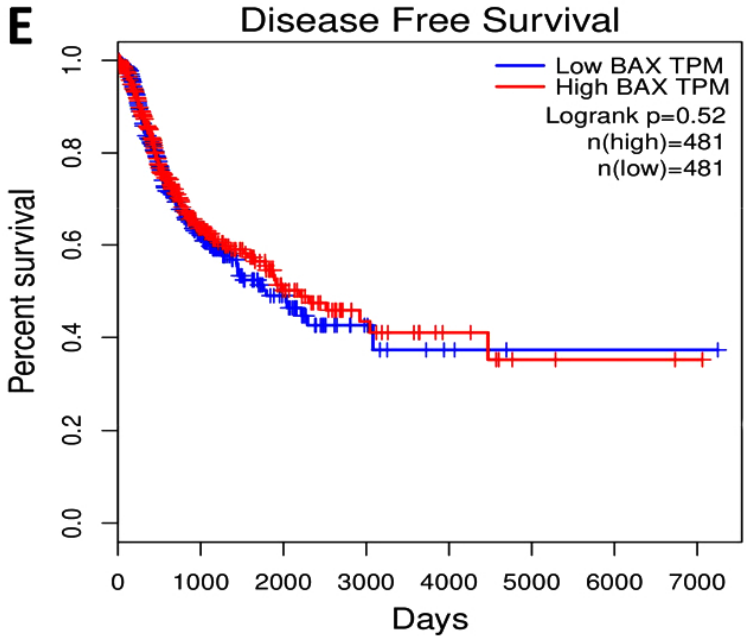
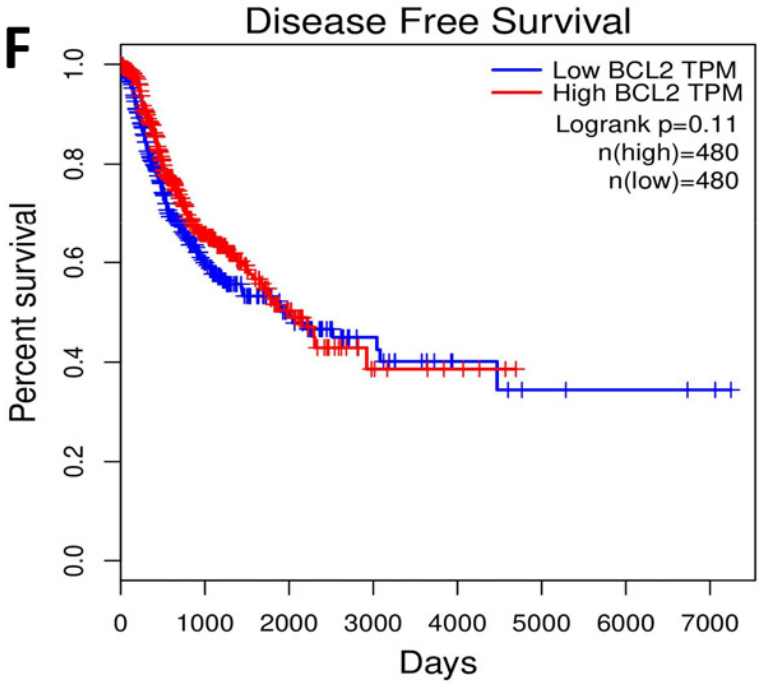

B
LUAD
(num(T)=483; num(N)=59)
LUSC
(num(T)=486; num(N)=50)
C
F value = 1.85
Pr(>F) = 0.137
Stage I
Stage II
Stage III
Stage IV
D
F value = 4.25
Pr(>F) = 0.00543
Stage I
Stage II
Stage III
Stage IV

Fig. 1 contd.....


E
Disease Free Survival
Low BAX TPM
High BAX TPM
Logrank p=0.52
n(high)=481
n(low)=481
Percent survival
0.0
0.2
0.4
0.6
0.8
1.0
0
1000
2000
3000
4000
5000
6000
7000
Days


F
Disease Free Survival
Low BCL2 TPM
High BCL2 TPM
Logrank p=0.11
n(high)=480
n(low)=480
Percent survival
0.0
0.2
0.4
0.6
0.8
1.0
0
1000
2000
3000
4000
5000
6000
7000
Days

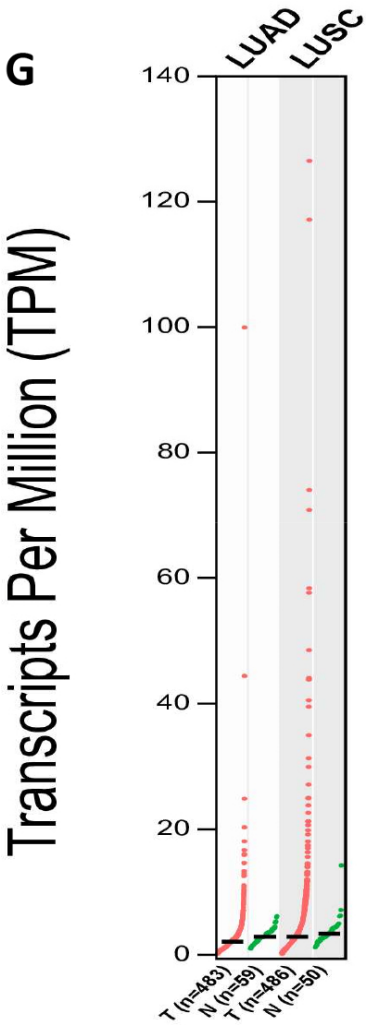

G
LUAD
LUSC
Transcripts Per Million (TPM)
0
20
40
60
80
100
120
140
T (n=483)
N (n=59)
T (n=486)
N (n=50)

Fig. 1 contd.....

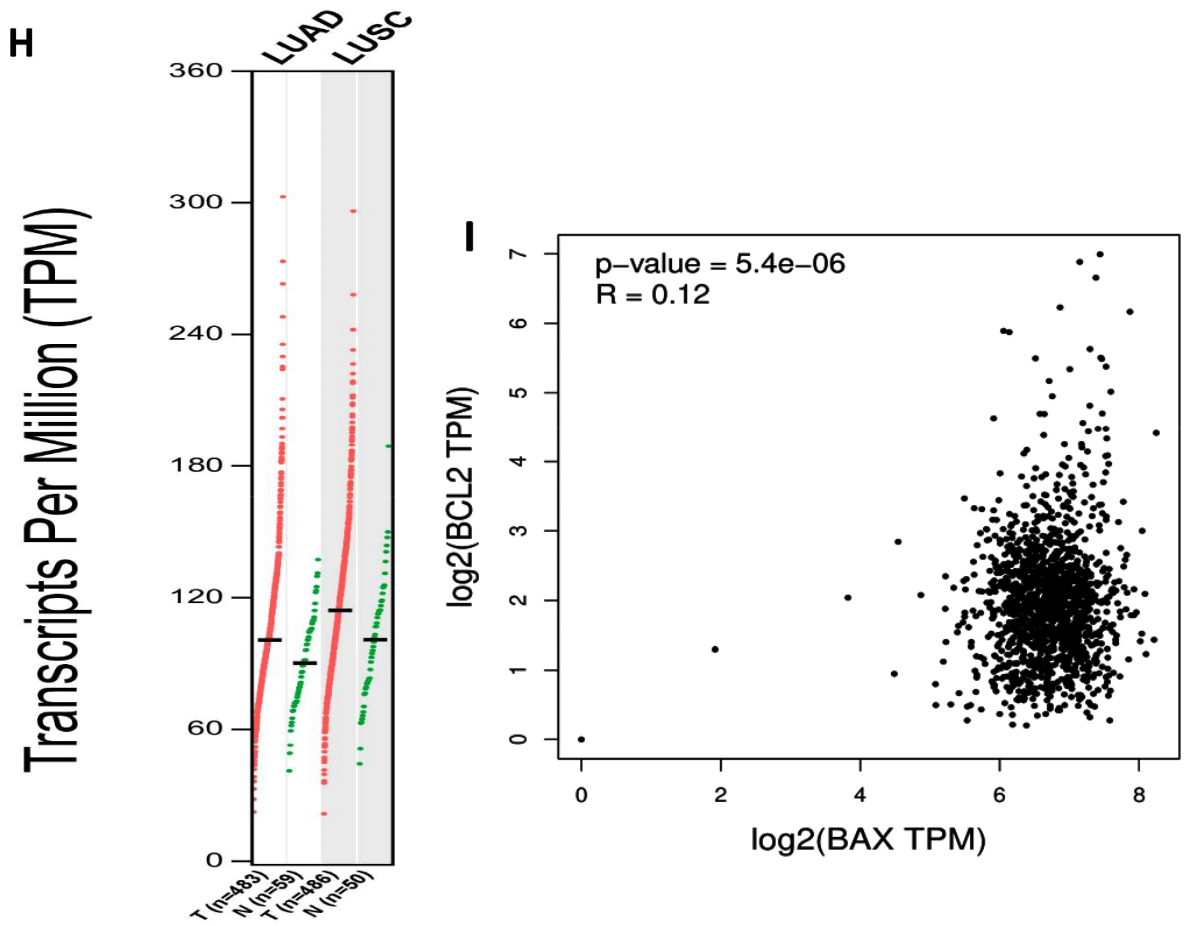


Fig. (1). BAX and BCL2 correlated with poor prognosis in LC. (**A**). BCL2 (**B**). BAX expression in LUAD, LUSC, and normal lung tissues based on GIPIA. (**C**, **D**). Data are represented as mean + SEM. Kaplan–Meier estimates of survival of patients with LC according to the BAX and BCL2 gene expression. Overall survival curve of the patients with high and low expression of LUAD and LUSC. The P-value was obtained from the log-rank test. (**E**, **F**). BAX and BCL2 expression in LC different stages. (**G**, **H**) the BAX and BCL2 expression in LUAD and LUSC compared to normal tissue with reference to transcripts per million (TPM). (**I**) The positive correlation between the expression of BCL2 and BAX.

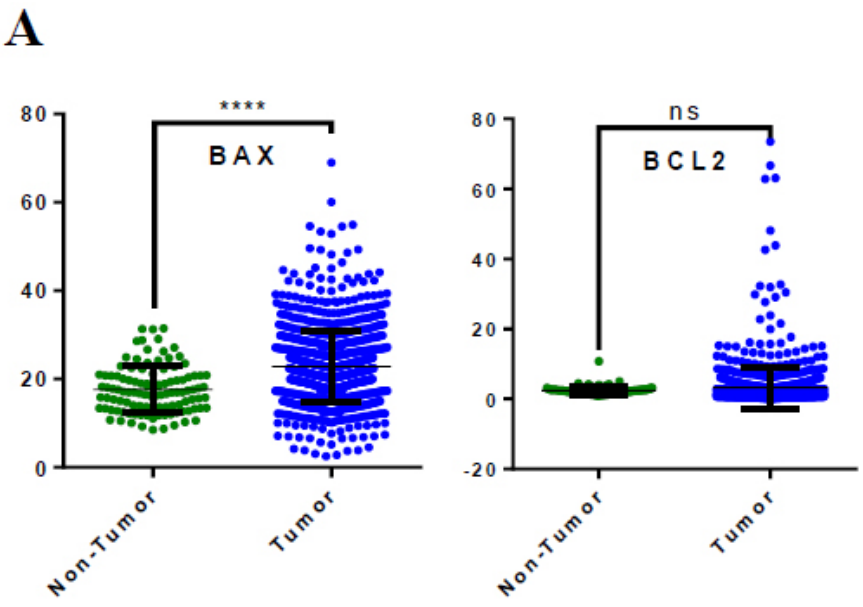
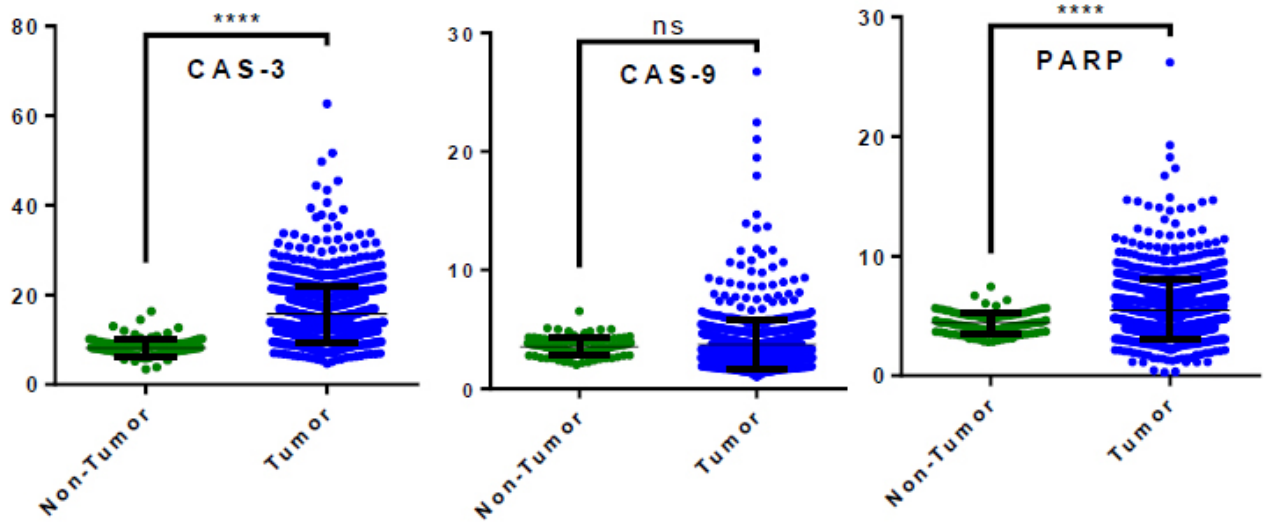
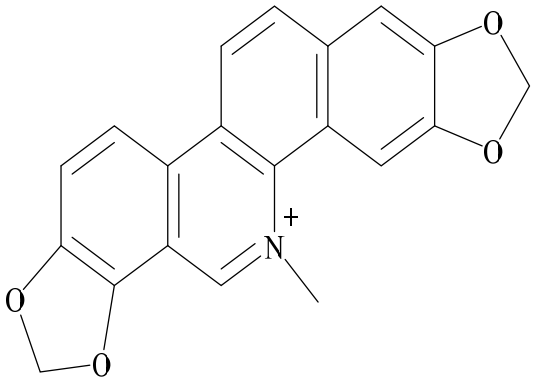


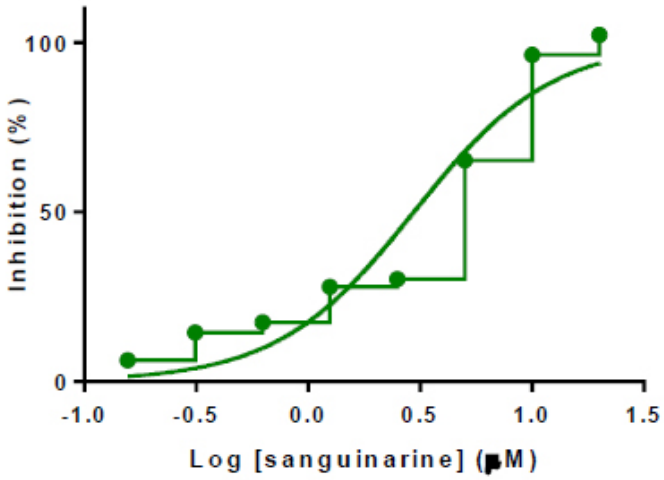
Fig. 2 contd.....





Cell	IC50(Mm)
A549	2.957
H522	3.520
H1299	2.128

C



A549

Fig. 2 contd.....

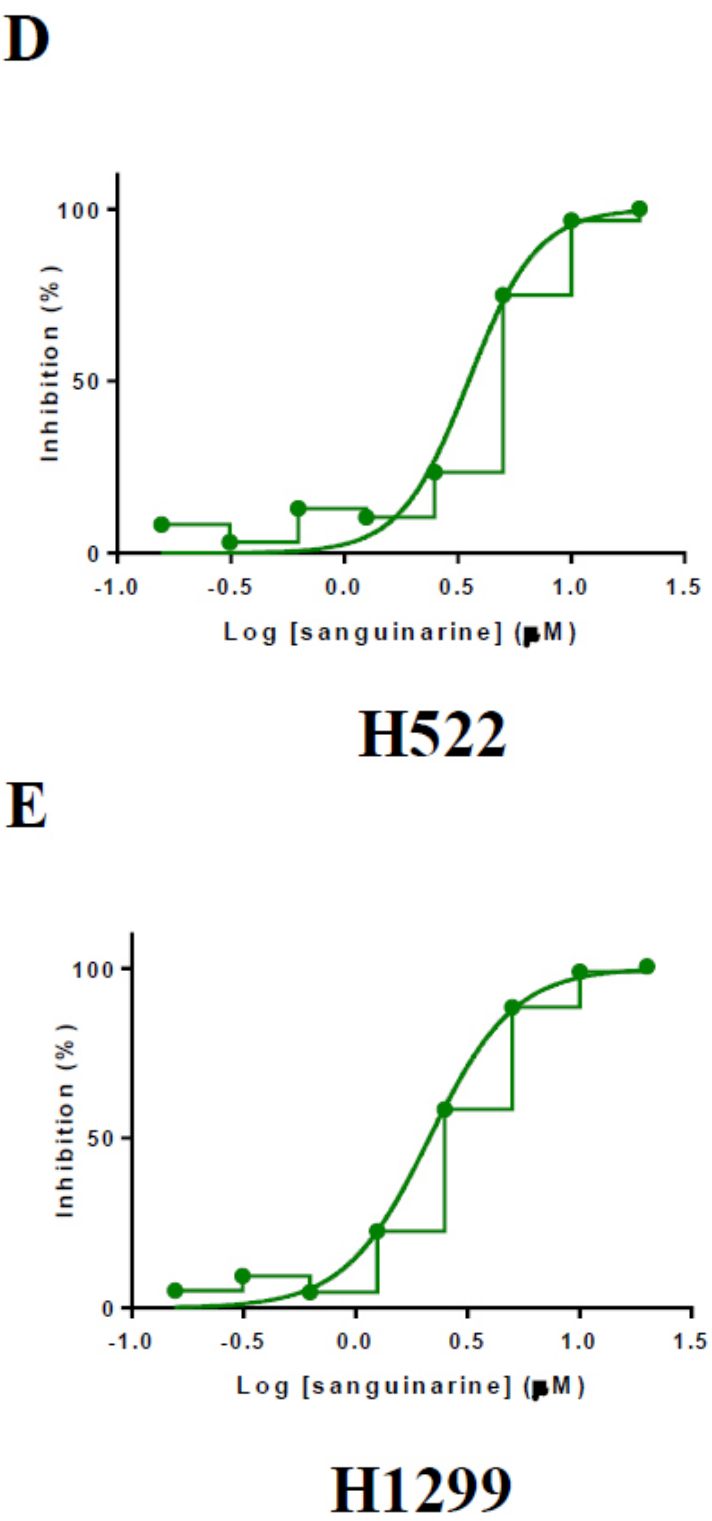


Fig. (2). Effect of SNG on cell proliferation in LC cell lines. (**A**). BAX, BCL2, CAS-3, CAS-9, and PARP expression in LC and normal liver tissues based on TCGA dataset (ns, non-significant, ****P < 0.0001). (**B**) sanguinarine chemical structure. (**C**-**E**) Sang inhibits the growth of LC cells. A549, H522 and H1299 cells were treated with increasing doses of SNG for 24 h, and cell proliferation assays were performed using MTT assay as mentioned under Materials and Methods. GraphPad Prism 7.01 (GraphPad Software, Inc.) was employed to compute nonlinear regression and the half-maximal inhibitory concentration of 50% (IC50).

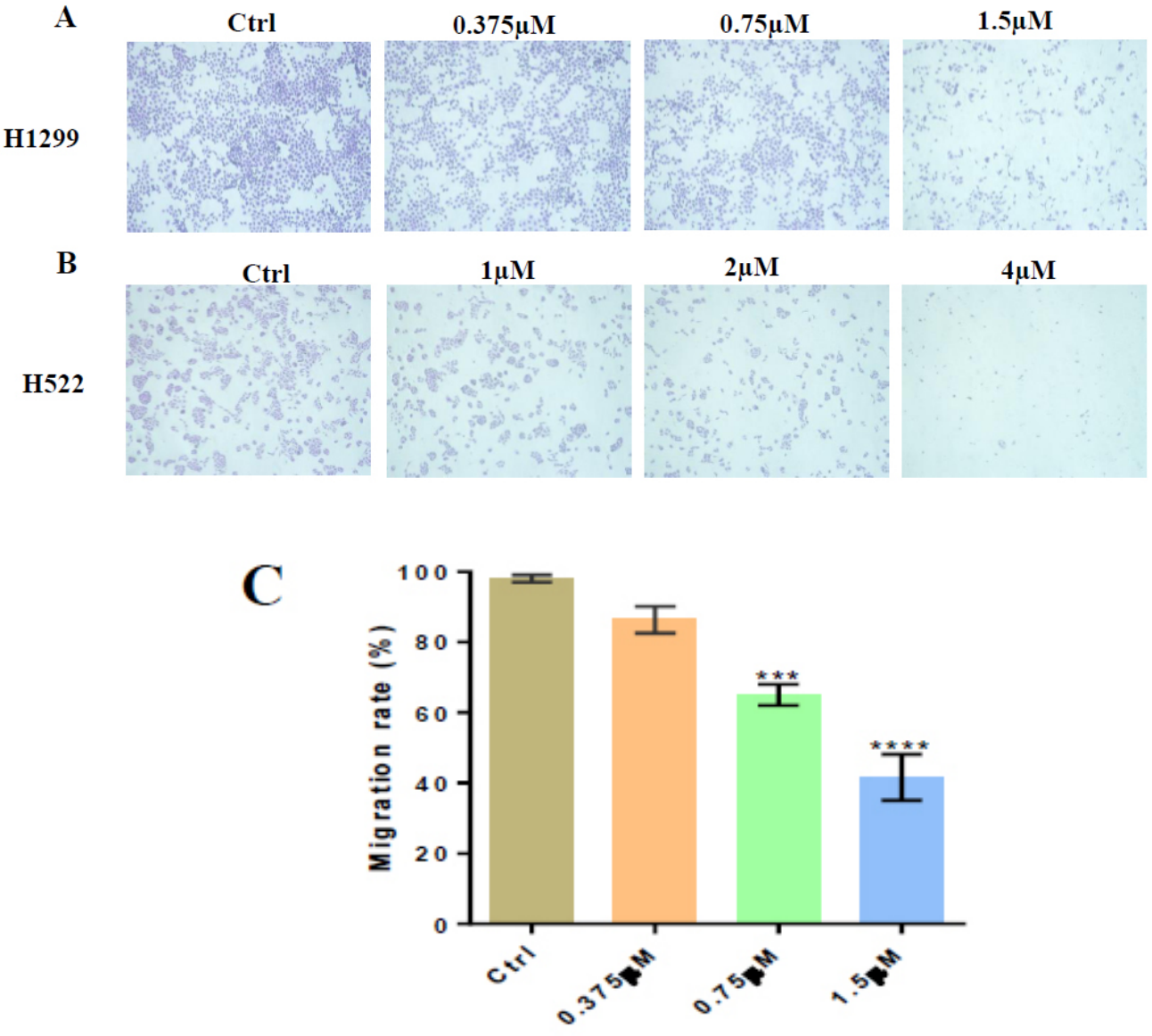
3.2. Sang Impedes LCC Proliferation

TCGA data confirmed that Bax is significantly highly expressed in 502 squamous lung cell carcinoma and 533 lung adenocarcinomas compared to non-tumour samples (Fig. **2A**).

Herein, we found that Sang (by using eight different concentrations: 20.0, 10.0, 5.0, 2.5, 1.25, 0.62. 0.31, and 0.16 µM) impedes the proliferation of LC cells (A549, H522, and H1299) in a dose-dependent manner, using MTT assay (Fig. **2B**). The IC50 values of Sang analysed in A549, H522, and H1299 cells were 2.957 µM, 3.520 µM, and 2.128 µM, respectively (Fig. **2C** - **E**).

3.3. Sang Induces Apoptosis in LCC

To evaluate the effect of Sang on apoptosis in LC, Sang-induced morphological changes of H522 and H1299 LCC were first analysed by microscopy (Fig. **3A** and **3B**, respectively) and then quantified (Fig. **3C** and **3D**, respectively). Sang could inhibit both LC cell lines in a concentration-dependent manner. In addition, Sang also limited the colony formation characteristics in H522 cells and H1299 cells (Fig. **3E** - **3H**). In a further step, to confirm apoptosis in LC H1299 and LC522 cells, and because tumour cell DNA condensed during this physio-pathological phenomenon, Hoechst staining was used [40]. We observed that the dye staining was stronger in function of the Sang concentration used for treating LCC. This strongly suggested and confirmed that apoptosis occurred in a Sang-dependent concentration manner (Fig. **4A** - **C**).



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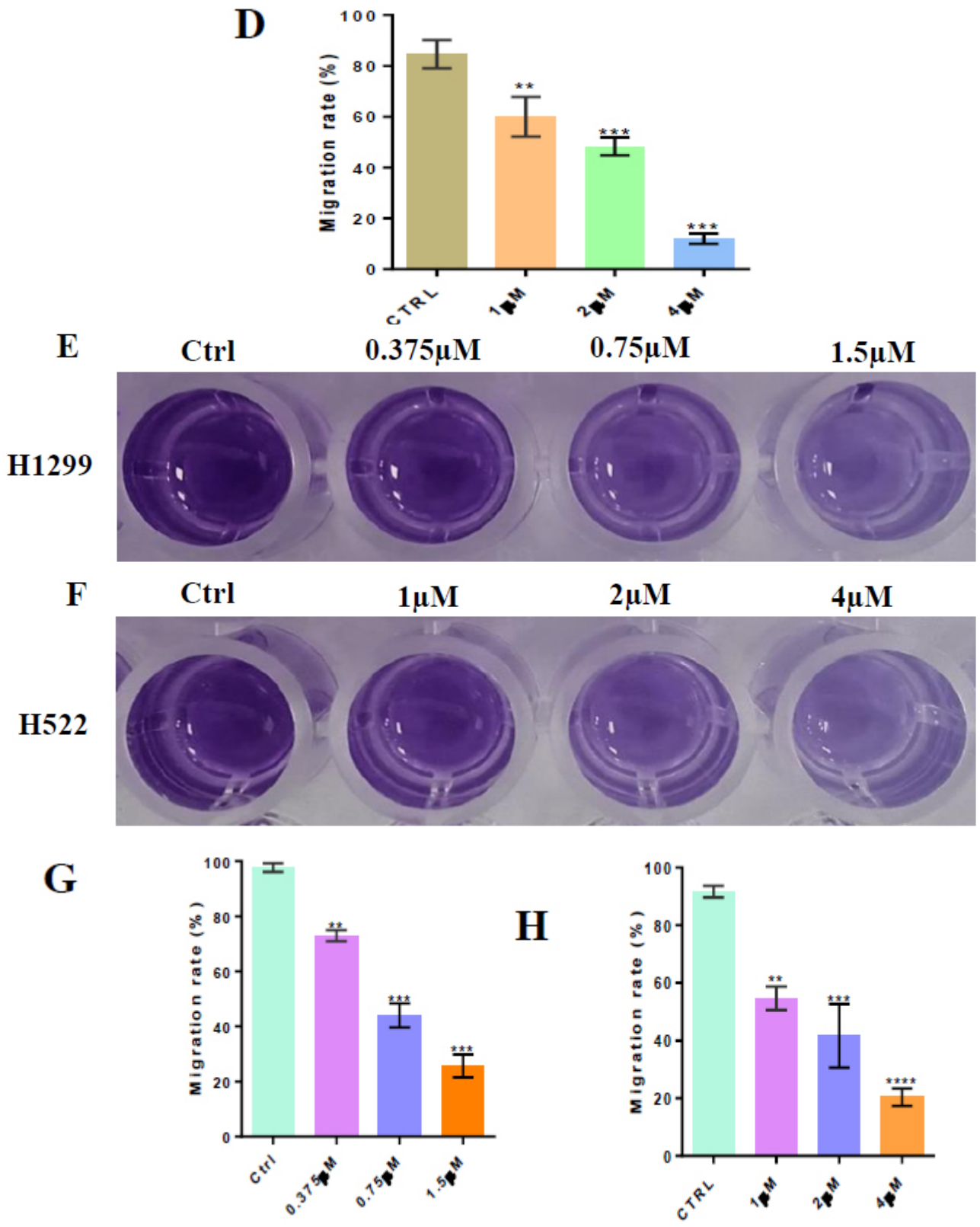
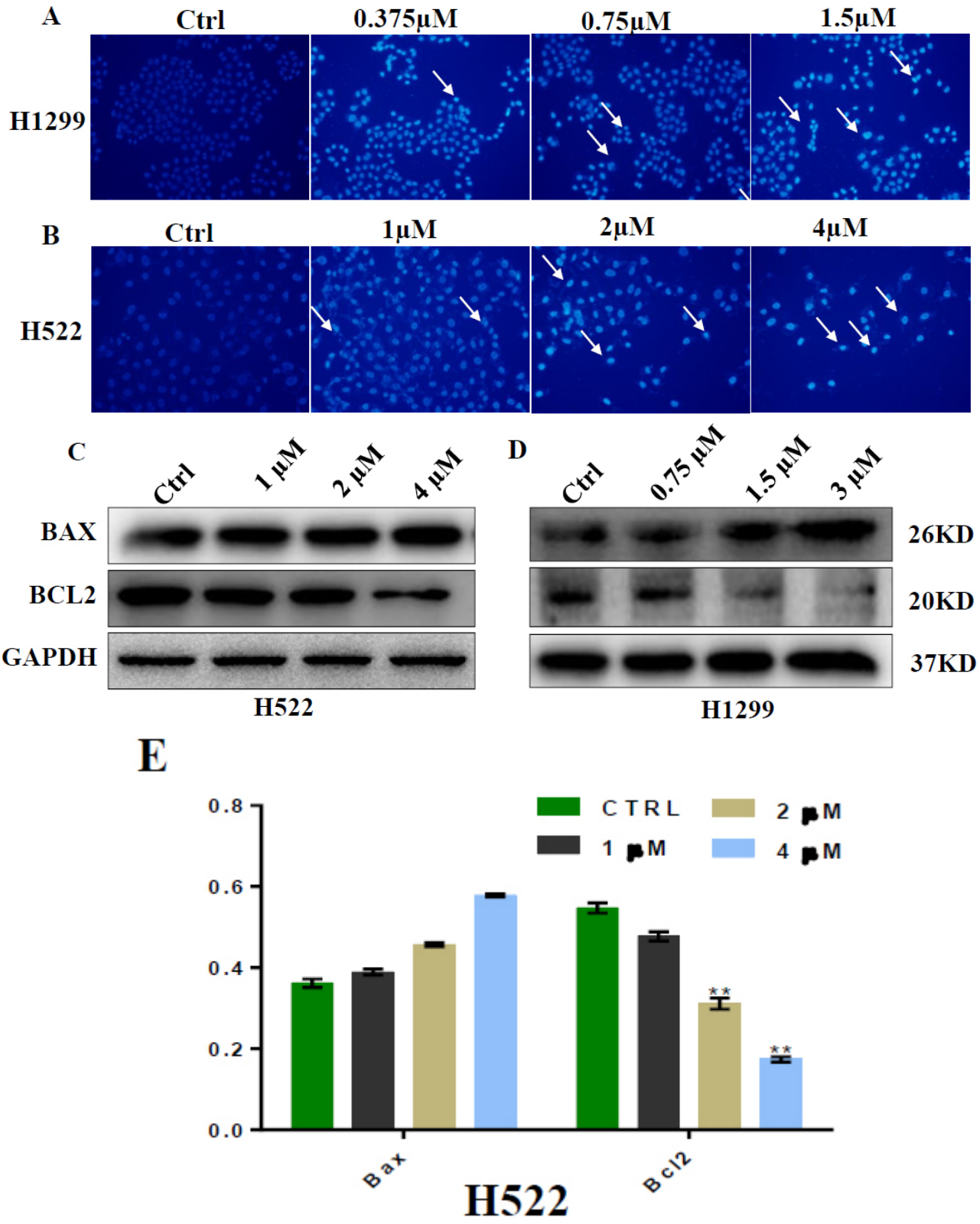


Fig. (3). Effect of SNG on lung cell morphology. (**A**, **B**). Lung cancer cell lines H1299 and H522 morphology were determined at various concentrations of treatment with Sanguinarine for 24 h, (**C**, **D**). Quantification of A and B, respectively. (**E**, **F**). Effect of Sanguinarine on the colony formation of H1299 and H522 cells. The colony formation (×200 magnification) was photographed, (**G**, **H**). Quantification of E and F, respectively. **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with untreated control cells.

Furthermore, the Sang-treated H1299 cells were analysed using flow cytometry, and the hypodiploid cell population was detected. At the highest concentration used, *i.e.*, 1.5 µM, Sang induced much more (about 12-fold) early apoptosis (58%) than late apoptosis (5%) in H1299 cells (Fig. **5A**); Sang induced much more (about 2-fold) early apoptosis (28%) than late apoptosis (14%) in H522 cells (Fig. **5B**). Thus, both LC cell lines have undergone apoptosis after treatment with Sang. Additionally, cell cycle analysis showed that Sang encouraged apoptosis and arresting cell cycle at the S (replication) phase in H1299 (Fig. **5C**) and H522 LC cell lines with 43% and 40% cells in the S phase, respectively (Fig. **5D**).



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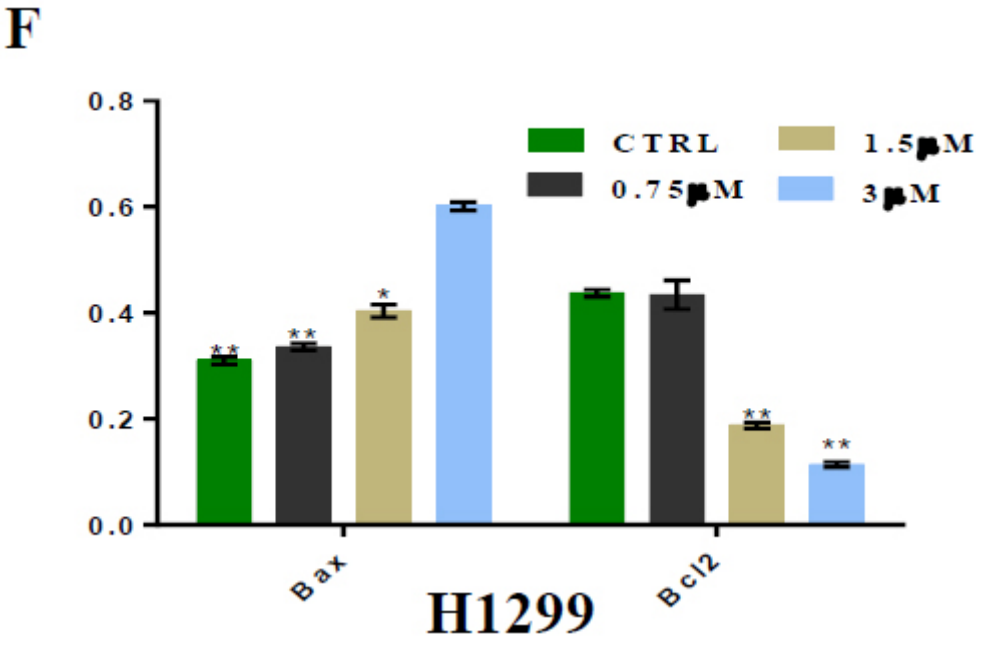


Fig. (4). Effect of Sang on apoptosis in LC cell lines. (**A**). (**B**) Hoechst staining pf H1299 and H522 at various concentrations, (**C**, **D**). Immunoblotting of apoptotic associated protein BAX and BCL2 expression in H522 and H1299 LC cells. (**E**, **F**) Quantification of (**C**, **D**). Quantification of (**E** and **F**), respectively. **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with untreated control cells.

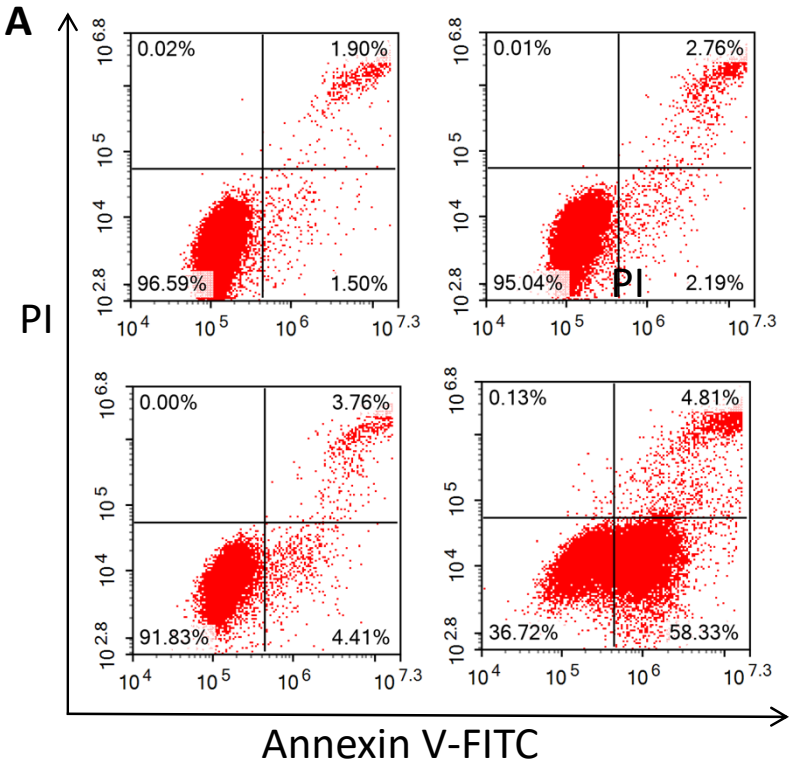
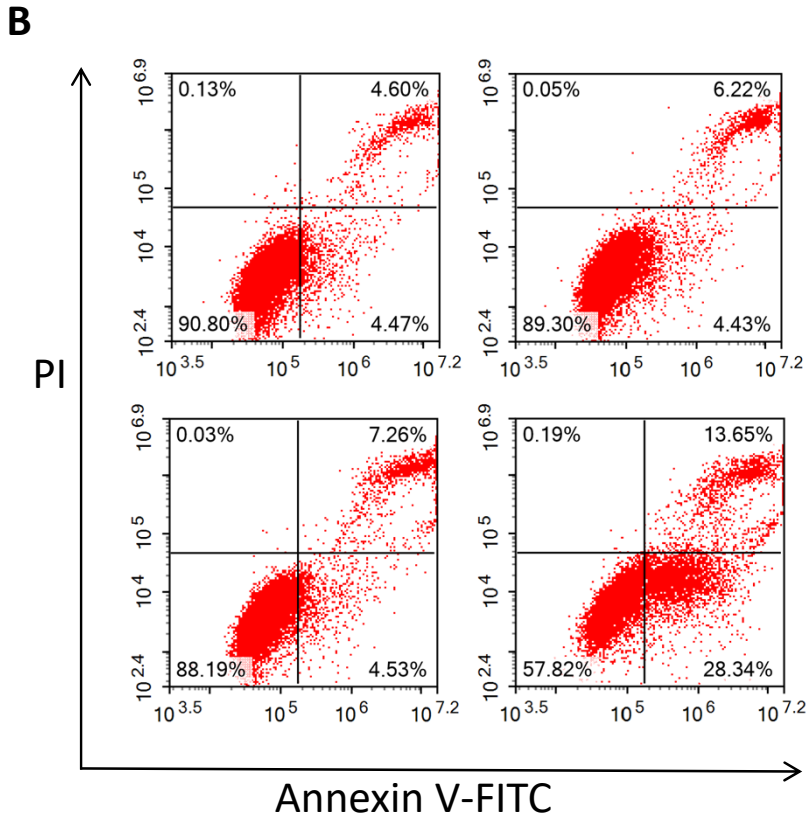


Fig. 5 contd.....


B
PI
Annexin V-FITC
0.13%
4.60%
90.80%
4.47%
0.05%
6.22%
89.30%
4.43%
0.03%
7.26%
88.19%
4.53%
0.19%
13.65%
57.82%
28.34%

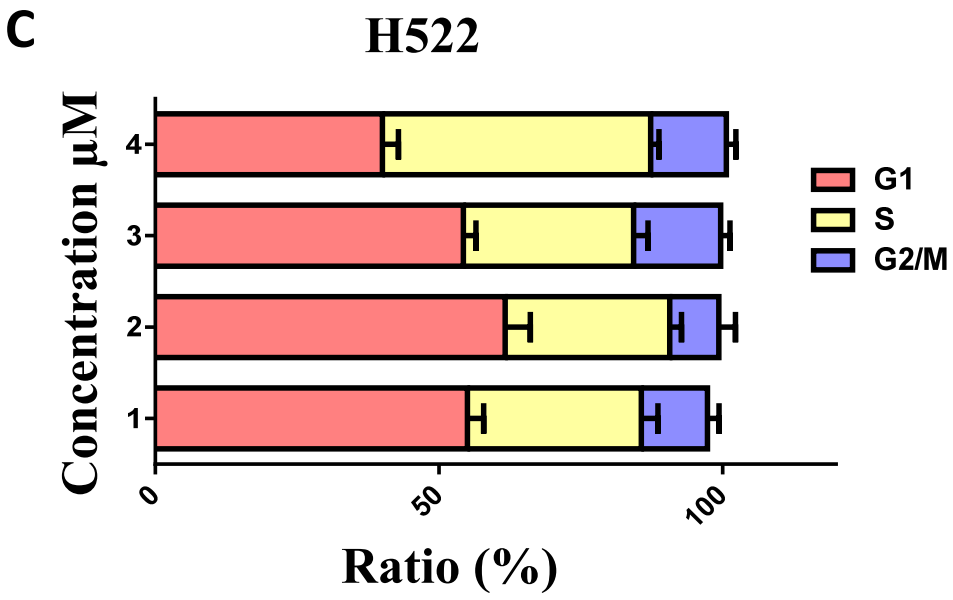

C
H522
Concentration µM
4
3
2
1
0
50
100
Ratio (%)
G1
S
G2/M

Fig. 5 contd.....

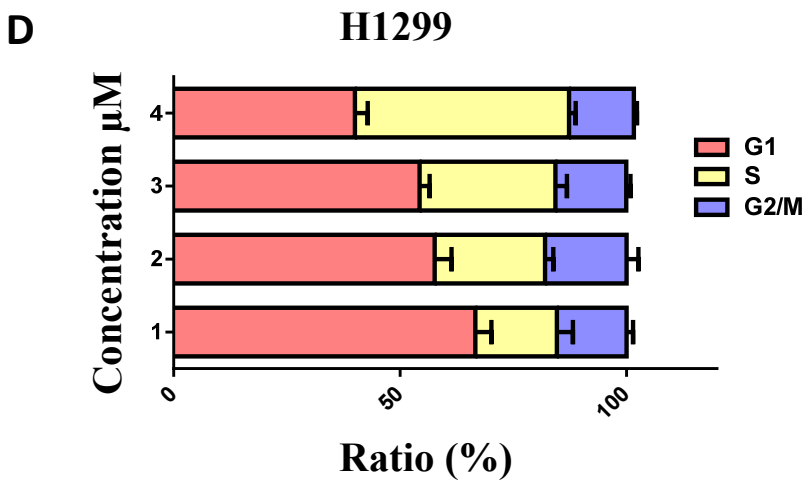


Fig. (5). Effect of Sang on apoptosis in LC cell lines. (**A**). Cell apoptosis was measured by Annexin V-FITC/PI staining in h460 and h1299 cells treated with Sanguinarine. The percentage of apoptotic cells is indicated. (**B**, **C**). Effect of Sanguinarine on H1299 and H522 cell cycle progression (**D**). Cells were treated with Sanguinarine for 48 h and stained with propidium iodide (PI). DNA content was then measured by flow cytometry. Data are represented as mean + SEM.

3.4. Sang Regulates Apoptosis-associated Proteins

Hematogenous metastasis is a key pathway of metastasis and invasion for LC. Presently, for antitumor treatment, anti-angiogenic therapy is the collective approach [44]. The development of tumour is angiogenic dependent. Blocking angiogenesis can impede tumor formation, while enhanced cell apoptosis associated with a reduction in angiogenesis for tumour remains non-angiogenic [45, 46]. Therefore, apoptosis-associated protein expression in the Sang-treated H522 cell line was analysed. At different concentrations of Sang treatment, in H522, it was found that Bax expression was enhanced and Bcl2 expression was decreased in a dose-dependent fashion. Furthermore, we determined apoptosis-associated caspase expression in Sang-treated H522 and H1299 cell lines. Western blot results confirmed that caspase-3, caspase-9 and PRAP expression were inhibited by Sang in H522 cells in a concentration manner.

Immunofluorescence assay for the H522 cell line also confirmed that Bax was highly expressed after treatment with Sang. Relative expression of Bax and Bcl2 genes showed that Sang at 3.0 µM for 24 h interfered with H522 cell apoptosis. Gene expressions of CAS-3, CAS-9, and PARP were inhibited during RT-PCR in Sang-treated groups.

3.5. Sang Inhibits Invasion and Migration in LCC

Amongst solid tumors, LC has a significant metastatic tendency; a greater part of LC patients were detected with metastatic disease [47]. LC is mostly detected during the last stage of the disease, and an elevated mortality rate is linked with metastasis [39]. Due to this reason, the mechanistic invasion and migratory capability of LCC are highly investigated to improve patients' prognosis and prevent metastasis [48, 49].

Herein, we investigated the effect of Sang on LCC migration using scratch and transwell assays. In scratch (migration) assay, Sang impedes the migration of H522 and H1299 cell lines in a concentration-dependent manner (Fig. **6A** and **6B**). We analysed the invasion of H522 and H1299 cell lines in Sang-treated cells using transwell assay at different concentrations for 48 h. Data showed that Sang inhibited the invasion of H522 in a concentration-dependent manner (Fig. **6C**, **D**).

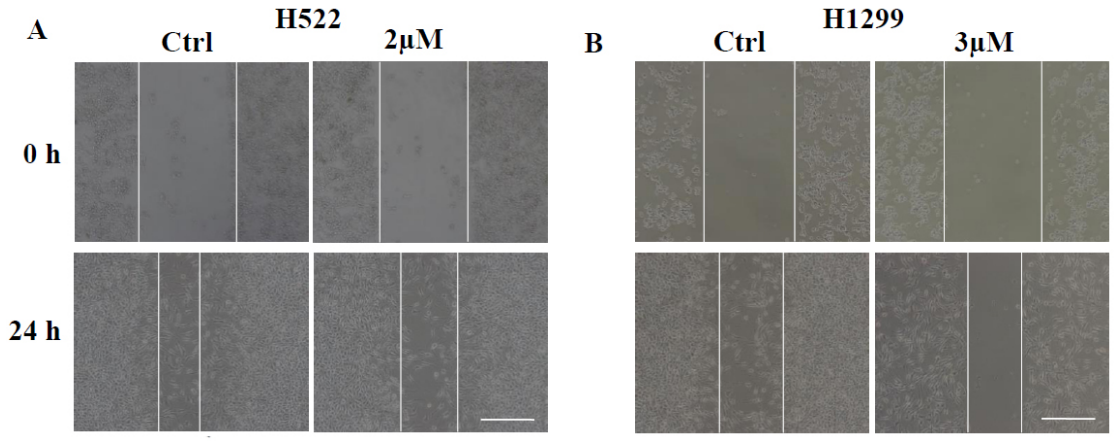


Fig. 6 contd.....

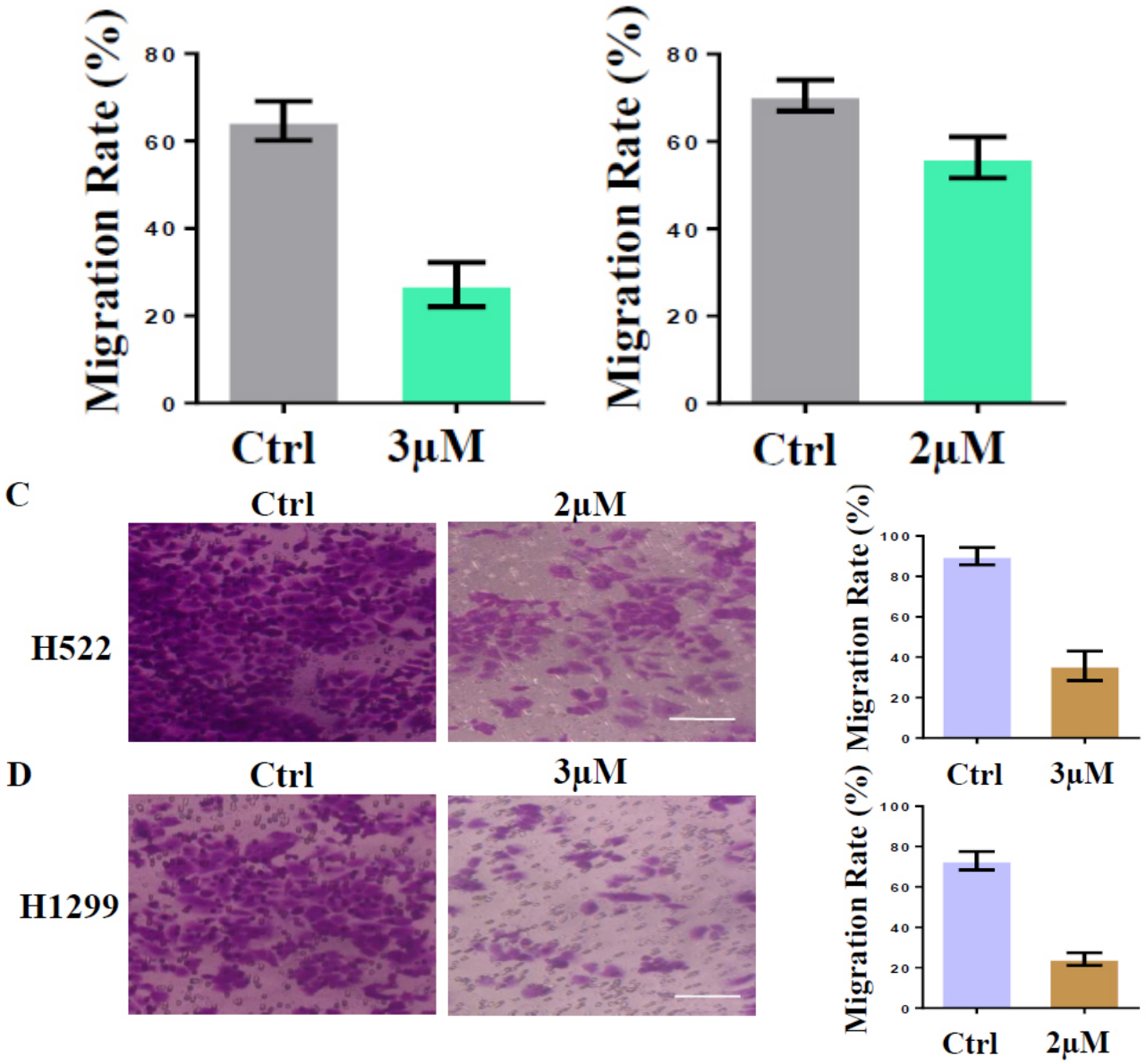


Fig. (6). Effect of Sang on lung cell migration and invasion. (**A**). (**B**) Scratch assay at various concentrations in H522 lung cancer cell lines at 24 h, (**C**). (**D**) Effects of Sanguinarine on the cell invasion assay in H522 cells (×200 magnification) were photographed. Right: Quantification of the data presented on the left. *P < 0.05, **P < 0.01, ***P < 0.001, compared with untreated control cells.

As angiogenesis is the pathway for metastasis and VEGF (Vascular Endothelial Growth Factor) is the main contributor to angiogenesis, we next checked the levels of VEGF expression in both Sang-treated H522 and H1299 cells using ELISA assay. We found that VEGF could significantly inhibit VEGF in H522 but not in H1299, indicating that Sang exerts an LC cell-specific metastatic activity.

Taken together, it was found that Sang can inhibit both LC cell migration and invasion in a concentration-dependent manner.

3.6. Sang Elevates ROS to Induce Apoptosis in LC

As confirmed by several research studies that ROS is involved in effecting the induction of apoptosis, we, therefore, examined the amount of intracellular ROS production, which was enhanced by Sang. To realize this, ROS enhancement was measured by DCFH-DA, which is an oxidative-sensitive dye. The results showed that 2 μM Sang treatment for 24 h enhanced ROS level compared to 1.0 μM and untreated group for 24 h of H522. These results showed that ROS is a critical factor for Sang-induced apoptosis in H522 cells (Fig. **7A - C**).

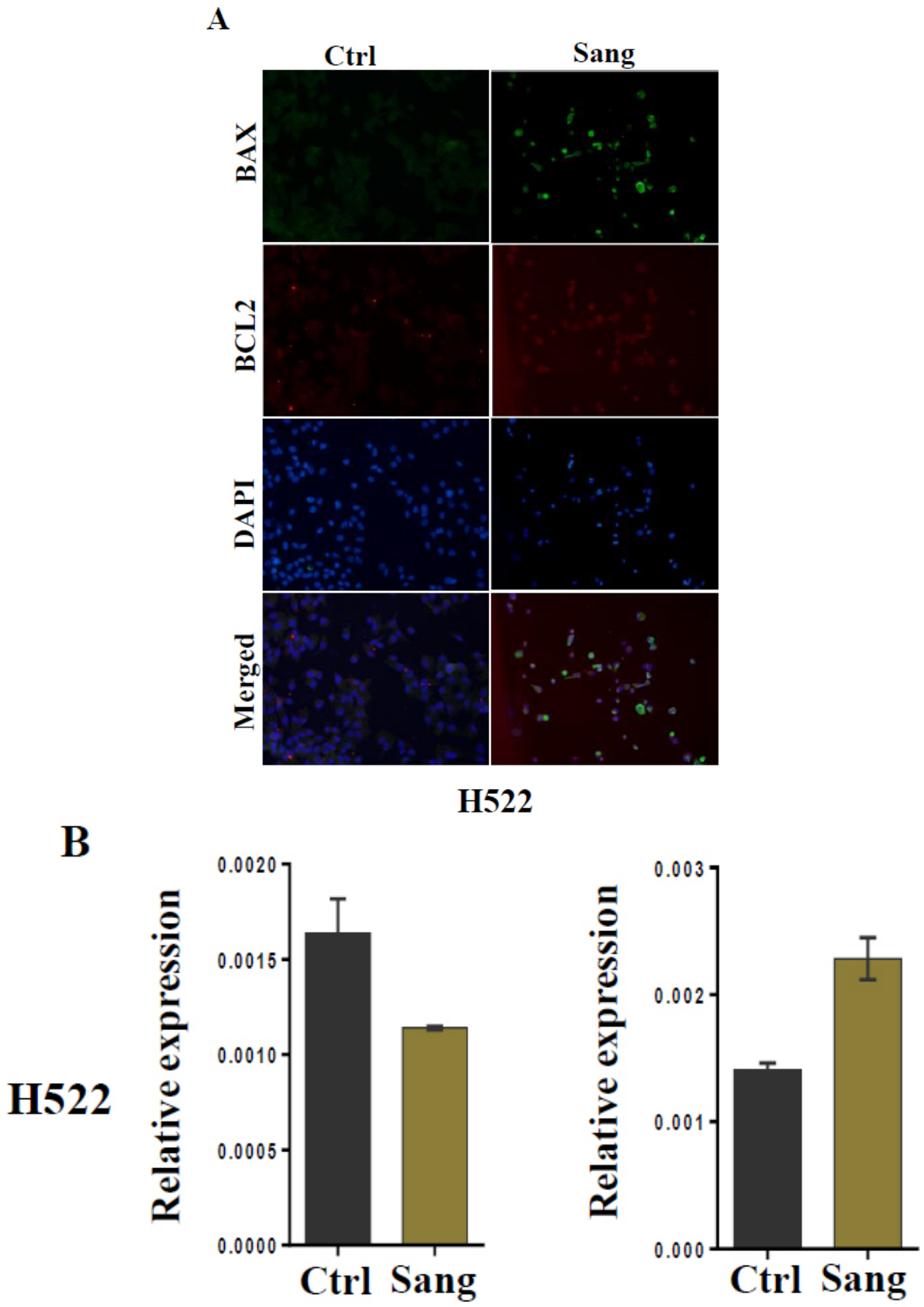

A
Ctrl
Sang
BAX
BCL2
DAPI
Merged
H522
B
H522
Relative expression
0.0020
0.0015
0.0010
0.0005
0.0000
Ctrl
Sang
Relative expression
0.003
0.002
0.001
0.000
Ctrl
Sang

Fig. 7 contd.....

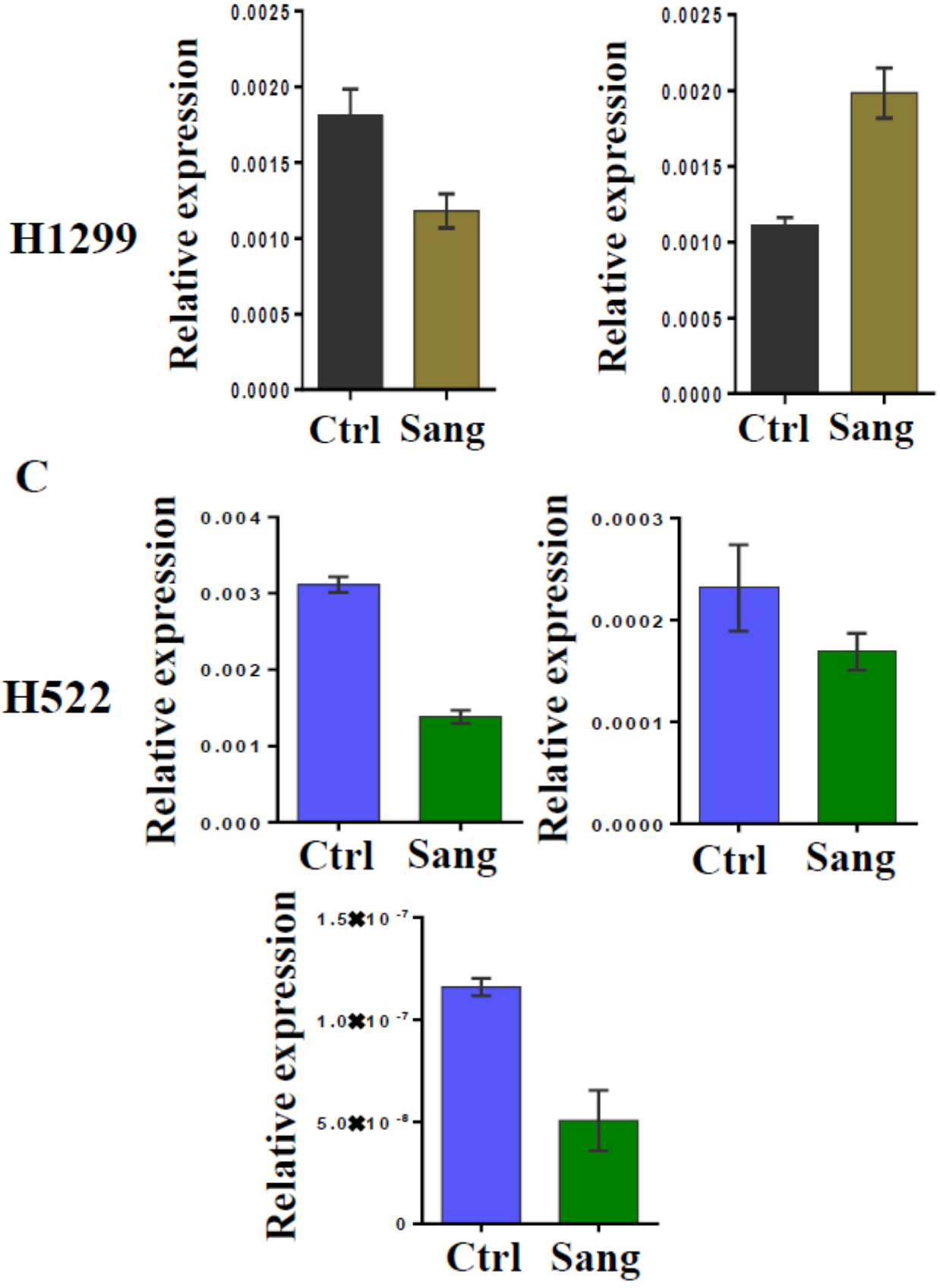

H1299
Relative expression
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Ctrl
Sang
Relative expression
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Ctrl
Sang
C
H522
Relative expression
0.004
0.003
0.002
0.001
0.000
Ctrl
Sang
Relative expression
0.0003
0.0002
0.0001
0.0000
Ctrl
Sang
Relative expression
1.5✖10 -7
1.0✖10 -7
5.0✖10 -8
0
Ctrl
Sang

Fig. 7 contd.....

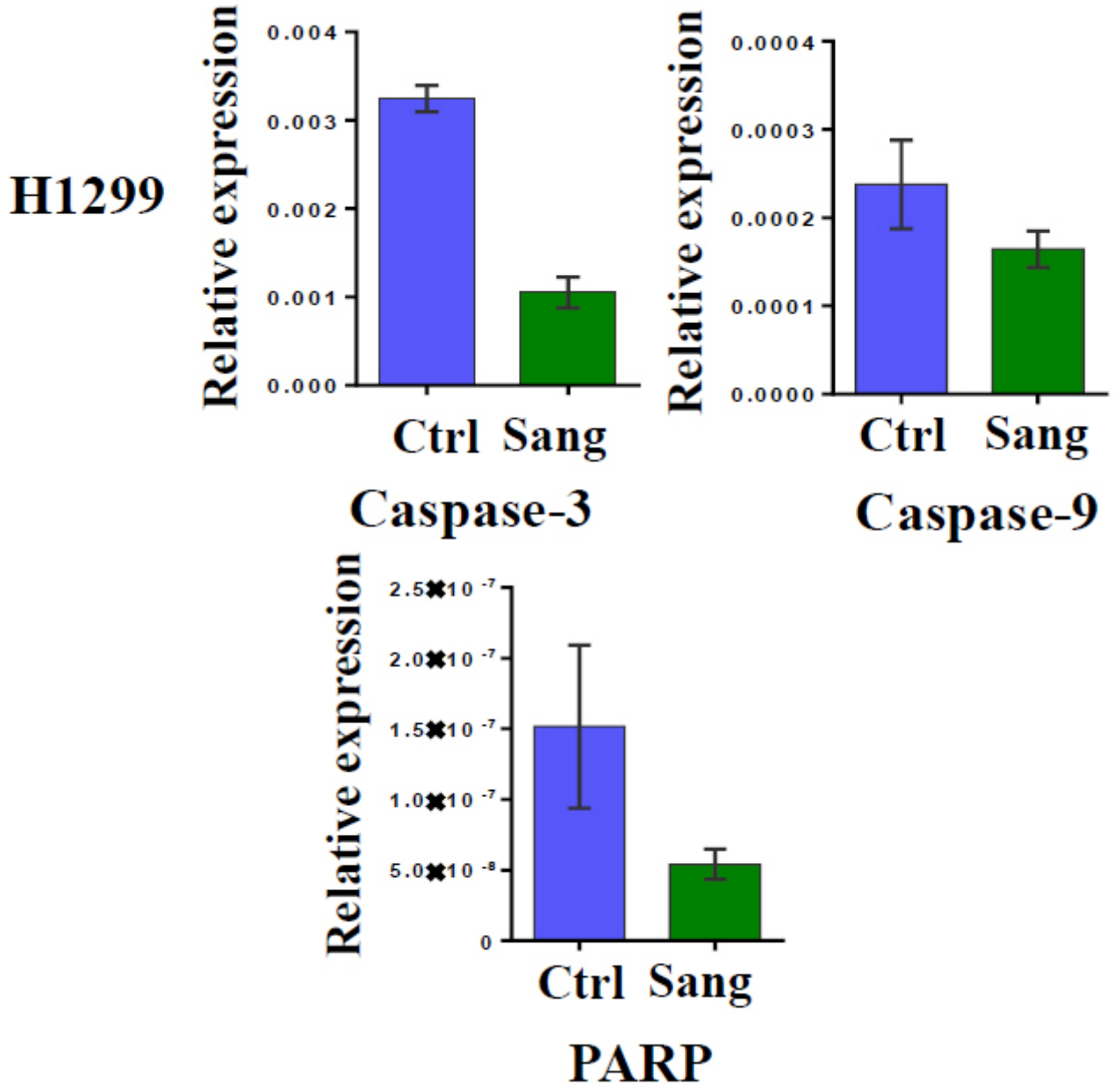


Fig. (7). Sanguinarine regulates BAX and BCL2-induced apoptosis in the H460 LC cell line *in vitro*. (**A**). H522 cells were treated with Sanguinarine for 24 h. BAX (green), BCL2 (red), DAPI (blue) staining, and 3-channel merged images indicated the translocation of BCL2 and BAX. Scale bars (×200 magnification), (**B**). Relative gene expression of BCL2 and BAX in H522 cell line, (**C**). Relative gene expression of CAS-3, CAS-9, and PARP in H522 cell lines.

4. DISCUSSION

The initial step of cancer progression and development is proliferation [50]. Proliferation is triggered by altered expression of the tumour-related protein. Regular activation of signal transduction pathways also activates cancer cell growth [51]. The formation of tumour is assumed to be associated with the obstruction of apoptosis after abnormal proliferation [52]. Low pro-apoptotic protein expression or anti-apoptotic protein overexpression can boost drug resistance and survival [53]. Such events can also be responsible for metastasis in LC [54]. In different types of tumour cells, Sang can induce cell apoptosis, but the cytotoxic effect and its underlying molecular mechanism in LC are poorly investigated (Fig. **8A** and **8B**).

In this study, we evaluated the impact of Sang on H522 and H1299 LC cell lines. Results determined that Sang-treated H522 and H1299 cell lines showed a markedly inhibitory effect by inducing apoptosis. Sang impedes cell development and presents a different therapeutic approach for the clinical treatment of LC by involving the caspase-dependent pathway. Natural compounds remain the option for treatment in patients with recurrent or advanced LC. However, long clinical studies need to recommend that the overall survival rate of LC has enhanced significantly (Fig. **8C - H**).

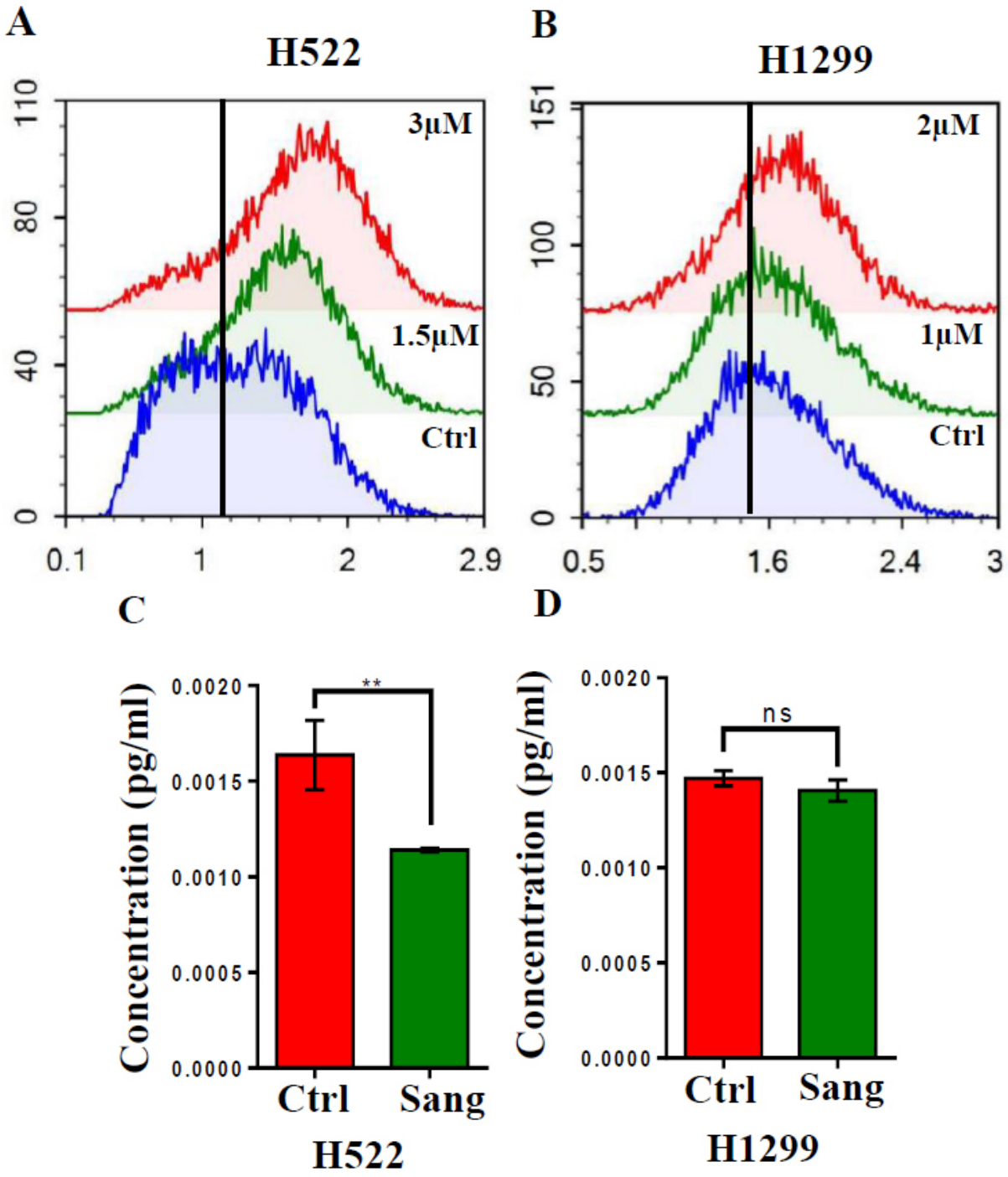

A
H522
3µM
1.5µM
Ctrl
110
80
40
0
0.1
1
2
2.9
B
H1299
2µM
1µM
Ctrl
151
100
50
0
0.5
1.6
2.4
3
C
Concentration (pg/ml)
0.0020
0.0015
0.0010
0.0005
0.0000
**
Ctrl
Sang
H522
D
Concentration (pg/ml)
0.0020
0.0015
0.0010
0.0005
0.0000
ns
Ctrl
Sang
H1299

Fig. 8 contd.....

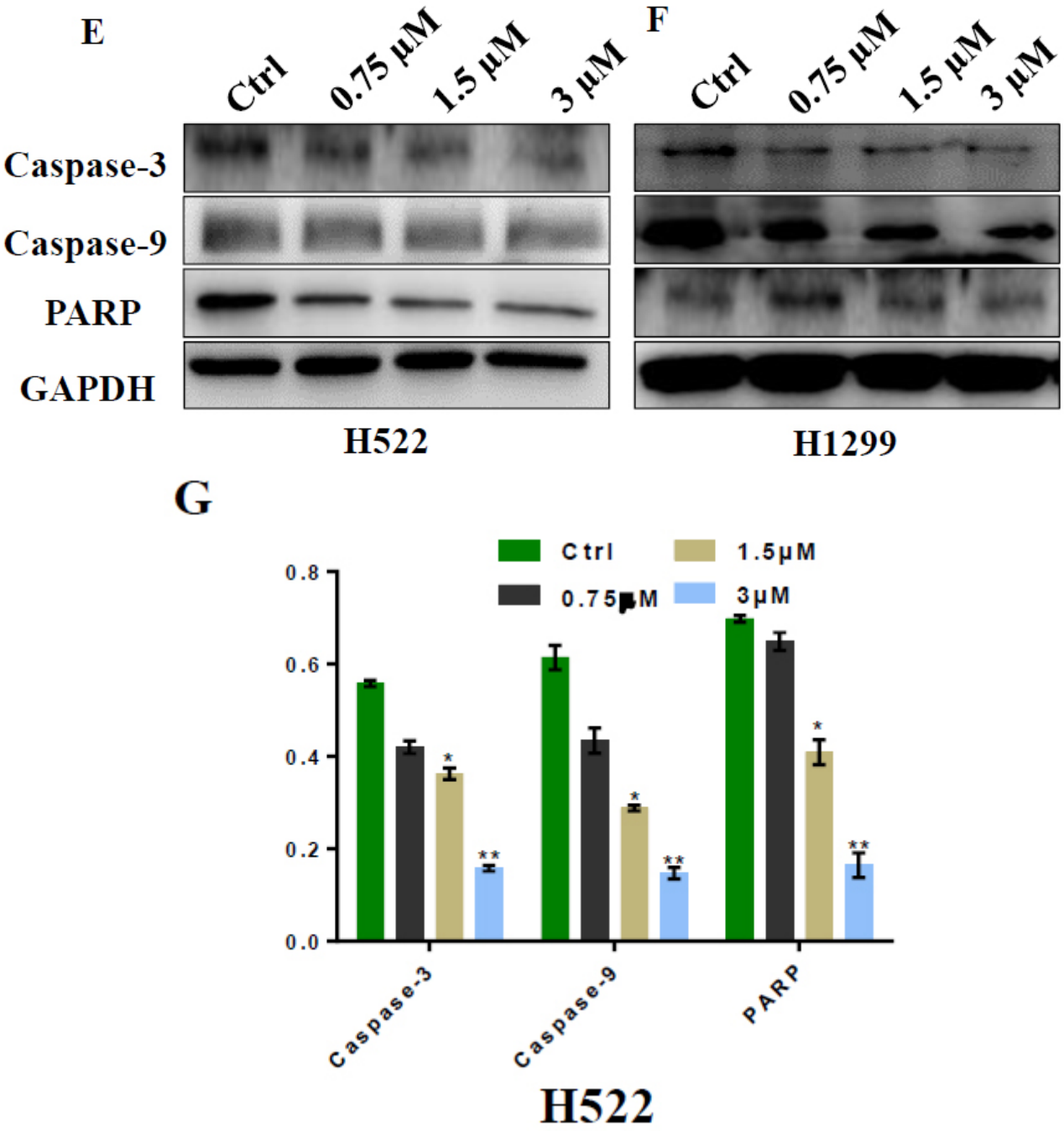

E
Ctrl
0.75 μM
1.5 μM
3 μM
Caspase-3
Caspase-9
PARP
GAPDH
H522
F
Ctrl
0.75 μM
1.5 μM
3 μM
H1299
G
Ctrl
1.5μM
0.75μM
3μM
0.8
0.6
0.4
0.2
0.0
Caspase-3
Caspase-9
PARP
H522

Fig. 8 contd.....

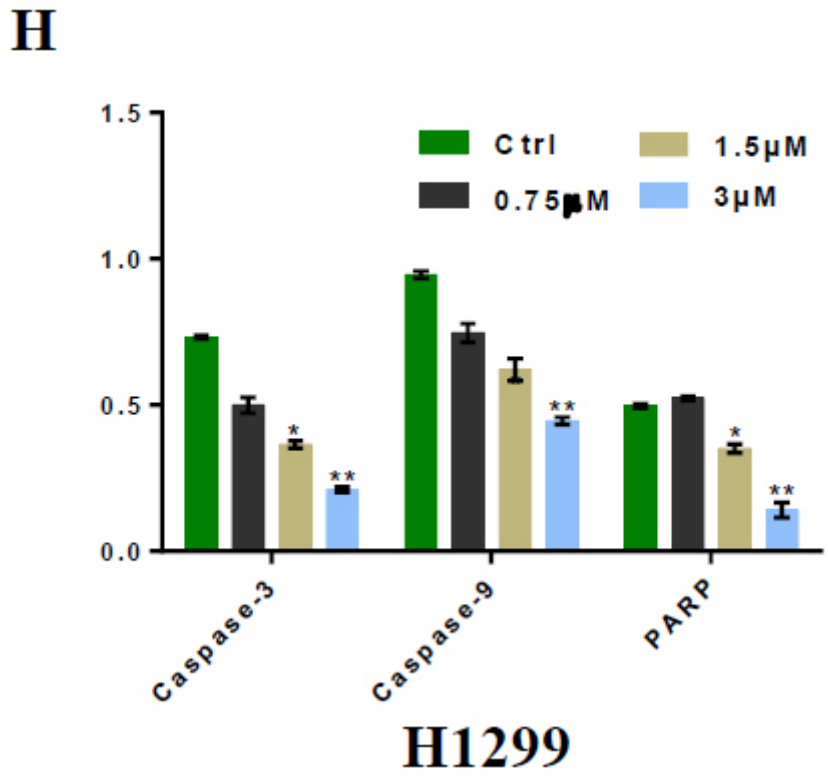


Fig. (8). Effects of Sang on intracellular ROS and VEGF in H522 LC cells *in-vitro*. (**A**). (**B**) ROS expression of Sanguinarine-treated H522 and H1299 cells at 1.5 µM and 3 µM and 2uM and 1 uM after 24 H of treatment, (**C**). (**D**) ELISA expression of VEGF after Sanguinarine treatment in H522 and H1299 cells, (**E**). (**F**) Immunoblotting of apoptosis-associated protein in H522 and H1299 at different concentrations (**G**). (**H**) Quantification of E. F. *P < 0.05, **P < 0.01, compared with untreated control cells.

Clinically, a large number of relapses occur in LC patients after first-line chemotherapy, and the toxic effect of chemotherapy is severe, which is linked to poor prognosis [55]. In chemotherapy treatment, Traditional Chinese Medicine (TCM) has gained a huge interest and attention recently [56 - 58]. Severely studies confirmed that Sang inhibits proliferation in various human cancer cells [57]. Our investigation endorses that Sang has a significant inhibitory influence on H1299 and H522 cell lines. Above mentioned investigation outcomes also supported the significant reduction in mRNA and protein expression of associated apoptosis markers. Apoptosis is a self-directed cell death that is involved in the expression, regulation, and activation of different proteins, which are regulated by genes. Clinically, cellular encouraging apoptosis is the imperative mechanism of chemotherapeutic agents for regulating tumour enlargement [59, 60]. Sang induces apoptosis in A549 cells by mediating the mitochondrial/caspase pathway [4]. We confirmed in other LC cell lines that Sang significantly induces apoptosis in both H522 and H1299 through PI/Annexin V-FITC staining. On the other side, Sang alleviated the permeability of mitochondria transition pores for the production of ROS, which ultimately directed to apoptosis. Usually, apoptosis is induced by death receptors. Moreover, stimulation of caspase-8 plays a key role in the apoptosis mechanism. Its key function is splitting and stimulating downstream caspase, including caspase-3 and caspase-9 and proapoptotic proteins, to encourage mitochondrial destruction and apoptosis [61].

Normal cellular processes produce ROS as a by-product, and it has been proposed that ROS elaborate in regulating the pathways and processes involved in the instigation of apoptotic induction. Hence, the constitutively raised levels of intracellular oxidative stress in ROS signaling make redox vulnerable to malignancy, which could be targeted by chemotherapeutic involvement by using redox modulators, where both pro-oxidant and antioxidant agents exhibit anticancer action [62]. The fluorescence microscope provides a visual way of observing the morphological changes. It has been observed that the H522 and H1299 treated cells with Sang induce apoptosis. The results confirmed that Sang inhibits cell proliferation and apoptotic induction, as proven by high apoptotic number and increased expression and activation of caspase-3 and caspase-7. The data demonstrated that Sang improves the anticancer effect by impeding proliferation and metastasis by inducing ROS-induced apoptosis in LC.

CONCLUSION

To conclude, the alkaloid Sang significantly impeded proliferation and encouraged apoptosis in LC in a concentration-dependent manner. Moreover, Sang reduced the expression of the anti-apoptotic protein Bcl-2 and enhanced the expression of the pro-apoptotic protein Bax in various LC cell lines. These studies highlight the potential use of Sang in LC treatment. However, more investigations are needed to discover the literal mechanism(s) of the responses induced by

Sang as well as to validate their efficacy in an animal model system. From these results, it has been confirmed that Sang inhibited LC *in vitro*. Therefore, it can be concluded that Sang holds a promising role in LC treatment.

AUTHORS' CONTRIBUTION

A.U, A.R, N.U, and H.I. contributed to conceptualization. A.U, S.S, T.U, M.Y.A, and S.E.E. contributed to selecting methodology. S.E.E, F.M., and M.Y.A gathered resources. A.U, T.A, H.I, and F.A participated in writing the original draft preparation. M.Y.A, S.E.E, H.I, T.A., and S.S contributed to writing the review and editing. A.U and H.I performed supervision. M.Y.A, S.E.E, and A.U participated in funding acquisition. All authors have read and agreed to the published version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used in this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Data that supports the findings of this study is available from the corresponding author upon reasonable request [A.U].

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the Publisher's website.

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