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Alpinia galanga induces caspase-dependent apoptotic cell death in human lung and cervical cancer cells

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ABSTRACT

Introduction: *Alpinia galanga* (L.) Willd is a rhizomatous, recurrent herb used to treat numerous diseases, including cancer. This study examines the efficiency of the different parts of *A. galanga* for their phytochemical, antioxidant, and anti-cancer properties.

Methods: The powdered leaf, stem, and rhizome of *A. galanga* were extracted using hexane, ethyl acetate and methanol; the phytochemical compositions of the extracts were characterized using high performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GC-MS). Their antioxidant potentials were evaluated using different assays, including 2,2-Diphenyl-1picrylhydrazil (DPPH), 2-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Superoxide anion (O2-), Hydroxyl radical (OH), and Phosphomolybdenum assays. The *in vitro* anticancer activity was studied using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Comet assay was used to determine the level of DNA damage. Inductions of morphological alterations were studied using the AO/Et-Br dual staining method. The ELISA kits were used to measure caspase activities.

Results: The cytotoxicity results showed that the various extracts had inhibitory and cytotoxic effects on cancer cell lines. The leaf hexane (LH) extract of *A. galanga* induced DNA damage with prominent increased tail length and tail moment followed by the induction of apoptotic cells and up-regulation of the caspase activities. HPLC and GC-MS analysis allowed the identification of bioactive compounds, recognized as apoptotic inducers in human cancer cells by activating caspase-dependent pathways.

Conclusion: This work reports for the first time the effect of LH extract of *A. galanga* in triggering apoptosis in A-549 and HeLa cells through the upregulation of caspase-8 and caspase-3 activities.

Implication for health policy/practice/research/medical education:
According to our research, the leaf hexane extract of *Alpinia galanga* has strong and safe anti-cancer effects with negligible cytotoxicity to normal cells, suggesting that the extract has great potential for use in the future and may be used as a source of compounds for medication development.

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Introduction

Alpinia galanga (L.) Willd is a recurring, rhizomatous herb used medically to cure a wide range of illnesses. It is also known in English as Greater Gangal (1) and grows naturally in woods and open warm areas in Indonesia (2). In India, it is cultivated in the Western Ghats and grows along the southwest India and eastern Himalayas (3). As a well-known traditional Chinese medicine, *A. galanga* is frequently prescribed for gastrointestinal disorders like stomach aches, dyspepsia, ulcers, diabetes, heart disease,

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kidney disease, and gastro-frigid vomiting, as well as for tumors, microbial infections, inflammations, and HIV (4,5). *A. galanga* has been demonstrated in numerous studies to possess a wide range of pharmacological properties, including anti-microbial, anti-tumor, anti-allergic, antiviral, anti-inflammatory, and gastro-protective properties (6,7). While the rhizome is the primary focus of the Zingiberaceae family scientific research, certain studies also indicate that the stem and leaves have strong pharmacological properties (8,9). According to Chan et al (10), the ferrous ion-chelating activity of *A. galanga* leaf extract is 20 times higher than that of rhizome extracts.

Apoptosis eliminates malignant cells while sparing surrounding normal cells and tissues, therefore compounds that can kill cancer cells through this process are very desirable (11). The ability of natural compounds with anti-tumor capabilities to eradicate cancer cells and reinstate medication sensitivity implies the potential benefits of natural compounds in cancer chemotherapy and radiation therapy (12,13).

As the second most deadly disease globally, cancer poses a serious threat to public health, accounting for over 190 000 cases and over 60 000 deaths from the disease in 2021. It is projected that the number of cancers reports in India will increase from 14 lakhs in 2022 to 15 lakhs in 2025, as per the Indian Council of Medical Research - National Cancer Registry Programme (ICMR-NCRP) report (14). In 2020, there were around 139 000 cases of cancer in India (15). According to reports from the National Centre for Disease Informatics and Research (NCDIR) and ICMR, Aizawl (the capital of Mizoram) has the highest rates of stomach cancer and lung cancer in Asia (15).

The literature survey revealed that no prior work was done to assess the anti-cancer activity and phytochemicals in the leaf, stem, and rhizome of *A. galanga* from a high-altitude mountainous tropical wet evergreen forest in Mizoram. The genotype, environmental adaptations (such as the presence of minerals in the growth locale), and geographic origin all have a significant impact on the type and quantity of bioactive anti-cancer compounds that are present in each plant organ (16,17). Since, Mizoram is afflicted with cancer, the current study was centered on characterizing the anticancer potential of *A. galanga*, which is a part of the dietary intake of the Mizo people.

Materials and Methods

Collection and extraction

The healthy leaf, stem, and rhizome of *A. galanga* L. (Willd) were collected during the dry season from Bairabi (24.185° N, 92.537° E), Mizoram, India. The specimen was identified by Prof. H. Lalramnghinglova, Dept. of Environmental Science, Mizoram University. The dried plant materials were sequentially extracted with hexane, ethyl acetate, and methanol using the maceration method and filtered and dried using a rotary evaporator. An herbarium specimen was placed in the Mizoram University (voucher number: MZUEVS29520) and Botanical Survey of India, B.S.I (E.R.C):133416 repositories.

Cell lines and culture medium

A normal cell (L-132) and two cancer cell lines of A-549 and HeLa were acquired from the National Centre for Cell Science in Pune, India. The culture medium used for stock cells included 10% inactivated fetal bovine serum (FBS), 100 µg streptomycin, 100 IU/mL penicillin, and 5 µg of amphotericin B per milliliter. The cells were kept at 37 °C in a humidified atmosphere with 5% CO_2 until confluent. The cells were separated using a trypsin solution (0.2 percent trypsin, 0.02% EDTA, and 0.05 percent glucose in PBS). Each experiment was conducted in 96 microtitre plates and the stock cultures were grown in 25 cm^2 culture flasks (Tarsons India Pvt. Ltd., India).

Phytochemical analysis

The qualitative phytochemical screening was done through standard protocols (18,19). The quantitative analysis was done for flavonoid (20), phenol (21), and alkaloid (22) contents and the results were shown as quercetin, gallic acid, and atropine equivalents, respectively.

Antioxidant activity

The antioxidant activity of the various parts of *A. galanga* was done as per our previous work (13) using different assays: DPPH (23); ABTS (24); Superoxide anion (25); Hydroxyl radical (26); Phosphomolybdenum assay (27). The results were calculated with standard reference - ascorbic acid equivalent.

Determination of the most cytotoxic extract

The selection of the extract having the highest cytotoxic result was done by MTT assay. The leaf hexane extract of *A. galanga* revealed the lowest IC_{50} value and the further experiments were carried out after separation of the cell cultures into three groups:

- MEM group: The cells remained untreated and served as the negative control.
- Dox group: Treated with IC_{50} concentration of doxorubicin (positive control).
- Treated group: Treated with IC_{50} concentration of leaf hexane extract (LH).

MTT assay

The cytotoxic activity of *A. galanga* against A-549, HeLa, AGS, HT-29 cell lines, and normal cell line (L-132) was assessed by MTT assay (28). Briefly, 10^4 cells were seeded in 96 well plates and retained in a CO_2 incubator (37°C, 5% CO_2, 95% humidified air) for 24 hours. Doxorubicin and the different extracts with the concentrations of 5, 10, 20, 40, 80, and 100 µg/mL were supplemented in each well. 20 µl of MTT was added after 72 hours of cell plating followed by incubation of 2 hours. The insoluble purple

formazan was dissolved with DMSO after removal of media followed by 4 hours of incubation. The absorbance reading was taken at 560 nm.

Comet assay

The HeLa and A-549 cells were allowed to adhere in 6-well plates and treated with the IC_{50} concentration of LH (13.2 µg/mL) for 24 hours, followed by trypsinization and centrifugation of the cells (29). After discarding the supernatant, the cells were amalgamated with 0.7% of low melting agar and affixed on slides coated with 1% agarose solution. The cover slips were removed and allowed to stand over-night after addition of the lysis buffer (0.5 M NaCl, 0.01 M Tris HCl, 1% SDS, 0.1 M NA_2EDTA, pH10), 1% Triton X, and 10% DMSO. The slides were electrophoresed at 70 mA (Buffer: 100 mM Disodium-EDTA, I M Sodium hydroxide) for 50 mins. After EtBr staining, the slides were kept in a neutralized buffer (IM Tris HCl pH7.5) and observed in a Fluorescence Microscope (EVOS® FL Cell Imaging System, Thermo Fisher Scientific). Open Comet v1.3.1 and Graph Prism 7 were used for image capture and analysis.

Apoptosis and morphological alterations

The cells were adhered in 6 well plates and doxorubicin; LH were treated using IC_{50} concentrations for 24 hours. Ice-cold 1XPBS (pH 7.4) was used for washing the cells followed by fixing of the cells in formaldehyde (4%) in 1X PBS at RT for 20 minutes. The cells were incubated with *AO/Et-Br* (2:1) stain in dark at RT for 10 minutes before observing under Fluorescence microscope and Apoptotic cum Necrotic indices were calculated by the following (13).

Apoptotic index (%) = Number of apoptotic cells scored X 100/ Total number of cells counted.

Necrotic index (%) = Number of necrotic cells scored X 100/ Total number of cells counted.

Evaluation of the activation of the caspase-3 and caspase-8 enzymes

ELISA kits (Bioassay Technology Laboratory, China) were used to assess the activity of caspase-3 and caspase-8. Apoptosis was induced in A-549 and HeLa cells by treating with LH at IC_{50} value for 48 hours. Doxorubicin (10 µg/mL) was used as positive control. The fold increase change was calculated by comparing with the untreated controls.

Gas chromatography-mass spectroscopy (GC-MS) analysis

Clarus 690 Perkin/Elmer (Autosystem XL) GC mass-detector with Elite 1 capillary column (100% Dimethyl polysiloxane; 123.5 × 678 m) was used. The temperature was fixed at 40 ˚C ramp 5 ˚C/min to 115 ˚C, held 5 minutes, ramp 5 ˚C/minutes to 140 ˚C, held 5 minutes, ramp 2 ˚C/min to 210 ˚C, held 8 minutes, and retained for 3 minutes. The temperature was gradually increased to 250 ˚C, at 5 ˚C/minutes for 9 minutes. The temperature of the injection port was kept at 250 ˚C, and the Helium flow rate was retained at 1.5 ml/minutes with 70 eV. Injection of the samples in a 10:1 split mode and 500–800 (m/z) scan range was used. The contact was 240 ˚C and the ion source was 230 ˚C. There was a 3-minute start time for the MS, a 75-minute end time, and a 3-minute solvent cut time. The NIST 17 online library Ver. 2.3 and PubChem Compound (NCBI) were matched with these substances.

High performance liquid chromatography (HPLC) analysis

Shimadzu Instrument (Shimadzu Corp, Kyoto, Japan) used with a C18 column (5 µm; 4.6×250 mm) and a diode array detector (DAD, SPD N 20A) to identify the bioactive chemicals. The gradient system started with 100% solvent A at 0.1 min, with 35% solvent B at 25 minutes, and a gradual increase in the concentration to 50% at 45 minutes and 100% at 65 minutes. Sterile water was used for dissolving the standard compounds; filtering was done by PVDF (0.45 µm), and 20 µL volume was eluted. The solvent systems used in the mobile phase were H_2O: C_2H_3N: CH_3COOH (solvent B- 48:51:4 v/v) and HPLC grade H_2O (solvent A). About 20 µL of sample was injected and 1 mL/min was maintained as the flow rate. The reference compounds were considered to attain the retention periods.

Statistical analysis

Microsoft Excel 2010 was used for performing statistical analysis. The IC_{50} value calculation was done using GraphPad Prism 7. The data were presented as mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used for experimental data calculation, and significance was considered at P value < 0.05.

Results

Phytochemical analysis

Alpinia galanga showed the presence of potent bioactive compounds (Table 1). The quantitative analysis revealed that rhizome methanol (RM) extract contained the highest flavonoids followed by leaf methanol (LM) extract. Stem ethyl acetate (SE) contained the highest alkaloids (Table 2).

Antioxidant property

DPPH

Based on the IC_{50} values, the leaf ethyl acetate (LE) showed the highest ability to scavenge DPPH (Table 3). The scavenging activities were effective in the order: Leaf ethyl acetate (LE) (6.44)> stem hexane extract (SH) (7.81)> leaf hexane extract (LH) (10.19)> RM (25.51)> rhizome hexane extract (RH) (33.37)> stem methanol extract (SM) (34.21)> LM (41.13)> rhizome ethyl acetate extract (RE) (45.19)> SE (55.02).

ABTS + cations

SH with an IC_{50} value of 1.18 µg/mL exhibited the highest

Table 1. Qualitative phytochemical analysis of the various *Alpinia galanga* extracts

		Qualitative phytochemical analysis								
Portion used	**Solvent**	**Phenols**	**Flavonoids**	**Alkaloids**	**Saponins**	**Quinones**	**Terpenoids**	**Tannins**	**Cardiac glycosides**	**Anthraquinones**
Leaf	Methanol	+	+	+	+	+	+	+	-	-
Stem		+	+	+	+	+	+	+	-	-
Rhizome		+	+	+	+	+	+	+	-	-
Leaf	Ethyl acetate	+	+	+	+	-	+	-	-	-
Stem		+	+	+	-	-	+	+	-	-
Rhizome		+	+	+	-	+	+	-	-	-
Leaf	Hexane	+	+	-	+	-	+	+	-	-
Stem		+	+	-	-	-	+	+	-	-
Rhizome		+	+	-	-	+	-	-	-	-

+: Present; -: Absent.

Table 2. Quantitative phytochemical analysis (mg/g of dry weight) of the different extracts of *Alpinia galanga*

		Phenolics	**Flavonoids**	**Alkaloids**
Leaf	Methanol	3.77±0.33	2.80±0.40	0.67±0.22
Stem		1.20±1.2	1.065±0.82	0.83±0.21
Rhizome		3.98±0.83	3.02±0.05	0.67±0.63
Leaf	Ethyl acetate	3.36±0.35	2.46±0.82	0.45±0.83
Stem		1.01±0.8	0.65±0.02	0.90±0.05
Rhizome		2.28±0.55	2.07±0.83	0.39±0.52
Leaf	Hexane	2.20±0.77	1.64±0.32	-
Stem		1.07±0.52	0.78±0.03	-
Rhizome		2.25±0.34	1.56±0.50	-

Results are expressed as mean± standard deviation (SD) of triplicate measurement.
+: Present; -: Absent.

Table 3. Antioxidant activity (IC_{50} values) of the different extracts of *Alpinia galanga*

		Antioxidant activity (IC_{50} values in µg/mL)				
		DPPH	**ABTS**	**O_2^-**	**OH**	**Phosphomolybdenum**
Leaf	Methanol	41.13±0.02	25.36±0.77	47.96±0.28	4.13±0.87	34.19±0.15
Stem		34.21±1.5	20.66±0.23	24.39±1.7	2.53±0.3	25.02±0.07
Rhizome		25.51±0.14	14.74±0.12	95.28±1.5	5.06±0.03	49.76±0.2
Leaf	Ethyl acetate	6.44±0.07	5.15±0.5	59.58±1.7	3.57±1.02	35.67±0.08
Stem		55.02±0.28	37.23±0.8	57.37±0.57	1.81±0.7	68.24±0.07
Rhizome		45.19±0.02	78.92±0.15	161.8±0.8	3.65±0.40	30.3±0.28
Leaf	Hexane	10.19±0.04	15.81±0.35	75.43±0.7	2.36±0.5	3.93±0.5
Stem		7.81±0.3	1.18±0.7	183.1±0.72	3±0.05	28.77±0.05
Rhizome		33.7±0.7	28.07±0.12	179.1±1.05	4.06±0.8	41.89±0.55

Results are expressed as mean± standard deviation (SD) of triplicate measurements.
DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; O_2^-: Superoxide; OH: Hydroxyl radical; IC_{50}: Half-maximal inhibitory concentration

ability to scavenge ABTS+ cations, followed by LE with IC_{50} value of 5.15 µg/mL. The radical scavenging activities were found in the order: SH (1.18)> LE (5.15) > RM (14.74)> LH (20.66)> LM (25.36)> RH (28.07)> SE (37.23)> SM (67.37)> RE (78.92) with respect to ascorbic acid (1.31 µg/mL) (Table 3).

Superoxide anion

SM, with an IC_{50} value of 24.39 µg/mL showed the highest superoxide radical activity. The scavenging activity were found effective in the order: SM (24.39)> LM (47.96)>SE (57.37)> LE (59.58)> LH (75.43)> RM (95.28)> RE (161.8)> RH (179.1)> SH (183.1) (Table 3).

Hydroxyl radical

SE (1.81 µg/mL) showed the highest hydroxyl scavenging activity and was effective in the order: SE (1.81)> LH (2.36)>SM (2.53)> SH (3.00)> LE (3.57)> RE (3.65)> RH (4.06)> LM (4.13)> RM (5.06) with respect to ascorbic acid (Table 3).

Phosphomolybdenum assay

LH with an IC_{50} value of 3.93 µg/mL showed the highest total antioxidant capacity. The detected order of IC_{50}: LH (3.93)> SM (25.02)> SH (28.77)> RE (30.3)> LM (34.19)> LE (35.67)> RH (41.89)> RM (49.76)> SE (68.24) with respect to ascorbic acid (Table 3).

Cytotoxicity

The results showed that the various extracts of *A. galanga* were capable of killing A-549, HeLa, AGS, and HT-29 cells but had no significant toxicity in normal and L-132 cells. Table 4 shows the cytotoxicity analysis (IC_{50} values) of *A. galanga* extracts on human cancer and normal cell lines. The morphological changes of the cells treated with 100 µg/mL of leaf hexane extract (LH) of *A. galanga* in A-549, HeLa, AGS, HT-29, and L-132 cells are shown in Figure S1a-e (see Supplementary file 1). The LH exhibited insignificant cytotoxicity against the L-132 cell line attaining an IC_{50} value of 81.76 µg/mL. In contrast, LH revealed an IC_{50} value of 13.2 µg/mL against the A-549 cells. The concentrations of the positive control (Doxorubicin) were 0.5, 1, 2, 4, and 8 µg/mL with an IC_{50} value of 2-4 µg/mL for all the cancer and normal cells (Table 4).

Apoptosis evaluation by DNA fragmentation detection using Comet assay

Using a visual score and software analysis, the percentage of tail DNA and moment were utilized as indices to assess the amount of DNA damage caused by the extract. Major decrease in the rate of migration was observed in untreated cells in comparison to positive control and LH (Figure 1). Treatment of the A-549 cells with LH revealed increased tail DNA (64 %) and tail moment (63.24 %) thereby inducing DNA damage while the untreated control revealed 4.74 % tail DNA and 0.11 % tail moment. Treatment of the HeLa cells with LH also revealed high tail DNA (65.3 %) and moment (63.2 %). Significant increased tail DNA (65.62 %) was observed upon the treatment with Doxorubicin, which was comparable to the level of DNA damage induced by LH (Figure 2).

Apoptotic cell death by LH

Fluorescence microscope observation showed that the amount of cell death was higher in the A-549 and HeLa cells than the normal cells, revealing an increased activation of apoptotic and necrotic cells over 48 hours (Table 5).

Compared to L-132 cells, treatment with the IC_{50} concentration of LH against A-549 and HeLa cells resulted in apoptosis and necrosis cells with high cell death at 48 hours (Figure 3). Similar to doxorubicin, the stimulation of apoptotic cells resulted in a much higher level of apoptosis than the untreated control. A-549 cells treated with 13.22 µg/mL of LH increased the apoptotic cells by 70.03 % and necrotic cells by 3.50 % compared to the control. An analysis of the extracts' activity using the normal cell line (L-132) revealed that the apoptotic and necrotic indices were not as significant as those of A-549 and HeLa cells. HeLa cells treated with 45.83 µg/mL of LH increased the apoptotic cells by 72.57 % and necrotic index by 5.83 %, but there was an insignificant increase in the normal cell line (L-132) treated with LH (Table 5).

LH and caspase-8 and -3 activities

LH induced cytotoxic effect by activating apoptosis, thereby up-regulating the activities of caspase-8 /3 in HeLa and A-549 cells (Table 5).

GC-MS analysis

LH recorded 18 compounds (Figure S2a) and SH recorded 12 peaks (Figure S2b) based on peak retention time and area (%) in comparison with known compounds in PubChem Compound (NCBI) and NIST library. The biological activities of the 11 phytocompounds identified in LH are shown in Table S1a. SH recorded 8 phytocompounds with known biological activity (Table S1b, Supplementary file 1).

Table 4. Cytotoxicity analysis (IC_{50} values (µg/mL) of *Alpinia galanga* extracts on human cancer and normal cell lines

Solvent	A-549			HeLa			AGS			HT-29			L-132		
	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome
Methanol	164.5 ± 0.28	38.86± 1.8	65.38 ± 1.8	77.76 ± 0.25	>180 ± 0.37	32.2± 0.37	81.52 ± 1.2	>180 ± 0.83	140.7 ± 0.53	150 ± 0.85	168.1 ± 0.23	33.44 ± 0.38	82.28± 1.97	148± 0.2	124± 0.85
Ethyl acetate	134 ± 1.23	79.75 ± 1.53	65.73 ± 0.55	>180 ± 0.03	73.7 ± 0.10	35.3 ± 0.057	39.31 ± 0.85	132.3 ± 1.52	101.8 ± 1.32	124.6 ± 0.35	>180 ± 0.76	70.46 ± 0.20	>180 ± 0.30	142 ± 0.52	>180±3.7
Hexane	13.2 ± 0.80	36.59 ± 0.90	48.66 ± 0.12	45.83 ± 1.23	41.6 ± 0.83	75.9 ± 0.87	15.87 ± 0.55	74.88 ± 0.53	115.3 ± 1.58	20.46 ± 1.35	43.23 ± 0.85	67.63 ± 0.15	81.76± 2.5	94.75± 3.7	133.6± 2.5
Dox	2.038±0.25			3.65±0.47			2.55±0.55			3.464 ± 0.78			3.448 ± 1.5		

Dox: Doxorubicin; A-549: Lung cancer cell; HeLa: Cervical cancer cell; AGS: Gastric cancer cell; HT-29: Colon cancer cell; L-132: Normal cancer cell
Results are expressed as mean± standard deviation (SD) of triplicate measurements.

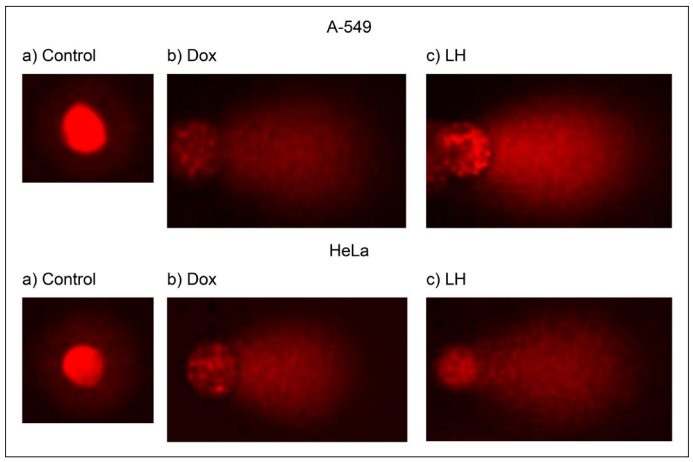


Figure 1. Fluorescence Comet figures observed in adenocarcinoma human alveolar basal epithelial cells (A549) and Henrietta Lacks (HeLa) cells. When comparing the treated groups to the untreated control, there was a noticeable increase in tail length and tail moment due to leaf hexane (LH)-induced DNA damage in A-549 and HeLa cells. Dox: Doxorubicin.

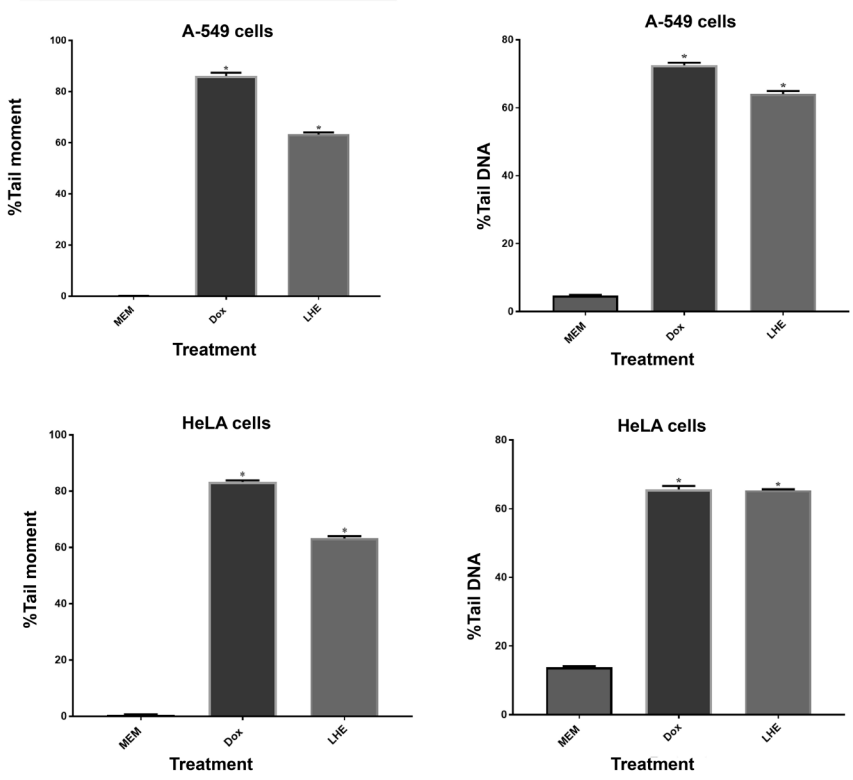


Figure 2. The DNA damage level induced by the leaf hexane extract (LHE) and doxorubicin (Dox) in Tail DNA (%) and Tail moment (%). The results are presented as mean ± SD (n=5). One-way ANOVA was done to test the significant level. *<0.05 between the control and treatment groups. MEM: Minimum essential media control; DOX: Doxorubicin; LHE: Leaf hexane extract.

HPLC analysis

The HPLC analysis revealed that LH contained 4 active substances: gallic acid, quercetin, catechin, and rutin (Figure 4a). SH recorded the presence of gallic acid (Figure 4b).

Discussion

The efficiency of the leaf, stem, and rhizome of *A. galanga* was analyzed for their phytochemical, antioxidant, and anti-cancer activities. Potent bioactive substances were detected in *A. galanga* (Table 1). Our results showed that the different extracts possessed potent antioxidant properties. LH exhibited higher potency to scavenge free radicals compared to other samples (Table 3). MTT assay serves as an analytic tool for chemotherapy as it is widely used in the assessment of cytotoxic drug therapy (30). Substances that can intentionally eliminate cancer cells without dealing significant damage to healthy cells are

Table 5. Effect of leaf hexane extract (LH) on caspase-3 and -8 expression and apoptotic activation

Treatment		HeLa cell			A-549 cell			L-132 cell		
		Control	Dox	LH	Control	Dox	LH	Control	Dox	LH
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Apoptosis (%)		2.03±0.70	76.63±2.31*	72.57±1.75*	2.51±0.95	79.40±2.06*	70.03±1.23*	2.77±0.67	67.62±1.01*	14.25±0.86
Necrosis (%)		1.63±1.22	14.76±1.34	5.83±1.05	1.50±0.08	13.35±1.24	3.50±1.44	1.14±1.31	13.25±1.27	8.32±0.87
Live (%)		96.34±1.22	16.05±0.57	8.62±1.34	95.99±1.01	7.24±1.24	10.97±1.44	94.82±1.55	17.67±0.68	72.54±0.87
Caspase (Fold change/ increase)	C8	0.00	4.01	2.24	0.00	11.14	4.57	-	-	-
	C3	0.00	2.25	2.25	0.00	10.48	5.78	-	-	-

Dox: Doxorubicin.
The findings are shown as mean ± SD (n = 5). *$P < 0.05$ compared to the respective control group (one-way ANOVA).

greatly valued (31). The different extracts of *A. galanga* were effective in eliminating cancer cells but not as effective as doxorubicin; however, they were less cytotoxic in the healthy cells (Table 4). Therefore, they complement the traditional use in treating cancer.

As stated by NCI, a plant extract with IC_{50} value ≤25 µg/mL is examined to acquire an active cytotoxic effect following a 48–72-hour incubation time (32,33). In this regard, LH was chosen for completing the remaining experiments. Earlier reports have shown the anti-cancer activities of aqueous and methanol extracts of *A. galanga* against the HeLa cell line (34). Our results are in accordance with earlier reports where the anticancer activity of *A. galanga* was evaluated using two different cell lines, A-549 and MCF-12A (35). Results from earlier research have stated that the ethanol extract of *A. galanga* exhibits cytotoxic properties against MRC-5, MCF-7, HepG2 (36,37), and HeLa cells (38). Lintao and Medina (39) also reported that the ethanol extract from the leaf of *A. elegans* was capable of inhibiting the growth of lung (A549), colorectal (HCT116), and liver (HEPG2) cancer cells. Total phenolic and flavonoid contents are prominently related to the efficiency of antioxidant activity (40). The stable radical scavenging and cytotoxic activities are directly related to phytochemical compounds present (41).

In eukaryotic cells, quantification of DNA strand breaks was done using Comet assay (42). Apoptosis evaluation by DNA fragmentation detection was done by this assay (Figures 1 and 2). Cell death occurs in cancer cells after cytotoxic drug treatment and apoptosis is the prominent fundamental process of the anticancer properties of numerous anticancer drugs in natural compounds (43). We examined whether LH exhibited cytotoxicity via activating apoptosis in A-549 and HeLa cells. The AO/EtBr staining is a dependable procedure for detecting apoptosis (44). Viable cells showed green fluorescence with a consistent nuclei structure whereas the cells in the early apoptotic stage were distinguished with disintegrated yellow chromatin nuclei. Round nuclei with orange chromatin were characterized as necrotic cells (45). In our experiment, the A-549 and HeLa cells exhibited a greater percentage of cell death compared to the normal cells, indicating a greater activation of necrotic and apoptotic cells for 48 hours (Table 5 and Figure 3). Death signaling pathways may be initiated by cytotoxic agents in susceptible target cells by inducing apoptosis. Apoptosis is the mechanism of killing cancer cells and is carried out by

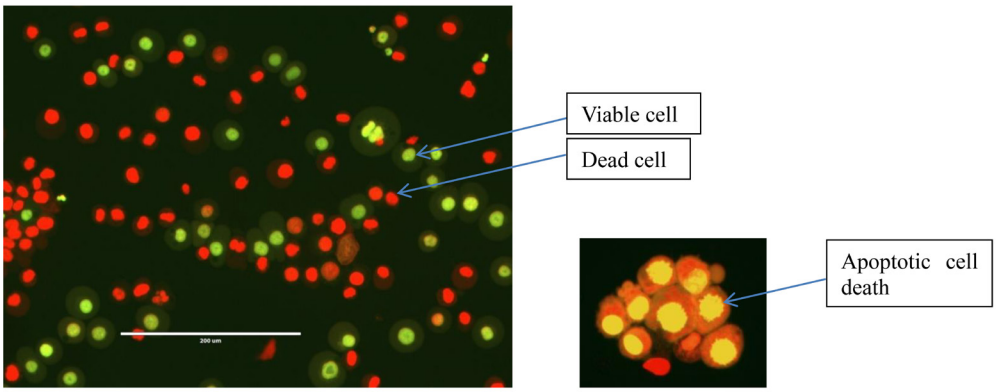
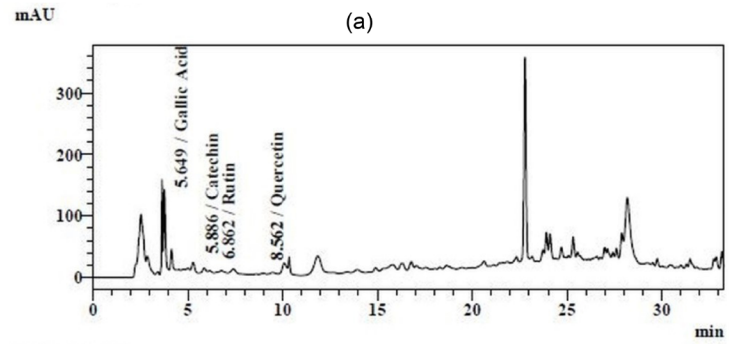
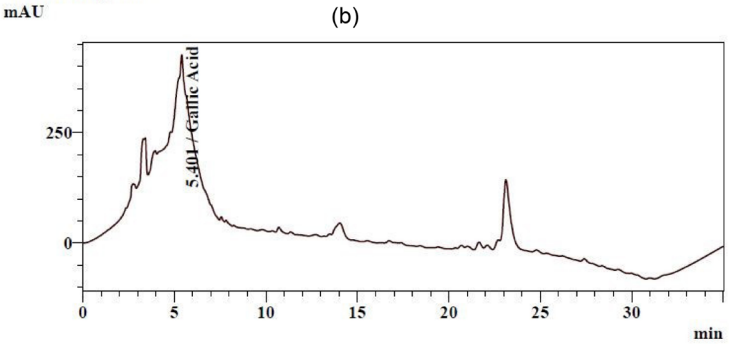


Figure 3. Activation of apoptotic cell death by leaf hexane extract (LH). Living cells stained with acridine orange and ethidium bromide revealed green fluorescence and a typical nuclear appearance. The nucleus of the cells showed orange fluorescence with disintegrated yellow chromatin nuclei in apoptotic cells. Dead cells showed red fluorescence.



<Peak Table>
PDA Ch1 275nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.649	370035	51498	0.000	mg/L	M	Gallic Acid
2	5.886	122700	13367	0.000	mg/L	M	Catechin
3	6.862	107462	5542	0.000	mg/L	M	Rutin
4	8.562	47462	25542	0.000	mg/L	M	Quercetin
Total		647659	95949				



<Peak Table>
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.401	9498570	216553	0.000	mg/L	M	Gallic Acid
Total		9498570	216553				

Figure 4. Identification of bioactive compounds in the leaf hexane (a) and stem hexane (b) extracts of *Alpinia galanga* via HPLC method.

a group of caspases (46). LH induced cytotoxic effect by activating apoptosis, thereby up-regulating the activities of caspase-8 /3 in HeLa and A-549 cells (Table 5), which is in line with the DNA fragmentation morphology. Samarghandian et al (36) reported that the rhizome of *A. galanga* induced apoptosis and cytotoxic effects in MCF-7 cells. Likewise, the methanol extract of the leaf, stem, and rhizome of *C. aeruginosa* triggered apoptosis through caspase-involved pathways in A-549 and HeLa cells (13). In another study, the aqueous extract of the rhizome of *A. galanga* showed cytotoxic activity against A-549, CRL2522, MCF-12A, CRL2321, and CRL2335 cells (35). The ethanol extract of *A. galanga* has also been reported to induce cytotoxicity against PC-3 cells (47). According to a study done by Hadjzadeh et al (48), the cell growth of AGS and L929 cells was inhibited by the aqueous extract of *A. galanga*.

Identification of the bioactive compounds with pharmacological properties was done for the best cytotoxic fractions using HPLC and GC-MS analysis. GC-MS data revealed the presence of many compounds that had previously been documented in previous publications. (49-51). Limonene, vitamin E-ɤ-tocopherol, and a sesquiterpene, β-elemene, detected in the leaf hexane extract (LH) had cancer-protective properties (Table S1a). Yu et al (52) and Jia et al (53) reported that limonene could induce apoptosis in human lung and colon cancer cells, presenting great potential as an anticancer agent. β-Elemene was also reported to trigger apoptosis following caspase-activated pathways in different cancer cells (54-56). The cytotoxic activities of ɤ-tocopherol on A549, HeLa, HT-29, PC-3, and LNCaP cells were reported by activating apoptosis involving caspase-independent pathways (57-59).

The HPLC study revealed that LH contained 4 active substances: gallic acid, quercetin, catechin, and rutin (Figure 4a), which were identified as apoptotic inducers through caspase apoptotic pathways in human cervical and lung cancer cells (60-71). SH showed the presence of gallic acid (Figure 4b). The apoptotic activities of *A. galanga* on HeLa and A549 cells should be by these potent phenolic and flavonoid compounds. GC-MS analysis showed that LH contained natural terpenoid and vitamin compounds capable of inducing apoptosis with high anticancer potential compared to the other plant parts studied.

Conclusion

Different extracts of *A. galanga* revealed that LH had potent cytotoxicity with low IC_{50} values of 13.2 µg/mL and 45.83 µg/mL in lung adenocarcinoma (A-549) and human cervical cancer cells (HeLa), respectively, exhibiting an increase in DNA fragmentation and apoptotic indices. Our findings inform for the first time that the leaf hexane extract of *A. galanga* is effective in triggering apoptosis in HeLa and A-549 cells through increased expression of caspase-8 (the initiator) and caspase-3 (the executor). Our conclusion supports the traditional use of this plant in treating cancer, as the results also showed insignificant cytotoxicity against normal cells. The compounds (flavonoids, phenolics) identified in this study could be attributed for the anticancer effect. GC- MS identified the presence of anti-cancer compounds (β-elemene and ɤ-tocopherol) in LH as a first report to further support its medicinal values. In sum, *A. galanga* might be a suitable plant for lung and cervical treatment and further studies are warranted.

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Conflict of interests

There is nothing to declare.

Ethical considerations

The authors have closely monitored ethical concerns related to data collection, analysis, authorship, review, plagiarism, and duplication of effort. Neither humans nor animals are involved in this investigation. As a result, ethical considerations regarding the usage of humans and animals are not necessary.

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Supplementary files

Morphological changes of cells treated with 100 µg/mL of leaf hexane extract (LH) in different cell lines (Figures S1a-1e) are available in Supplementary file 1, along with GC-MS supplementary Tables S1a-1b and Figures S2a-2b.

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