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ORIGINAL ARTICLE

Identification of Compounds of *Aristolochia tagala* and Apoptotic Activity in HeLa Cells

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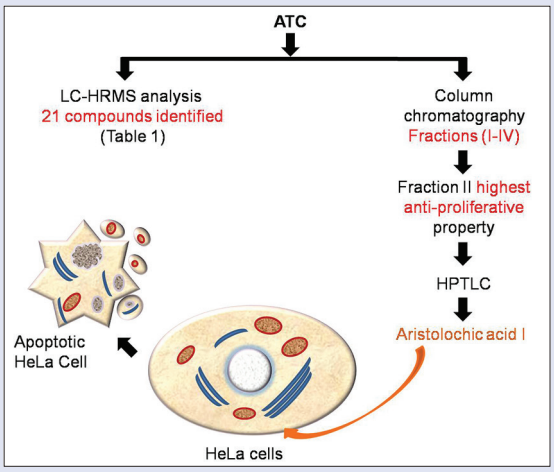
ABSTRACT

Background: Plants contain secondary metabolite used as drugs/medicines for the treatment of various diseases. *Aristolochia tagala* is used for the treatment of several diseases. Our study reported the chemopreventive potential of crude aqueous-methanol extract against diethylnitrosamine-induced hepatocellular carcinoma in BALB/c mice. A few articles have reported the presence of pharmacologically active compounds. **Objective:** Identification of biologically active compounds can give an insight into the mechanism of action of *A. tagala* and its potential development into modern drugs for the treatment of various diseases including cancer. **Materials and Methods:** Aqueous methanol extract (ATC) was prepared from roots of *A. tagala* and fractionated by column chromatography. The compounds present in ATC were identified and characterized by liquid chromatography (LC)–high-resolution mass spectrometry. ATC as well as the fractions (FI–FIV) were tested for their cytotoxic effect in HeLa cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, and apoptotic events were analyzed by flow cytometry. The fraction that showed the most efficient cytotoxic effect against HeLa cells was purified by high-performance thin-layer chromatography. Purified compounds were again assayed for their apoptotic and cytotoxic effect. The most active compound was identified and characterized by electrospray ionization high-resolution mass spectrometry and LC–tandem-mass spectrometry. Statistical analysis was carried out using one-way anova followed by Tukey's multiple comparisons test. **Results:** A total of 21 compounds were identified; aristolochic acid I, aristolactam IIIa, β-sitosterol, kaempferol, and stigmasterol were previously reported in *A. tagala and* other compounds in other species of *Aristolochia,* and some compounds were reported to have anticancer, anti-inflammatory activities. From our study, compound S7 showed the highest cytotoxic and apoptotic activity and was identified as aristolochic acid I. Aristolochic acid earlier has been reported to have antitumor and anticancer effects, but lately, it has also been reported to have nephrotoxic effect. **Conclusions:** *A. tagala* was found to contain a number of compounds with reported biological activity. This plant and its related species can, therefore, be exploited for the extraction and isolation of these compounds with no toxicity.

Key words: Apoptotic activity, *Aristolochia tagala,* aristolochic acid I, HeLa, liquid chromatography–high-resolution mass spectrometry, Liquid chromatography tandem-mass spectrometry

SUMMARY

- 21 compounds were identified in aqueous-methanol extract of *A. tagala*. Caffeoylquinic acid, isocorydine, β sistosterol, stigmasterol, aristolochic acid, kempferol, dehydrooxoperezinone have been reported to have anticancer as well as other medicinal properties.
- Four major fractions (I–IV) were obtained from column chromatographic separation.
- Fraction II (F II) showed highest anti-proliferative activity in HeLa cells.
- S7 purified compound showed highest anti-proliferative and apoptotic activity and the compound was identified as aristolochic acid I.



Abbreviations used: ATC: Aqueous-methanol extract, AA-I: Aristolochic acid I, CQA: Caffeoylquinic acid, $CHCl_3$: Chloroform, MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, ESI: Electrospray ionization, FITC: Fluorescein isothiocyanate, HCC: Hepatocellular carcinoma, HPLC: High-performance liquid chromatography, HRMS: High-resolution mass spectrometry, LC-MS/MS: LC-tandem-mass spectrometry, MeOH: Methanol, PBS: Phosphate buffered saline, PVDF: Polyvinylidene difluoride, RF: Radio frequency, *R*F: Retardation factor, XEVO-TQD: Xevo triple quadrupole

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INTRODUCTION

Traditional system of medicine used whole plant or crude extracts of the herbal plant which are minimally prepared to treat various forms of diseases. Evidently through time, traditional system of treatment has been found to be successful and has been a source of remedy for several patients throughout generations.[1] With the advancement of science and technology, it was found that the constituents that exhibit these medicinal properties are the secondary metabolites present in plants. The secondary metabolites are synthesized by the plants for its adaptation in the surrounding environment and act as a defense mechanism against infections as well as predators.[2] The same species and genus of plants

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will have similar metabolites synthesized; slight variation of metabolites within the species/genus can occur when grown in different conditions. Many such secondary metabolites present in plants have been extracted, isolated, purified, and modified and currently used as drugs/medicines for the treatment of different types of pathophysiological condition.[3,4]

Aristolochia tagala is one such plant that has been used in traditional medicine for the treatment of various diseases. The plant has been studied for several biological activities and was reported to have antiproliferative, anti-inflammatory, and antioxidant properties.[5,6] We have reported the potential anticancer activity of aqueous-methanol extract of *A. tagala* in diethylnitrosamine-induced hepatocellular carcinoma (HCC) in BALB/c mice.[7] The anticancer/antitumor activity has also been reported by Anilkumar *et al*., Garg *et al*., and Angeles *et al*. against different cancer cell lines.[8-10] The phytochemical constituents and compounds present in *A. tagala* have been reported by only a few[11-14] though compounds present in related species have been identified and characterized extensively.[15,16] From our findings, it was of interest to identify the metabolites present in the aqueous-methanol extract that exhibits medicinal properties and to characterize the active compounds responsible for the apoptotic activity in cancer cell. The identification of these metabolites or active compounds will give us an understanding into the possible mechanism of action, improved efficacy, and reduce toxicity through dose regulation.

MATERIALS AND METHODS

Separation and purification of phytochemical compounds

The phytochemical compounds present in the roots of *A. tagala* were separated by fractionation of the crude aqueous-methanol extract. Fractionation of the aqueous-methanol extract of roots of *A. tagala* afforded 38 fractions which were pooled into four fractions (I–IV) based on their absorption maxima (λ_{max} nm).[11] Each fraction was assayed for the cytotoxic activity in HeLa cells. Fraction II (F II) which showed the highest cytotoxic activity was subjected to subsequent separation and purification by high-performance thin-layer chromatography in $CHCl_3$-MeOH (9:1) and 1% acetic acid solvent. After the plates were developed, they were removed from the chamber and viewed under the CAMAG Ultraviolet Visualizer at 254 nm and 366 nm and photographed. The spots observed were marked, and the compounds were scrapped off the plate. Each spot was again rechromatographed and scrapped off to ensure proper purification.

Desorption of compounds from the sorbent (silica)

Compound-rich sorbent (silica) was transferred to a beaker. Solvent ($CHCl_3$-MeOH; 1:1) was added, and the suspension was stirred using a magnetic stirrer for 30 min to facilitate leaching of compounds to solvent. The process was repeated twice. The final washing was carried out with methanol and 2% acetic acid to recover maximum quantity of each compound. The solvent was left standing for 30 min, pipette out into glass tube, and centrifuged at 800 g for 5 min to remove the silica. The solvent-rich compounds were then filtered through a 0.45-µ polyvinylidene difluoride (PVDF) to completely remove any remaining silica. The solvent was evaporated to dryness to obtain the compounds.[17]

Analysis of cytotoxicity by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) proliferation assay

The cytotoxic effect of the compounds of *A. tagala* was carried out in HeLa cells. The cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with heat-inactivated 10% fetal bovine serum, 100 µg/mL of antibiotics (penicillin and streptomycin), and incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C.[18] For the cytotoxic assay, cells were seeded in 96-well plates at the density of 1.5×10^3 cells/well. After 24 h of incubation at 37°C, cells were treated with increasing concentrations of the different fractions and compounds of *A. tagala* prepared in neat media (media without serum and 0.5% dimethyl sulfoxide) for 48 h. Cells treated with neat media only served as a control group. Cell viability was assessed by adding 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution in phosphate-buffered saline (PBS) to a final concentration of 5 mg/mL. The plates were then incubated at 37°C for an additional 4 h, and the MTT-formazan crystals were solubilized in dimethyl sulfoxide (100 µl) at 37°C for 30 min. The absorbance values of the solution in each well were measured at 570 nm using a microplate reader (Thermo Scientific Multiskan FC microplate photometer, USA).[18] Relative cell viability (%) was expressed as a percentage relative to the untreated control cells. All MTT experiments were performed in duplicate and repeated at least three times.

Annexin V-fluorescein isothiocyanate apoptosis assay

Cells were seeded in 24-well plates at the density of 0.1×10^6 cells/well. Cells were treated with S7 at a dose of 0.079 mg/mL and S10 at a dose of 1 mg/mL as described above. Cells were then trypsinized, centrifuged at 2000 g, and washed with 1X phosphate buffered saline (PBS) three times. Following centrifugation, cells were processed for apoptosis analysis by resuspension in buffer solution provided in the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit PF032 (Calbiochem) and proceeded as directed by the manufacturer. Cells were then incubated for 10 min with Annexin V-FITC and further incubated for 5 min with propidium iodide. Cells were analyzed using the BD LSRFortessa™ cell analyzer and BD FACSDiva 8.0.1 software, plotting at least 10,000 events per sample.

Morphological study by fluorescence microscopy using hoechst

Cells were seeded at a density of 0.3×10^6 on coverslips surface placed in 6 wells plates overnight. They were treated with S7 and S10 compounds for 24 and 48 h. Coverslips were removed carefully and placed in another 6 wells plate. They were fixed in 90% ethanol for 10 min at room temperature (rt). Cells were then washed with ×1 PBS thrice and stained with Hoechst for 15 min at rt in dark. The coverslips were mounted in slides using glycerol: PBS (1:1) mountant. The cells were then viewed under Nikon Eclipse 80i microscope at ×40 and photographed with Nikon DS-Ri1.[19]

Identification and characterization of compounds of *Aristolochia tagala* by liquid chromatography–high-resolution mass spectrometry and Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Liquid chromatography–high-resolution mass spectrometry (LC-HRMS) identification of compounds present in *A. tagala* was carried out in Agilent 6520 Quadrupole time-of-flight MS system hyphenated with an Agilent 1200 high-performance LC (HPLC) system (Agilent Technologies; Santa Clara, CA, USA). 1 mg/mL stock solution of aqueous-methanol extract was prepared in methanol and filtered through a 0.22-µm polyvinylidene difluoride (PVDF) membrane. The extract was further diluted to 0.5 mg/mL for analysis.

High-performance liquid chromatography conditions

The compounds were separated out in SunFire C18 column (250 mm × 4.6 mm, 5 μm) maintained at 25°C. The flow rate was set at 1.5 mL/min; a splitter was connected to allow 0.6 mL/min into the electrospray ionization (ESI) interface of the mass spectrophotometer. A sample volume of 10 μL was injected automatically by the autosampler. The mobile phase consists of acetonitrile (A) and 5 mM ammonium acetate buffer (B). The gradient program was 95% B for 0–6 min, 70% B for 6–12 min, 40% for 12–20 min, 20% for 20–26, and 95% for 26–30 min.[20]

Mass spectrometric conditions

The ion source for mass spectrometric analyses was ESI operated in positive mode. Nitrogen was used as drying and collision gas. The heated capillary temperature was set to 350°C and nebulizer pressure to 45 psi. The drying gas flow rate was 13 L/min. VCap, fragmentor, skimmer, and octapole radio frequency (RF) peak voltages were set to 3500 V, 150 V, 65 V, and 750 V, respectively, in the ion source parameters. Detection was carried out within a mass range of m/z 100–1000 and resolving power above 15,000 full width at half maximum. Mass Hunter software version B.04.00 build 4.0.479.0 (Agilent Technology) was used for the chromatographic and mass spectrometric analyses, including the prediction of chemical formula and exact mass calculation.[21]

LC tandem MS (LC-MS/MS) of isolated compound S7 was carried out in Waters Xevo triple quadrupole (XEVO-TQD) system hyphenated with waters alliance 2695 HPLC system (Waters, USA). 1 mg/mL stock solution of the compound S7 was prepared in methanol and filtered through a 0.22-μm PVDF membrane.

Liquid chromatography conditions

LC analyses were performed on a 250 mm × 4.6 mm, 5 μm, SunFire C18 column (Waters, USA), and the column temperature was set at 30°C. A volume of 20 μL of sample was injected automatically by waters alliance 2695 autosampler. The mobile phase used, and gradient program was the same as LC-HRMS analysis as described above.[20]

Mass spectrometry conditions

MS2 detection was carried on an XEVO-TQD (Waters, USA). ESI source was used for the analysis. The desolvation and cone gas flow were at 950 L/h and 30 L/h, respectively, capillary voltage 3500V, cone voltage 30V, source temperature 125°C, and desolvation temperature 350°C. Collision energy for MS/MS analysis was 10 eV. The range for the full ESI scan was set from 150 to 1000 in m/z, and the range for daughter ion scan was set from 50 to 400 in m/z. Precursor ions at m/z 359 was selected for daughter ion scan mode. Data acquisition and processing were carried out using MassLynx V4.1 SCN 714 software.[22]

Data and statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, Inc., USA) using one-way anova followed by Tukey's multiple comparisons test. Data are presented as mean ± standard deviation. Statistically significance was set at $P < 0.05$.

RESULTS

The separation and final purification of F II of *A. tagala* yielded thirteen compounds resolving at different retardation factor (*R*F) values. The compounds obtained is shown in Figure 1. Further purification was not carried out to avoid loss of compounds.

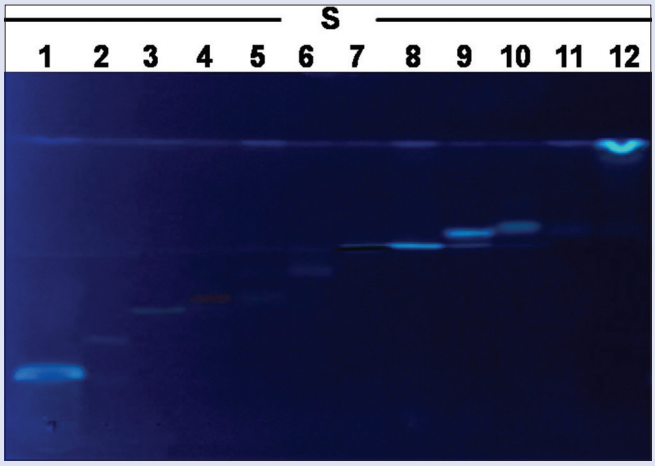

Figure 1: High-performance thin-layer chromatography profile of partially purified compounds of F II subjected to $CHCl_3$:MeOH (9:1 v/v, 1% acetic acid) viewed at 366 nm

In vitro cytotoxic activity

The cytotoxic activity of the crude aqueous-methanol extract (ATC) of *A. tagala* and the fractions (I–IV) was determined by MTT assay in HeLa cells. The result showed that the fractions (I–IV) were able to inhibit the proliferation of HeLa cells more than ATC in a dose-dependent manner and F II exhibited the highest inhibition with an inhibitory concentration $(IC)_{50}$ value of 0.320 mg/mL [Figure 2a]. Most of the semipurified compounds did not show any inhibition even at 4 mg/mL which is the highest dose selected. Only three compounds showed cytotoxic activity within the selected dose. Compound (S7) having an *R*F value of 0.43 (intense yellow crystalline powder) showed the highest antiproliferative activity followed by compound (S10) having *R*F value 0.59 (faint green crystalline powder). The IC_{50} value of S7 and S10 was 0.079 mg/mL (231.483 μM) and 1.074 mg/mL, respectively [Figure 2b].

Annexin V-fluorescein isothiocyanate apoptosis assay

The two compounds S7 and S10 were selected for apoptotic studies. The annexin V-FITC apoptosis assay was carried out for 24 h and 48 h posttreatment with compounds. Compound S7 (0.079 mg/mL) was able to induce apoptosis more effectively than S10 (1 mg/mL), and the effect was more pronounced after 48 h of treatment where about 32.3% and 12.15% of cells have entered early and late apoptotic stage, respectively [Figure 3]. From the apoptosis and cytotoxic activity assay, it was evident that S7 was the most effective. S7 was then identified using ESI-HRMS and LC MS/MS.

Morphological study by fluorescence microscopy

The effect of the compounds on cell viability was also evident when viewed under a microscope. There is a reduction in the number as well as the size, and the cells appeared to be shrunken and have lost their shape. The morphological changes in Hoechst stained cells indicated events of apoptosis where nuclear condensation, membrane blebbing, and nuclear fragmentation (marked with arrow) were observed. The control cells did not exhibit any of the above morphological changes, the nuclei were less stained in bright blue, and the color was homogeneous [Figure 4].

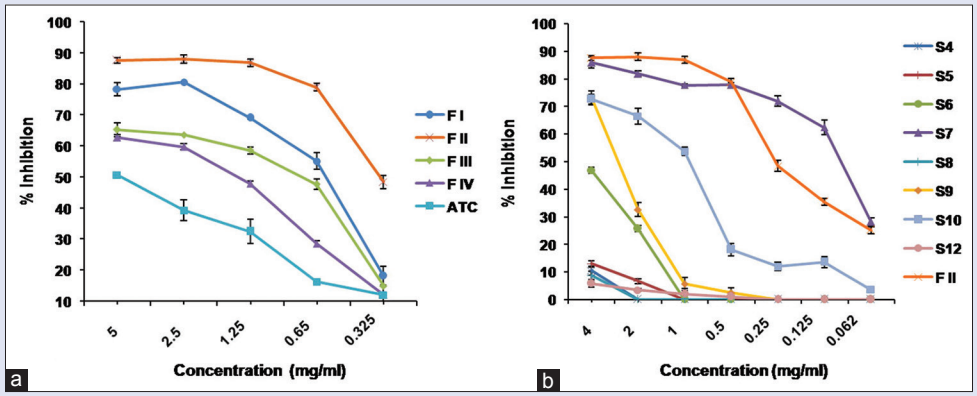

Figure 2: Percentage of inhibition of proliferation of HeLa treated with (a) varying concentrations of fractions (I–IV) and crude (ATC) of *A. tagala* and (b) varying concentrations of different compounds isolated from *A. tagala*

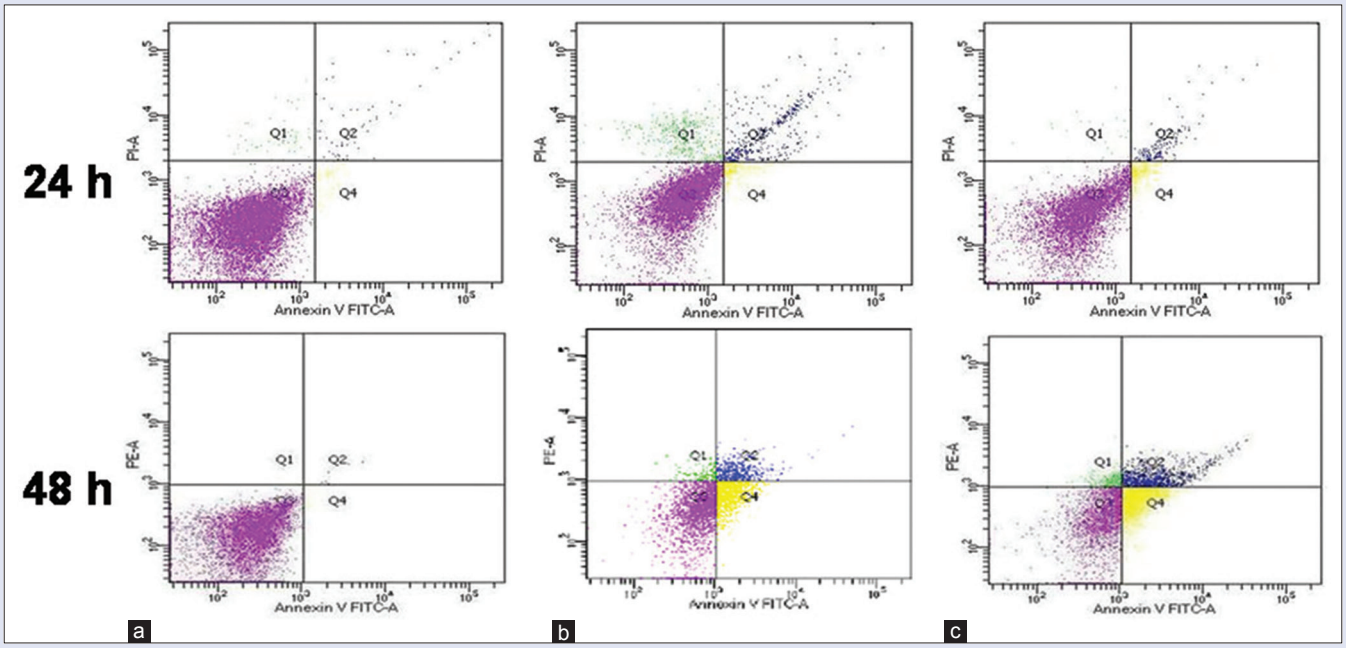

Figure 3: Representative fluorescence activated cell sorting pictogram of HeLa cells at 24 h and 48 h (a) untreated HeLa cells (b) treated with S7 (0.079 mg/mL) and (c) treated with S10 (1 mg/mL). Annexin-V positive cells (early apoptotic cells, lower right quadrant) and Annexin V/Propidium Iodide double positive (late apoptotic cells upper right quadrant)

Liquid chromatography -electrospray ionization high-resolution mass spectrometry and liquid chromatography tandem mass spectrometry analysis

HRMS techniques have been used for the identification of compounds from natural product; the high-resolution mass spectra generated gives the exact mass measurement in which the molecular formula of the compound can be determined, and the accuracy threshold for confirmation of the elemental composition was set at 5 ppm[23] which is widely accepted and reliable in the identification of compounds. The compounds identified in the LC-HRMS analysis of crude aqueous-methanol extract of *A. tagala* are listed in Table 1. They were compared to compounds listed in databases KNApSAcK (http://kanaya.naist.jp) and chemical database – Dictionary of Natural Products (http://dnp.chemnetbase.com).

The identification of partially purified compound S7 of *A. tagala* that showed maximum apoptotic activity against HeLa cells was identified by ESI-HRMS. The HRMS spectra of S7 showed adduct ions formation [M + Na] + at m/z 364.0453 and [2M+Na] + at m/z 705.1006 [Figure 5]. Further, LC analysis also showed adduct ions formation [M + NH_4] + at m/z 359 in ESI + mode and [M-H]$^-$at m/z 340 ESI-mode [Figure 6a and b]. LC-MS/MS of the ammonia additive precursor ions of S7 at m/z 359 gives daughter ions of m/z 342, 324, and 298 [Figure 6c]. The HRMS and LC-MS/MS confirmed the identity of the compound to be aristolochic acid I (AA-I).[24]

DISCUSSION

Many of the compounds present in *A. tagala* have been reported for various biological properties. Compounds such as aristolochic acid I, aristolactam IIIa, beta-sitosterol, kaempferol, and stigmasterol were previously reported in *A. tagala*[13-15] while other compounds have only

Table 1: Identification of compounds from the crude aqueous-methanol extract of Aristolochia tagala

Peak number	RT (min)	Molecular formula	HRMS, *m/z*, $[M+H]^+$		Error Δppm	Compound name
			Calc	Obs		
1	1.6	$C_{18}H_{15}NO_4$	310.1074	310.1082	0.36	Aristolactam C IIIa
2	1.8	$C_{15}H_{14}O_4$	259.0965	259.0962	1.3	Dehydrooxoperezinone
3	3.5	$C_{16}H_{22}N_2O_6$	339.1459	339.1551	0.36	Pyriferine A
4	4.4	$C_{20}H_{23}NO_4$	342.17	342.1706	−1.67	Isocorydine
5	4.4	$C_{20}H_{23}NO_5$	358.164	358.1644	1.54	Lagesianine A
6	6.5	$C_{15}H_{10}O_6$	287.055	287.0559	3.24	Kaempferol
7	6.54	$C_{29}H_{28}O_{15}$	617.1497	617.1501	0.59	3,5-Di-O-caffeoylquinic acid
8	6.7	$C_{22}H_{19}NO_9$	442.113	442.1137	1.2	Aristolactam IIIa; N-b-D-Glucopyranosyl isomer
9	8.3	$C_{22}H_{19}NO_9$	442.113	442.1137	1.2	Aristolactam IIIa; O-β-D-Glucopyranoside isomer
10	8.6	$C_{17}H_{11}NO_7$	342.0608	342.061	−0.42	Aristolochic acid I
11	8.8	$C_{15}H_{18}O_8$	326.0785	326.0790	0.58	4-O-beta-D-glucosyl-4-coumaric acid
12	10.28	$C_{15}H_{27}NO_6$	318.1908	318.1911	0.85	Leptantine
13	10.9	$C_{15}H_{22}O_2$	235.169	235.1691	0.74	Madolin W/K/A/R
14	11.6	$C_{12}H_{18}O_2$	195.138	195.1376	1.63	Perillyl acetate
15	15.47	$C_{29}H_{50}O$	415.2142	415.2141	3.76	Beta sistosterol
16	16.1	$C_{20}H_{30}O_3$	319.2268	319.2272	1.37	Δ-13,14-2-Oxokolavenic acid
17	20.6	$C_{16}H_{24}O_2$	249.1849	249.1853	1.56	Madolin L/M/S
18	21.2	$C_{15}H_{22}O$	219.1743	219.1746	−1.19	3-Oxoishwarane
19	23.7	$C_{15}H_{20}$	201.1638	201.1639	0.76	Aristolactone
20	25.4	$C_{29}H_{48}O$	413.3778	413.3775	0.78	Stigmasterol
21	26.1	$C_{29}H_{48}O_2$	429.3727	429.3728	0.25	Stigmastane-3,6-diol; (3b, 5a, 6a, 24R)-form, Diketone

RT: Retention time

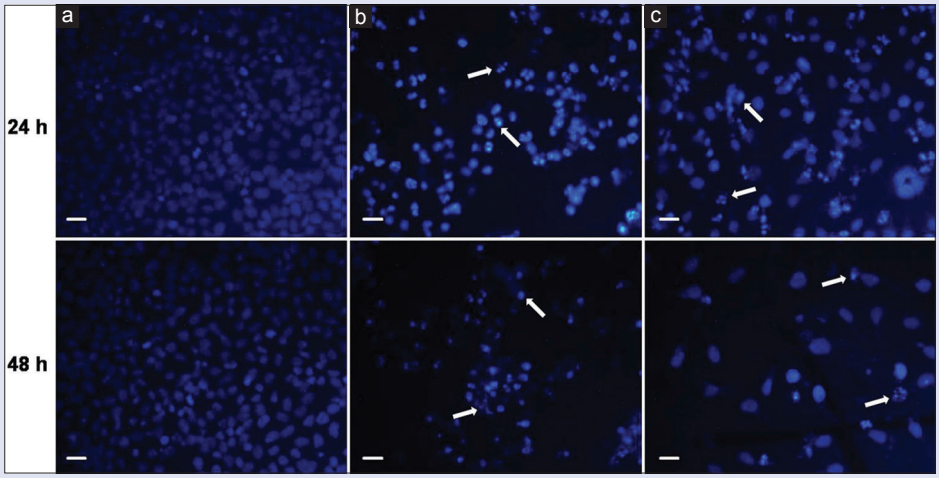

Figure 4: Fluorescence microscopic study of the morphological evaluation of HeLa cells stained with Hoechst after treatment with (b) S7 and (c) S10 for 24 h and 48 h compared to untreated (a), bar = 5 μm

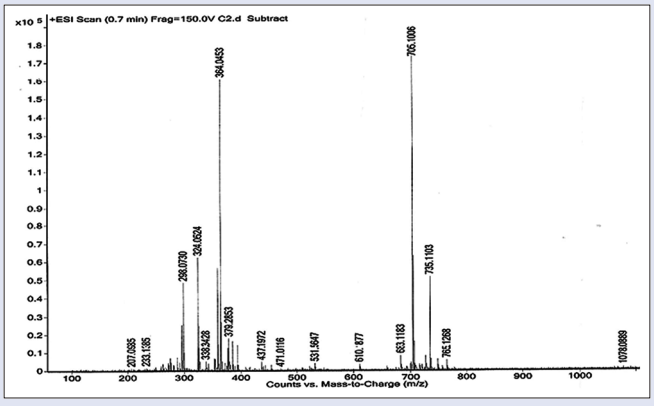

Figure 5: A full high-resolution mass spectrometry spectrum of AA-I from m/z 100 to m/z 1000, showing adduct ions formation (M + Na) + at m/z 364.0453 and (2M+Na) + at m/z 705.1006

been reported in other species of *Aristolochia* genus[15-25] and other plants.[44,45]

Caffeoylquinic acid (CQA) and its derivative are a phenylpropanoids that have been reported to have antioxidant, antibacterial, anticancer, and antihistaminic activity. A derivative of CQA has also been found to have neuroprotective effect on $A\beta_{1-42}$-treated SH-SY5Y cells.[26] Various studies have reported the anticancer activity of plant steroids, beta-sitosterol and stigmasterol.[27-30] Isocorydine an alkaloid monomer has been reported to induce apoptosis in HCC cell lines, decreased tumor volume of xenografts mice, it also enhanced the efficacy of treatment of doxorubicin when used in combination in xenograft models. The structural modification was reported to significantly improve the biological activity of this alkaloid.[31-33] Kaempferol extracted from this plant has been reported to have anti-inflammatory activity in carrageenan-induced paw edema rats and stimulated macrophages,[13] and a number of other studies on the anticancer and anti-inflammatory activity have also been reported.[34-37] Dehydrooxoperezinone was reported to displayed anti-HIV activity in

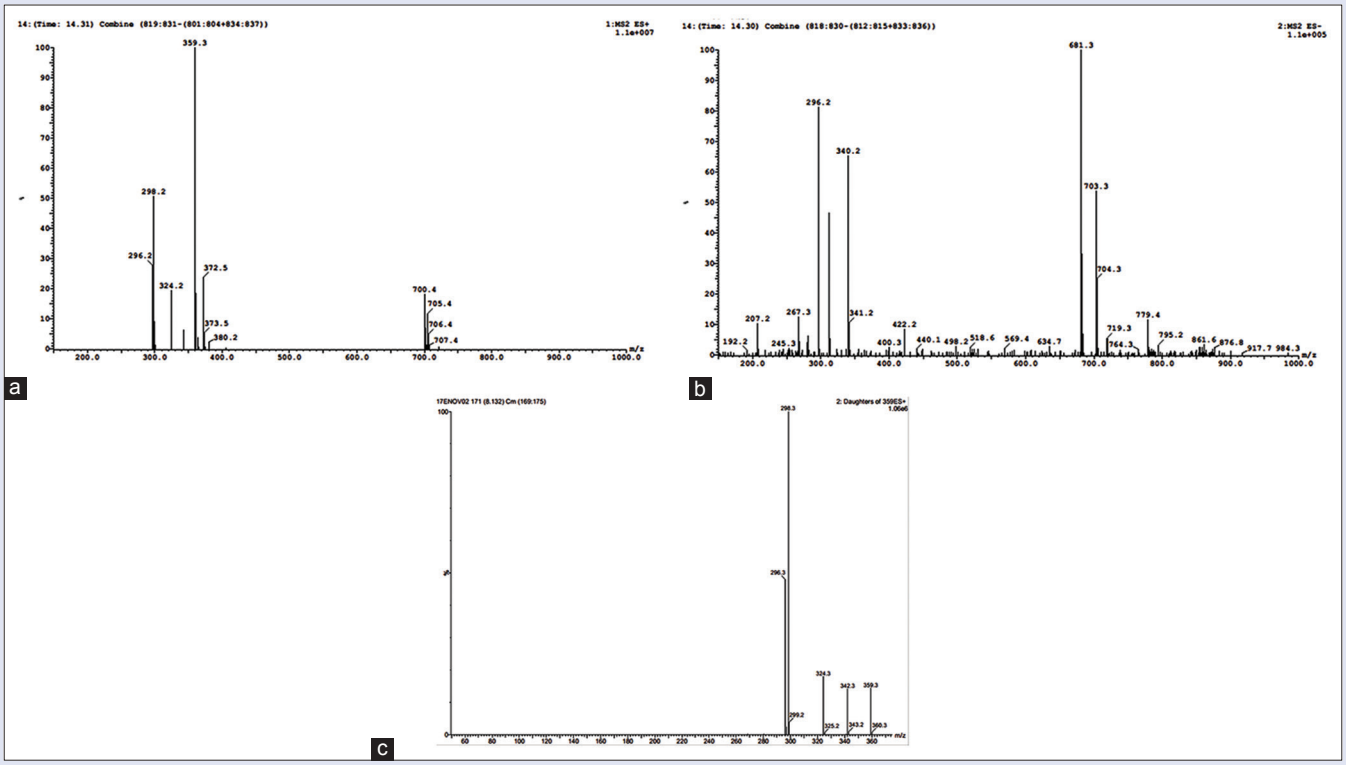

Figure 6: A full mass spectrum of AA-I from m/z 100 to m/z 1000, (a) electrospray ionization + mode showing considerable intensity of $(M + NH_4)$ + ions at m/z 359, (b) electrospray ionization mode showing $(M-H)^-$ ions at m/z 340, (c) daughter ion spectra of the ammonia additive precursor ions of AA-I at m/z 359. Distinctive fragments of ions of AA-I were observed at m/z 298

acutely infected H9 lymphocyte cells.[16] Aristolochic acid has earlier been reported to have antitumor effect in methylcholanthrene-induced mice and scarcoma-37 implanted mice[38,39] and anticancer effect in 4-nitroquinoline 1-oxide-induced oral cancer Wistar rats.[40] Since several compounds present in this plant have been reported for its anticancer and other biological properties, it was of interest to identify the compound (s) responsible for the apoptotic and antiproliferative activity against HeLa cells.

A. tagala has been used in traditional medicine for various diseases, the present of the active constituent aristolochic acid I though it showed apoptotic effect against HeLa cells and has been reported to have antitumor and anticancer properties is a concern since the compound have also been reported to have nephrotoxic effect.[41] Various reports on the use of this plant and other genus in low dose have been shown to have no toxicity.[40-43] We have also not observed any toxicity symptom in our studies with the crude extract at low doses.[7] The used of this plant at low doses of administration may have not produced any side effects as the concentration of AA-I may be very less in the whole crude extract. The present of other compounds may have also counter effect the toxicity of AA-I.

CONCLUSIONS

Traditional system of treatment has been found to be successful in the treatment of various diseases and has been a source of remedy for several patients. Although these forms of treatment may have been quite successful, we cannot rule out the possible side effects of using the crude extracts containing several compounds in them that can exert different types of physiological reactions. Identification of compounds present in herbal plants is, therefore, required to eliminate any unwanted outcomes and to understand the efficacy, toxicity, and mechanism of action of each compound. *A. tagala* has been used in traditional medicines for the treatment of various diseases, and the present of compounds such as isocorydine, kaempferol, and beta-sitosterol must have contributed to their biological property. Although there are reports of the anticancer property of aristolochic acid previously and there are also reports of its nephrotoxicity, the used of *A. tagala* in its crude form is still a concern. However, since there are also a number of other compounds with biological activity, this plant and its related species can be exploited for the extraction and isolation of these compounds which shows no toxicity.

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Conflicts of interest

There are no conflicts of interest.

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