A New Annulenol from *Aconitum heterophyllum* with Bioactivity and Cytotoxicity against Vero and Lung Cell Line

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ABSTRACT: In this research work, a detailed phytochemical and biological investigation of *Aconitum heterophyllum* was accomplished. The cold methanolic root extract of *A. heterophyllum* was subjected to VLC for fractionation over silica gel, which led to the isolation of two compounds 1,1,8,8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-1-ol (**1**) and β-stigmasterol (**2**). The compounds were characterized by analysis of their 1D and 2D NMR spectral data. Compound **1** is first reported from this plant. The crude extract was screened for antimicrobial activity against several gram-positive and gram-negative bacteria, and fungi using disk diffusion method where significant inhibitory activity was observed. The extract was also tested for cytotoxic property using brine shrimp lethality bioassay, which revealed moderate activity in comparison with the standard anticancer drug, vincristine sulphate. Antioxidant property of the crude methanol extract of *A. heterophyllum* was determined by the scavenging of the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical and found moderate antioxidant activity (IC_{50} = 53.25 µg/mL) when compared to standard antioxidant. Total phenolic and flavonoid content and polyphenolic compound content of the crude extract were determined by HPLC. The isolated compounds **1** and **2** showed dose-dependent antiproliferation activity against the vero and lung A549 cells.

Key words: *Aconitum heterophyllum*, Annulenol, Antiproliferation, Vero and lung cell line, Antimicrobial, Antioxidant, Phenolic, Flavonoid.

INTRODUCTION

Aconitum is the genus (Family: Ranunculaceae) having 400 species and found mostly in the northern temperate area (Asia, Europe, and America), whereas over 200 species are found in China.[1] Plants under this genus are used for the treatment of a variety of diseases such as ailments, oedema, rheumatic fever, aching joints, fainting, diarrhea, bronchial asthma, gastroenteritis, different malignancies, and several endocrine problems (i.e., unusual menstruation).[2,3] Medicinal plants are well known for their anti-inflammatory, analgesic, and anti-arrhythmic diterpenoid alkaloids and non-alkaloidal chemicals (fatty acids, flavonoids, phenolic acids, phenylpropanoids, terpenoids, polysaccharides, and steroids).[4-7] *A. heterophyllum* is an important medicinal plant distributed between 2800-4500 m and found in Alpine regions of the Himalaya.[8] The root tubers of *A. heterophyllum* are used as traditional medicine for various therapeutic actions (anti-arthritic and anodyne). This plant used as ayurvedic medicinal moiety in the traditional treatment of bronchial asthma, diarrhea, fainting, gastroenteritis, infections, inflammation, irregular menstruation,

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hepatoprotective activity, oedema, painful joints, and rheumatic fever[9, 10]. *Aconitum* species are known for their similar diterpenoid alkaloid (C18, C19, C20, and Bis-subtypes), which has anti-inflammatory, analgesic, anti-inflammatory, and anti-arrhythmic properties. *Aconitum* plants also contain non-alkaloidal compounds such as flavonoids, phenylpropanoids, phenolics, terpenoids, steroids, free fatty acids, and polysaccharides[11]. Because no chemical screening of *Aconitum heterophyllum* has been reported, we used this medicinal plant to identify its chemical components and examine biological activities.

MATERIALS AND METHODS

Collection and preparation of plant. The dried root of *Aconitum heterophylum* was collected from local source in Dhaka, Bangladesh. It was then recognized by a scientific officer, Bangladesh National Herbarium, where a voucher specimen (DABC 45232) has been deposited.

Extraction of the plant materials. The coarse powder of the root (1.0 kg) was soaked in a mixture of MeOH and H_2O_2 (3.0 L) for 10 days with intermittent shaking and stirring, filtered using a rotary evaporator. The resulting filtrate was concentrated at 50^{o}C.

Vacuum liquid chromatography of crude methanol extract for phytochemical screening. A column (length 27.0 cm and diameter 20.4 cm) was packed to a height of 12.3 cm with fine silica gel (Kiesel gel 60 G) as the packing medium and then washed with *n*-hexane to permit compact packing under reduced pressure. The MeOH soluble extract (15.0 g) was adsorbed onto silica gel to form a free-flowing powder, which was then applied on top of the adsorbent layer. The column was eluted with *n*-hexane and the polarity of the solvent was steadily enhanced by adding increasing amount of DCM and MeOH. Compounds **1** and **2** were isolated from the VLC fraction eluted with *n*-hexane-DCM (95:5) and *n*-hexane-DCM (55:45) respectively.

Screening for antimicrobial activities. Methanolic crude extracts was tested for antimicrobial activity using four pathogenic bacterial strains (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus megaterium* BTCC 18 and *Pseudomonas aeruginosa* ATCC 27833), as well as two fungal strains (*Aspaergillus niger* and *Aspaergillus flavus*). Kanamycin and ketoconazole (30 µg/disk) were employed as positive controls for bacteria and fungi respectively, whereas solvent disk was utilized as a negative control. After incubation at 37°C for 18-24 hrs for bacteria and at 28°C for 48-96 h for fungus, the diameter of the zone of inhibition was measured.

Evaluation of cytotoxic activity. The poisonous characteristics of plant extractives against *Artemia salina* was revealed by using the brine shrimp lethality test. The test sample was dissolved in DMSO and after serial dilutions to solutions of reach concentrations of 400-3.13 g/ml was achieved. Test tubes containing shrimps of simulated distilled water and the test solutions were kept at room temperature for 24 hrs. Plot of percentage of shrimp mortality against the logarithm of sample concentrations was used to determine the test samples' LC_{50}. In this experiment, vincristine sulphate was employed as a positive control to compare the cytotoxicity of the test materials.

Antiproliferative activity of isolated compounds. On human alveolar basal epithelial cells (A549 lung cancer) and vero cell lines, the anticancer potential of isolated compounds was explored. These cells have been grown in T75 culture flasks in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum and 1% Pen Step solution {Penicillin (10000 units/ml) + Streptomycin (10000 g/ml)} for 48 hrs at 37°C in 5% v/v CO_2 to achieve an 80% confluence level. The viable cells were determined by using the Trypan blue exclusion method in an automated cell counter for determination of cell density. Under a T25 culture flask containing the culture medium, an aliquot containing suspension was seeded and incubated for 24 hrs at 37°C in v/v CO_2. The test sample stock solution (compounds **1** and **2**) was produced in DMSO at a concentration of 1.0 mg/mL and filter

sterilized using a disk filter (0.2 m). The sample solutions were added to the cultured T25 flask in triplicate at three different concentrations and incubated for another 24 hrs at 37°C in v/v CO_2. DMSO was used as a negative control in this case. After 24 hrs, the flasks were harvested using trypsin-EDTA solution, and cells were counted in an automated cell counter using the Trypan blue exclusion method to determine the anti-proliferative activity. The antiproliferative activity of the test samples on the cells was measured by using an IC_{50} value, which was calculated by using the following linear relationship between the percentage of dead cells and sample concentration.

Antioxidant activity examination. DPPH radical scavenging technique was used to test the antioxidant properties of the crude methanolic extract. To obtain a final concentration of 400–3.13 g/l, 2.0 mL of different concentrations (800–6.25 g/ml) of extracts were combined with DPPH solution (0.05 mM and 2.0 ml). The absorbance was measured spectroscopically at 517.0 nm after 30 minutes of incubation in the dark. The experiment was repeated three times and the results were averaged. The percentage of inhibitions were then plotted against concentration, and IC_{50} was derived from the graph. Positive controls included ascorbic acid and tert-butyl-1-hydroxytoluene (BHT). The scavenging activity was calculated as follows:

$$\text{Scavenging ability } (\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Assessment of total phenolic and flavonoid content. The Folin–Ciocalteu reagent was used to determine the total phenolic content of the methanolic crude extract. In distilled water, a stock solution of crude extract (1.0 ml) was produced. Ten times diluted Folin–Ciocalteu reagent (5.0 ml) was added with crude extract (1.0 ml) in a test tube, which was maintained for 3 minutes before addition of 7.5 percent (w/v) sodium carbonate (5.0 ml) solution. This combination was left for another 60 minutes in the dark before being tested for absorbance at 650 nm. Using the same approach, a calibration curve for gallic acid was created at concentrations of 15.63-500 µg/ml. The total phenolic content was calculated as shown in the equation and presented as mean ± standard deviation.

$$\textit{Total Phenolic Content (mg of gallic acid equivalent/gm of extract)} = \frac{\textit{concentration of gallic acid from calibration curve (mg/ml) x Volume of extract in stock solution (mL)}}{\textit{Weight of extract in stock solution (gm)}}$$

The aluminum chloride colorimetric technique was used to determine the total flavonoid content of the crude extract. In a test tube with MeOH (3.0 mL), a stock solution of crude extract (1.0 ml) was taken. It was then treated with a 10% $AlCl_3$ solution (0.2 ml) and left to stand for 6 minutes before being treated with a potassium acetate solution (1.0 M and 0.2 ml) and distilled water (5.6 ml). After allowing the mixture to sit for 15 minutes, the absorbance was measured at 510.0 nm. Using the same approach, a calibration curve for quercetin was created at concentrations ranging from 12.5 to 100 µg/ml.

$$\textit{Total Flavonoid Content (mg of quercetin equivalent/gm of extract)} = \frac{\textit{concentration of quercetin from calibration curve (mg/ml) x Volume of extract in stock solution (mL)}}{\textit{Weight of extract in stock solution (gm)}}$$

Quantitative analysis of polyphenols by HPLC. The crude extract was analyzed using a reverse phase chromatographic (HPLC) system (Shimadzu, Japan) with a binary solvent delivery system (LC-20A), an autosampler (SIL-20A HT), a column oven (CTO-20A), and a photodiode array detector (SPD-M20A), all of which were controlled by software (LabSolutions, Shimadzu). At 33 °C, separation was carried out on a Luna C_{18} (4.6 mm × 250 mm, 5.0 µm) column (Phenomenex, USA). Table 1 showed the mobile phase that was used as gradient elution of solution A (1% acetic acid + acetonitrile) and solution B (1% acetic acid + water).

Table 1. Gradient elution program.

Time (minutes)	Solution A (%)	Solution B (%)
0	5	95
1-20	25	75
21-30	40	60
31-35	60	40
36-40	30	70
41-45	5	95
46-50	5	95

Performance of statistical analyses. GraphPad Prism version 9.00 for Windows, GraphPad Software, was used to conduct statistical analysis for the antiproliferative investigation. One-way ANOVA with Dunnett's multiple comparisons test was used to compare the variability of the treated and control samples. When values were less than 0.05, differences between samples were considered significant. The means, SEM, and SD were used to express all other numbers.

RESULTS AND DISCUSSION

Spectroscopic analysis of 1,8, 8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-1-ol (1). Compound **1** was obtained as white fine needles and was soluble in DCM and $CHCl_3$. It was not visible under UV light when developed on a TLC plate. After spraying the TLC plate with vanillin-H_2SO_4 reagent followed by heating it yielded a purple color {R_f = 0.55, toluene-ethyl acetate (45:55)}. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) revealed 15 carbon resonances, while 12 out of the 15 carbons were coupled to protons according to the HSQC experiment. The ^{1}H-NMR and DEPT-135 together with the HSQC data revealed the existence of two methines (δ_H 1.35, δ_C 49.5, C-10, δ_H 1.76, δ_C 49.8, C-11) and three methyl (δ_H 1.19, 3H, δ_C 27.2, C-12, δ_H 0.69, 3H, δ_C 16.3, C-13 and δ_H 1.19, 3H, δ_C 27.2, C-14). In addition, the spectrum showed seven methylene groups including one exomethylene group (δ_H 4.43, 4.70, δ_C 105.4, C-15). In the ^{13}C NMR spectrum the carbon at δ =72.9 revealed the presence of an oxygenated carbon. The structure compound **1** was assigned after analysing one and two-dimensional NMR spectra such as COSY, HSQC, and HMBC. The C2-C3-C11-C4-C5-C6 component of this structure was determined by tracing cross peaks in the COSY spectrum. HMBC correlations revealed the structure of compound **1** (H14/C1, H14/C10, H2/C10, H2/C11, H3/C10, H15/C5, H5/C7, H7/C9, H13/C8, H13/C10, H9/C12). The above spectral data are consistent to characterize compound **1** as 1, 8, 8-trimethyl-4-methylene-deca-hydro-1*H*-cyclopenta-[8]-annulen-1-ol (Figure 1 and Table 2), which appears to be new. However the mass spectral data will be helped to confirm the molecular formula of 1.

Stigmasterol (2). Compound **2** was obtained as white needles and was soluble in CH_2Cl_2 and $CHCl_3$, not visible under UV-Visible light when developed on TLC plate, spraying with vanillin-H_2SO_4 reagent followed by heating it produced purple color, R_f = 0.36 (toluene-ethyl acetate, 90:10). The ^{1}H-NMR and ^{13}C-NMR spectra of compound **2** (Table 3) were identical to those recorded for β-stigmasterol. Moreover, its spectral data were identical to published values of β-stigmasterol.[12] Thus, it was characterized as β-stigmasterol.

Antimicrobial screening evaluation. The disk diffusion approach[13-16] is well known for in vitro screening of antimicrobial test agents. Most of the test organisms showed weak to moderate

antibacterial activity when exposed to the crude methanol extract. At a concentration of 500.0 µg/disk, the zone of inhibition produced by crude methanol extract was determined to be 8-10 mm (Table 4).

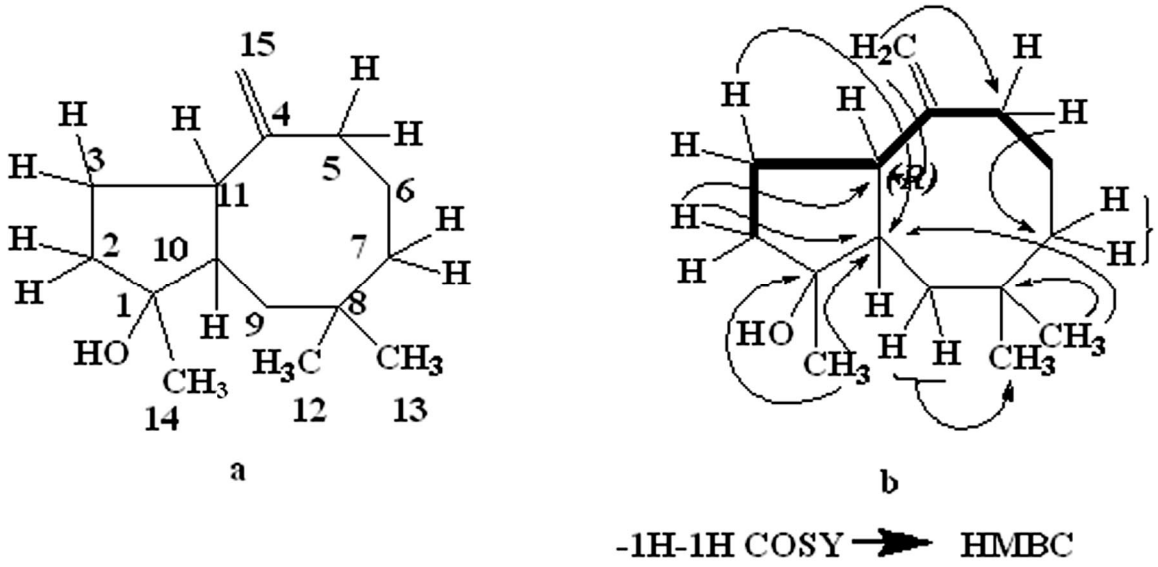

Figure 1. (a) Structure and (b) HMBC correlation observed for 1,8,8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-1-ol (1).

Table 2. NMR spectral data of 1,8,8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-1-ol (1).

No.	δ_H	δ_C	COSY	HMBC
1	---	72.9	---	---
2	1.51 (1H, m) 1.29 (1H, dd, *J* = 3.0, 9.4 Hz)	41.1	H2 (1.29)	C10 (49.5), C11 (49.8)
3	1.60 (1H, d, *J* = 3.0 Hz)	25.1	H2 (1.29), H3 (1.12)	---
	1.12 (1H, dd, *J* = 12.2 Hz)		H11 (1.76)	C10 (49.5)
4	---	151.2	---	---
5	1.98 (1H, bq, *J* = 10.4 Hz)	36.9	H5 (2.29), H6 (1.60), H15 (4.43), H15 (4.70)	C4 (151.2), C7 (22.4), C11 (49.8), C15(105.4),
	2.29 (1H, bd, *J* = 12.8 Hz)		H5 (1.98), H6 (1.60)	C4 (151.2), C7 (22.4), C11 (49.8)
6	1.61 (2H, d, *J* = 3.0 Hz)	23.5	---	---
7	1.25 (1H, m) 1.64 (1H, m)	22.4	---	C9 (41.9)
8	--	35.9	---	---
9	1.41 (2H, s)	41.9	---	C12(27.2)
10	1.35 (1H, dt, *J* = 2.8, 12.4 Hz)	49.5	---	---
11	1.76 (1H, d, *J* = 12.0 Hz)	49.8	H3 (1.12), H3 (1.60), H15 (4.43), H15 (4.70)	C3 (25.1), C4 (151.2), C5 (36.9), C15(105.4),
12	1.19 (3H, s)	27.2	---	---
13	0.69 (3H, s)	16.3	---	C8 (35.9), C9 (41.9)
14	1.19 (3H, s)	27.2	---	C1 (72.9), C10 (49.5)
15	4.43 (1H, d, *J* = 1.2 Hz, 15-H_b)	105.4	H11 (1.76), 15H (4.70)	C5 (36.9), C11 (49.8)
	4.70 (1H, d, *J* = 1.2 Hz, 15-H_a)		H11 (1.76), 15H (4.43)	

Table 3. ^{1}H-NMR spectral data of β-stigmasterol.

No.	δ_H (**2**)	δ_H (Literature value)[12]	δ_C (**2**)	δ_C (Literature value)
1			37.3	37.3
2			31.7	31.7
3	3.53 (1H, m)	3.53 (1H, m)	71.8	71.8
4			42.3	42.2
5			140.8	140.8
6	5.35 (1H, m)	5.36 (1H, m)	121.7	121.7
7			31.9	31.9
8			31.9	31.9
9			50.2	50.2
10			36.5	36.5
11			21.1	21.1
12			39.8	39.7, 39.8
13			42.3	42.3
14			56.8	56.8, 56.9
15			24.3	24.3, 24.4
16			29.2	28.2, 28.9
17			56.1	55.9, 56.1
18	0.67 (3H, s)	0.70 (3H, s)	12.0	11.8, 12.0
19	1.0 (3H, s)	1.02 (3H, s)	19.4	19.4
20			40.5	36.1, 40.5
21	0.91 (3H, d, *J* = 6.4 Hz)	0.94 (3H, d)	21.2	18.9, 21.1
22	5.15 (1H, dd, *J* =15.1, 8.5 Hz)	5.16 (1H, dd, *J* = 15.0, 6.5 Hz)	138.3	33.9, 138.3
23	5.01 (1H, dd, *J* =15.1, 8.5 Hz)	5.03 (1H, dd, *J* = 8.4, 15.2 Hz)	129.3	26.1, 129.3
24			51.2	45.8, 51.2
25			31.9	29.2, 31.9
26	0.86 (3H, d)	0.84 (3H, d)	21.2	19.8, 21.2
27	0.86 (3H, d)	0.86 (3H, d)	19.8	19.0
28			25.4	23.1, 25.4
29	0.82 (3H, t, *J* = 8.0 Hz)	0.81 (3H, t)	12.2	12.2

Table 4. Antimicrobial activity of crude methanol extract of *Aconitum heterophyllum*.

Microorganisms	Methanolic crude extract (mm)	Kanamycin (mm)
Bacillus megaterium	8.33 ± 0.47	18.67 ± 0.47
Staphylococcus aureus	9.00 ± 0.82	20.33 ± 0.47
Escherichia coli	9.00 ± 0.82	24.67 ± 0.47
Salmonella typhi	9.00 ± 0.82	24.00 ± 0.82
Aspergillus niger	13.33 ± 1.25	26.00 ± 0.82

Examination of cytotoxic activity. Brine shrimp lethality bioassay is considered as a simple and rapid test for determining general pharmacological activity[17]. Methanolic crude extract exhibited moderate activity as compared to the standard (Table 5). This bioassay has a positive relation with cytotoxic or pesticidal activity indicating the presence of bioactive compounds in plants.

Antioxidant activity. Antioxidant activity of naturally occurring compounds in higher plants has long been recognized. There has recently been a

surge of interest in oxygen containing free radicals in biological sciences, as well as their possible roles as causative agents in the etiology of several chronic diseases. As a result, researchers are concentrating their efforts on the protective biochemical effects of naturally occurring antioxidants in the cells of creatures that have them[18]. Antioxidants can be found in the body's enzymes, vitamin supplements, and industrial additives. To avoid free radical damage, they are commonly added to metals, oils, foods, and other things. The stable radical l,1-diphenyl-2-picrylhydrazyl was used to measure the free radical scavenging activities (i.e., antioxidant capacity) of the plant extracts[19]. Free radical scavenging activity of the crude extract of root fractions of methanol extract of *A. heterophyllum* was tested. In comparison to BHT and ascorbic acid, the crude extract showed scavenging activity with IC_{50} values of 53.25 µg/mL in this study (Table 6). Our findings are in line with a prior study on the antioxidant activity of methanolic crude extract of *A. heterophyllum*[20].

Table 5. LC_{50} values of the crude extract of *Aconitum heterophyllum*.

Test sample	LC_{50} value (µg/ml)
Vincristine sulphate	3.16
Crude extract of *Aconitum heterophyllum*	251.19

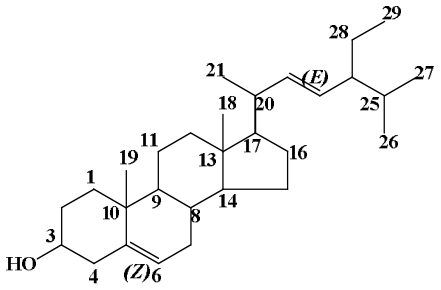

Figure 2. β-stigmasterol (2)

Table 6. IC_{50} values of the crude extract of *Aconitum heterophyllum*.

Test sample	IC_{50} value (µg/ml)
Ascorbic acid	2.81
BHT	1.93
Methanolic Crude extract of *Aconitum heterophyllum*	53.25

Total phenolic and flavonoid content. Plant phenolic secondary metabolites are known to protect plants against several biotic and abiotic stresses[21]. There are several classes of plant phenolic compounds with potential medicinal activity against neurodegenerative diseases, inflammation, cancer, diabetes, hyperlipidemia[22]. Plant phenolic metabolites may reduce oxidative damage by lipid peroxidation, which in humans may induce mutagenesis, carcinogenesis, ageing, and atherosclerosis[23]. These important classes of plant constituents were measured qualitatively (Table 7) and quantitatively (Figure 3) by HPLC analysis. Our study suggested that rutin and kaempferol are present in the highest amount the crude plant extract (Table 8). Rutin, a flavanol glycoside has several biological activities such as anti-inflammatory, antioxidant, anticancer, antidiabetic, antimicrobial, and antiallergic activities [24]. Whereas kaempferol, a natural flavanol, also has several important biological activities for example cytotoxic and anti-inflammatory activities[25]. The presence of these phenolic compounds is suggestive of the plant's antioxidant and anti-inflammatory potential.

Table 7. Total phenolic and flavonoid content of *Aconitum heterophyllum*.

Total content	Equivalent of dry extract
Phenol	43.25 ± 3.86 mg of gallic acid
Flavonoid	21.96 ± 0.05 mg of quercetin

Table 8. Total phenolic and flavonoid content of *Aconitum heterophyllum*.

Compounds	Amount in mg per 100 gm of dry extract
Catechol	30.58
Caffeic acid	15.16
Rutin hydrate	125.21
trans-Ferulic acid	21.72
Rosmarinic acid	12.35
Quercetin	26.78
Kaempferol	66.01

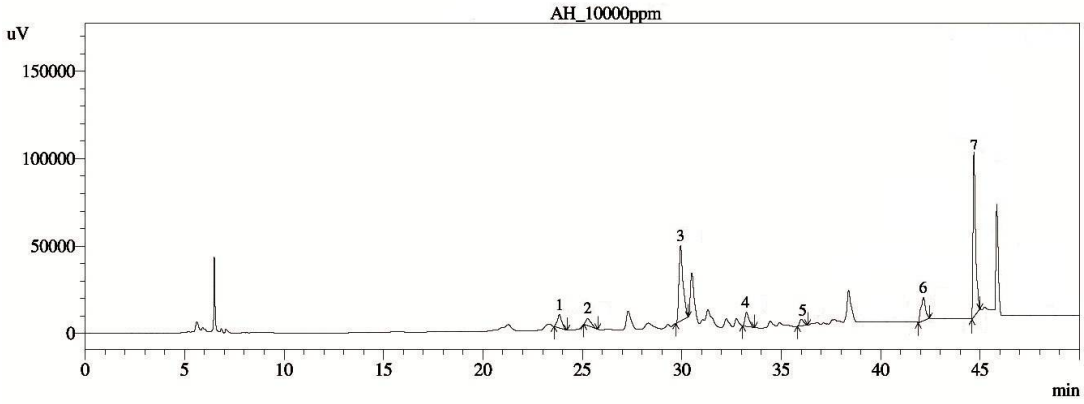

Figure 3. HPLC analysis of phenolic and flavonoid content of *Aconitum heterophyllum.*

Antiproliferative activity of isolated compounds. Human lung cancer cell A549 cells and vero cells were exposed to incremental concentrations of compounds **1** and **2** for 24 h. The cell viability was revealed by counting the dead cell numbers using an automated cell counter. It was found that compounds **1** and **2** effectively prevented the proliferation of tumor cells in a concentration dependent manner (Figure 4). The antiproliferative effect of compound **2** was statistically significant (Figures 5 and 6) at concentration of 20 µg/mL for both vero cell (78.97 % viable cells, $p < 0.0001$) and lung A549 cell (88.3 % viable cells, $p < 0.001$). Compound **1** showed weak activity (Figures 7 and 8) in both vero cell (86.3 % viable cells, $p < 0.01$) and lung A549 cell (88.4 % viable cells, $p < 0.01$) at the tested highest concentration of 20 µg/ml.

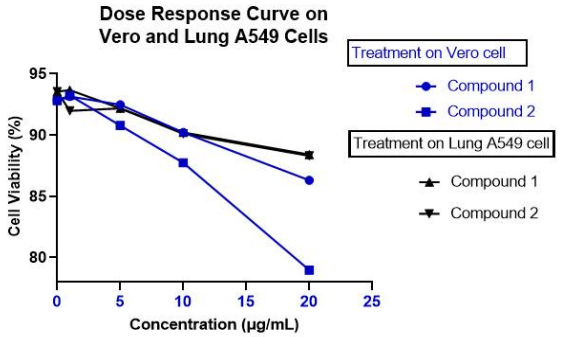

Figure 4. The dose response curve of compounds 1 and 2 for treatment on Vero and Lung A549 cells.

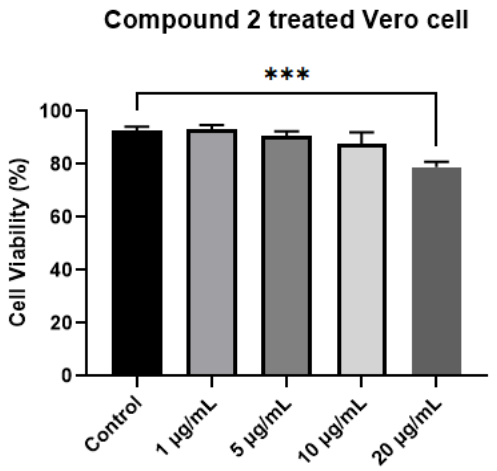

Figure 5. Efficiency of Compound 2 on Vero cell.

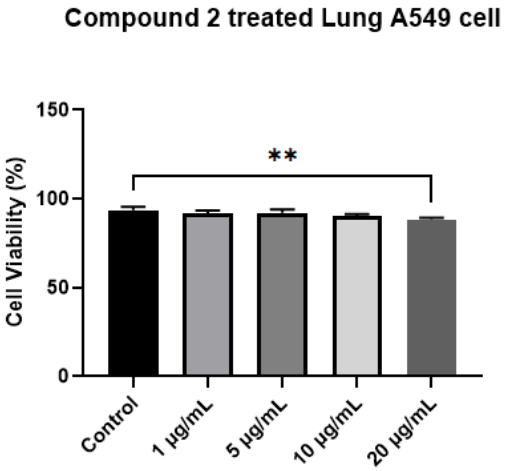

Figure 6. Impact of compound 2 on Lung A549 cell.

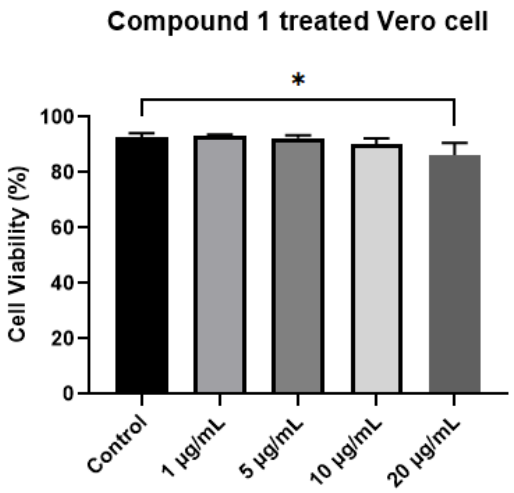

Figure 7. Influence of compound 1 on Vero cell.

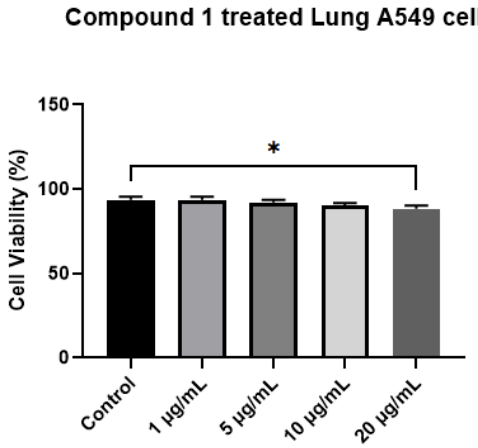

Figure 8. Impact of compound 1 on Lung A549 cell.

CONCLUSION

Researchers are currently focusing their efforts on the creation of novel medications from plant sources that may be used to treat a variety of ailments. These versatile medicinal plants are a unique source of a wide range of chemical substances. It is true that *Aconitum heterophyllum* has therapeutic value (anti-microbial, antioxidant, cytotoxic, and anti-proliferative), however it is now time to investigate the efficacy in the molecular level using various methodologies. Antioxidant, antibacterial and cytotoxic effects were observed in the revealed chemicals from this plant against a variety of pathogenic microbes. Exploration of these compounds could be an excellent way to generate pharmaceuticals at a commercial scale with high efficiency while also obtaining a pharmacophore that could be beneficial in drug design. The findings of this research will lead to more work on medicinal plants in general, with a present project focusing on the isolation, identification, and bioactivity screening of medicinal plants from Bangladesh.

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