Original Article

Active Fractions of Dichloromethane Extract of *Artemisia turanica* Inhibit Proliferation of HeLa and KB Cell Lines via Apoptosis Induction

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Abstract

Purpose: *Artemisia* is an important genus of Iranian flora. The current study on the aerial parts of *A. turanica* was conducted to determine the most potent extract and its fractions in the cytotoxic assays. **Materials and Methods:** The cytotoxic effects of 13 fractions (1–13) from dichloromethane extract on three cancer cell lines (KB, HeLa, and U87MG) were assessed. Preliminary phytochemical analysis of more potent cytotoxic fractions was carried out using thin-layer chromatography (TLC) and different spray reagents. **Results:** Dichloromethane extract showed the best bioactivity against cancerous cell lines. Fractions 4, 6, 7, and 9 of this extract had potential effective components in the inhibition of the proliferation of KB cancer cells. In addition, fractions 4 and 6 were able to inhibit the growth of HeLa cell line. The active fractions decreased the mitochondrial membrane potential level, and played a critical role in caspase-3 and 9 activation and generation of reactive oxygen species. The cytotoxic activity of these fractions was found to be not significant toward U87MG cells. TLC analysis suggested the probable presence of terpenoids as the main component of most of the selected fractions. **Conclusion:** The species is suggested as the potential source of cytotoxic phytochemicals.

Keywords: *Apoptosis, Artemisia turanica, cytotoxicity, HeLa, KB, U87MG*

Introduction

The genus *Artemisia* belongs to the tribe Anthemideae (family Asteraceae) with about 500 species, which is mainly distributed in the temperate zones of Asia, Europe, and North America.[1] About 34 *Artemisia* species have been identified in Iran.[2] One of them, *Artemisia turanica* Krasch., grows in the northeastern region of the country.[3] The major volatiles of the leaves and aerial parts of *A. turanica* are mono- and sesquiterpenoids, including among others, α-thujone, 1,8-cineole, chrysanthenone, and davanone.[4,5] The antimicrobial effects of methanolic extract,[6] the leishmanicidal activity of ethanolic extract,[7] and the antimalarial activity of various extracts of *A. turanica*[8-10] have been reported. The plant species is known as a source of antioxidant phytochemicals such as isomers of isochlorogenic acid.[11] Some studies have demonstrated the *in vitro* neuroprotective and the *in vivo* hepatoprotective potential of *A. turanica*.[12,13] Despite many reports about the cytotoxic and apoptotic properties of various extracts of different species of the genus *Artemisia*,[14-16] a few studies have demonstrated the cytotoxic and apoptogenic activities of *A. turanica* extracts.[17,18] To the best of our knowledge, there is no previous study on bioassay-guided fractionation and preliminary phytochemical analysis of more potent cytotoxic fractions of the plant species. This motivated us to examine cytotoxic and apoptotic effects of different fractions of dichloromethane (DCM) extracts toward two HeLa and KB and U87MG cell lines.

Methods and Materials

Plant material

The aerial parts of *A. turanica* Krasch. were collected from Samie Abad, Torbat-e Jam (Razavi Khorasan province, Iran) and were identified by Dr. Valiollah Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimen (No. 12572) has been deposited at the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Extraction and fractionation

Powdered and dried aerial parts of *A. turanica* (340 g) were successively extracted using

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petroleum ether (40:60), DCM, ethyl acetate, ethanol, and ethanol-to-water (1:1 v/v) at room temperature, respectively. In our preliminary study, because of the potent cytotoxic activity of DCM extract, the concentrated defatted DCM extract (35 g) was fractionated by normal phase vacuum liquid chromatography on silica gel using successive step gradients of heptane-to-ethyl acetate (8:2 to 0:10) and ethyl acetate-to-methanol (8:2 to 0:10). The procedure afforded 13 fractions (1–13) from DCM extract of *A. turanica*.

Thin-layer chromatographic analysis of selected fractions

Thin-layer chromatography (TLC) separations of fractions 4–11 were performed using commercially available plates (silica gel 60 F_{254}-precoated TLC plates; Merck, Germany) and suitable solvent systems at room temperature. For the detection of the main compounds, different spray reagents (Dragendorff, potassium hydroxide, vanillin-sulfuric acid, and Liebermann–Burchard reagents) were used. The characteristic components were also detected via observing the developed chromatograms under long- and short-wave ultraviolet (UV) light (366 and 254 nm, respectively).[19]

Cytotoxic study

HeLa, KB, and U87MG cells were purchased from Pasteur Institute (Tehran, Iran), kept at 37°C in a humidified atmosphere (95%) containing 5% CO_2, and cultured in Dulbecco's modified Eagle medium (DMEM/F12) supplemented with 10% v/v heat-inactivated fetal bovine serum, penicillin, and streptomycin 1%. The cytotoxic potential of prepared fractions was examined against cell lines by colorimetric assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).[20]

Intracellular reactive oxygen species measurement

Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA). Briefly, KB and HeLa cell lines were treated by fractions 5, 6, 7, 9 and fractions 4, 6, 7, 8, 9, 10, 11, respectively, and also, the controls in each cell line were exposed to certain concentration of dimethyl sulfoxide. The concentration of fractions used is based on IC_{50}, which is shown in Table 1. After washing with phosphate-buffered saline, the cells were incubated with 20 µL of DCF-DA at 37°C for 30 min and were prepared to be lyzed with Triton X-100. Fluorescence intensity was measured by using microplate reader (ELX 800; BioTek, Winooski, VT) with excitation wavelength at 448 nm and emission wavelength at 528 nm.

Estimating activities of caspase-3, 8, and 9

The activity of caspase-3 was determined by the Sigma colorimetric caspase-3 kit, according to the manufacturer's instruction. The activity of caspase-8 and 9 was determined as previous procedure.[21] Briefly, the labeled substrate DEVD-pNA was cleaved by existing caspase-3 into the chromophore substance (*p*-nitroanilide [pNA]) resulting in the enzyme activity measurement spectrophotometrically. The signal intensity was proportional to the enzyme activity. The concentration of fractions used is based on IC_{50}, which is shown in Table 1.

Table 1: The IC_{50} values (µg/mL) of isolated fractions from dichloromethane extract of *Artemisia turanica* in human cancer cell lines

Fractions	Cancer cell lines		
	KB	HeLa	U87MG
1	120	26	>125
2	101	38	>125
3	100	50	>125
4	49	23	>125
5	25.5	26	>125
6	24	23	>125
7	25	23	>125
8	50	19	>125
9	25	22	>125
10	37.5	20	>125
11	35	20	>125
12	75	56	>125
13	90	>125	>125

Mitochondrial membrane potential measurement

To define the mitochondrial function, a key indicator of cell health, Rhodamine 123, a cationic dye, was used to monitor changes in mitochondrial membrane potential (MMP); the earliest change occurred through apoptosis that led to a loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity. The concentration of fractions used is based on IC_{50}, which is shown in Table 1.

Statistical analysis

All the data were analyzed using one-way analysis of variance using GraphPad Prism software (version 9; San Diego, CA). Differences between the mean ± SEM of samples were considered significant at $P < 0.05$. The IC_{50} values were generated from the MTT results using GraphPad Prism software.

Results

Thin-layer chromatographic analysis of selected fractions

The developed chromatograms of almost all selected fractions (except for fraction 11 with weak quenching) showed zones with prominent fluorescence quenching under short-wave UV light. After spraying ethanolic potassium hydroxide reagent, the developed chromatograms of fractions 4–6 showed neither change in color of spots in daylight nor intensification of red fluorescence in UV 366 nm. No characteristic fluorescence under UV 366 nm was noticed in fractions 7–9. The blue and violet fluorescence under long-wave UV were observed for fractions 10 and 11, respectively. Similarly, after spraying ethanolic potassium hydroxide reagent, no change in fluorescence of

fractions 10 and 11 was observed. The sprayed TLC plates with vanillin-sulfuric acid and Liebermann–Burchard reagents after heating for 10 min at 100°C were inspected in UV 366 nm or visible and the presence of a number of spots with visible colors was noticed in all of the selected fractions. No colored spots in the TLC plates of selected fractions were observed on spraying Dragendorff reagent.

Cytotoxic activity of fractions

HeLa, KB, and U87MG cell lines were treated with different fractions of DCM extract of *A. turanica* at concentrations between 0 and 125 µg/mL. According to IC_{50} concentration for each individual cell line, some potent fractions have been selected. A glance at Table 1 showed that fractions 5–7 and 9 were potent to kill more than 50% of cancerous cells and successfully reached IC_{50} against KB cells. Moreover, fractions 4, and 6–11 exhibited outstanding antiproliferative effects against HeLa cells. As an example, fraction 8 was able to kill 50% of HeLa cells at 20 µg/mL. According to poor IC_{50} results for U87MG, we preferred to abandon this cell line and continue the following process with two remaining cell lines [Table 1]. The IC_{50} values were much higher than those observed in KB and HeLa cell lines at the same incubation period.

The effect of the selected fractions on reactive oxygen species generation

To measure oxidative stress induced by active fractions, fluorescent dye DCF-DA was used to measure ROS production. As shown in Figure 1, most of the fractions had a significant effect on ROS level. Fractions 6, 7, 9, and 11 were able to increase significantly the level of ROS compared with the control group in HeLa cells. Moreover, fraction 7 with a 74% increase in ROS level caused significant ROS enhancement and induced oxidative damage in KB cells.

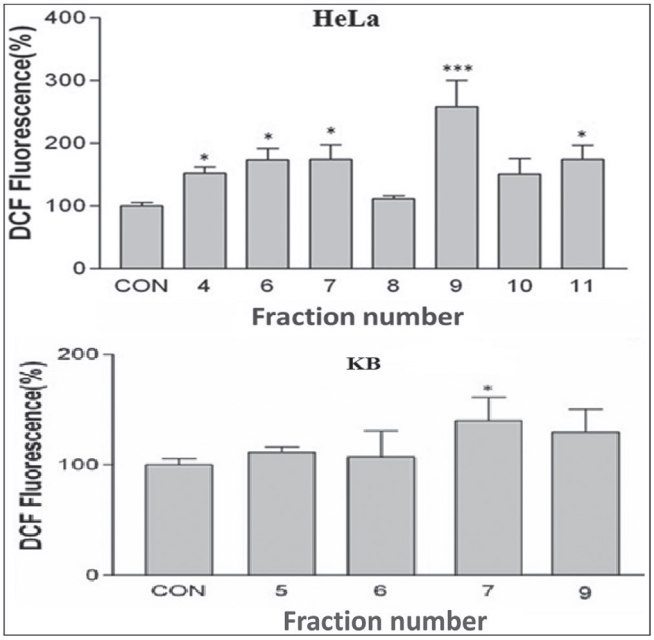

Figure 1: The effect of different fraction of dichloromethane (DCM) extracts on reactive oxygen species (ROS) overproduction in HeLa and KB cell line. (Mean ± SEM, *n* = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control group

Determining the activities of selected fractions on caspase-3, 8, and 9

In the current study for improvement of MTT results and also characterizing the type of mechanism of cell death involved in our experiments, the activity of caspases was evaluated. The obtained results for KB cell line [Figures 2–4] showed that fractions 6, 7, and 9 played the most critical role in caspase-3 activation. To determine which apoptotic pathway is activated by fractions, we evaluated the activation of caspase-8 and 9, the apical proteases in extrinsic and intrinsic pathways, respectively. Our results identified that fractions 6 and 7 significantly increased caspase-9 activations, and conversely, none of them had a significant role in the caspase-8 activity. According to the results for HeLa cells, fractions 4, 6, 7, and 9 caused stimulating caspase-3 activity; fractions 4 and 6 caused the same effect on caspase-8; and fractions 4, 6, 7, 8, and 9 had the same effect on caspase-9.

The effects of selected fractions on mitochondrial membrane potential collapse

To evaluate the role of mitochondria in the apoptosis induced by the most potent fractions, Rhodamine 123 was used. Fractions 7 and 9 decreased the MMP of KB cell by 29% and 8.02%, respectively, and fractions10, 9, 4, 6, 7, and 11 of DCM extracts decreased the MMP of HeLa cells by 31%, 21%, 17%, 19%, 14%, and 20%, respectively. Thus, they were able to reduce the MMP of these cells at their IC_{50} concentrations [Figure 5].

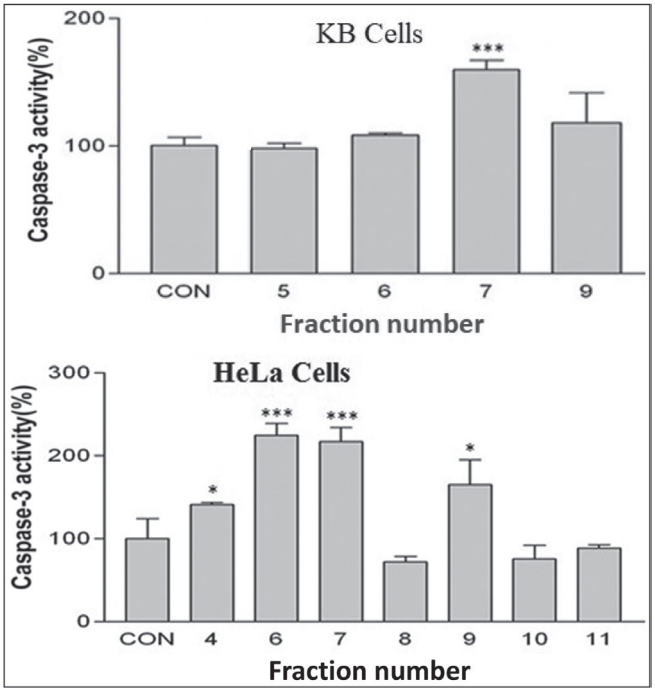

Figure 2: Caspase-3 activity in HeLa and KB cell lines. Caspase-3 activity measured by colorimetric detection of *p*-nitroanilide and expressed as percentage of control. (Mean ± SEM, *n* = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control group

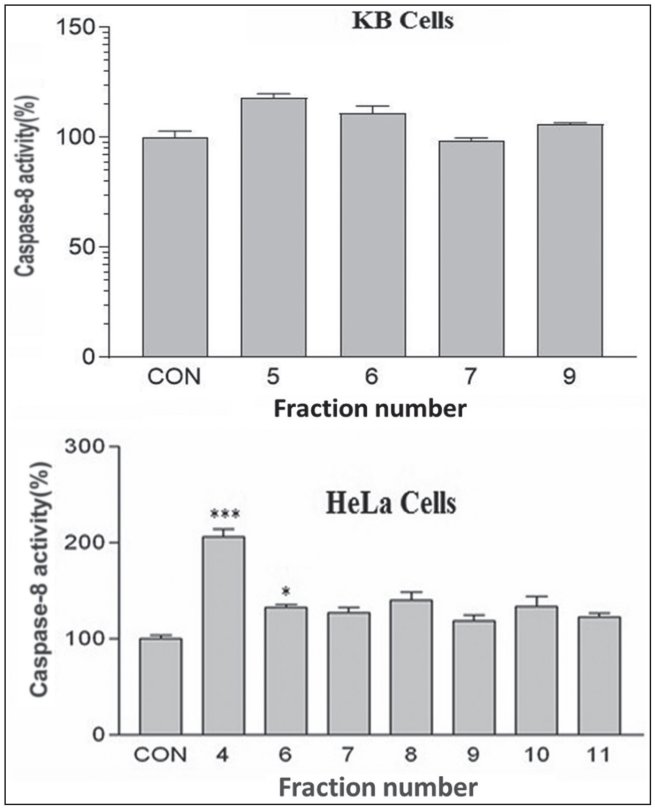

Figure 3: Caspase-8 activity in HeLa and KB cell lines. Caspase-8 activity measured by colorimetric detection of *p*-nitroanilide and expressed as percentage of control. (Mean ± SEM, *n* = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control group

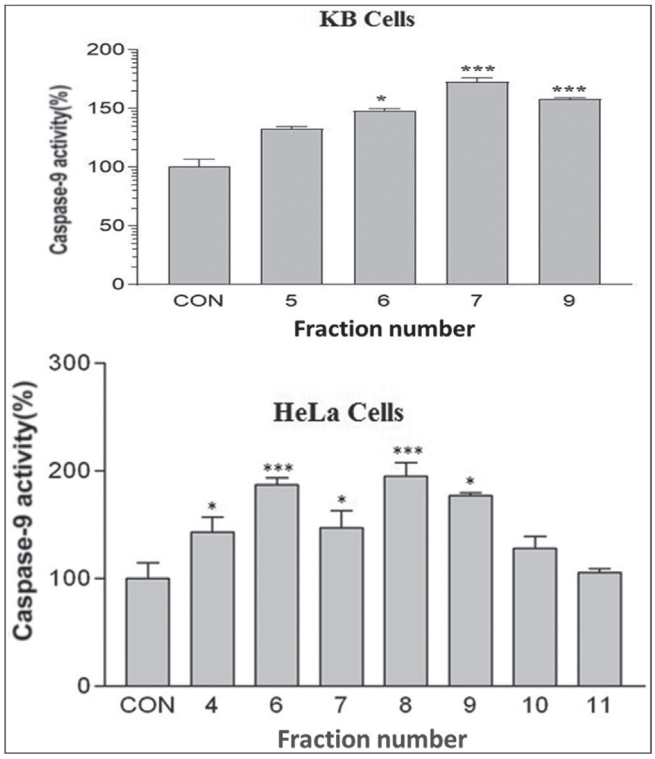

Figure 4: Caspase-9 activity in HeLa and KB cell lines. Caspase-9 activity measured by colorimetric detection of *p*-nitroanilide and expressed as percentage of control. (Mean ± SEM, *n* = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control group

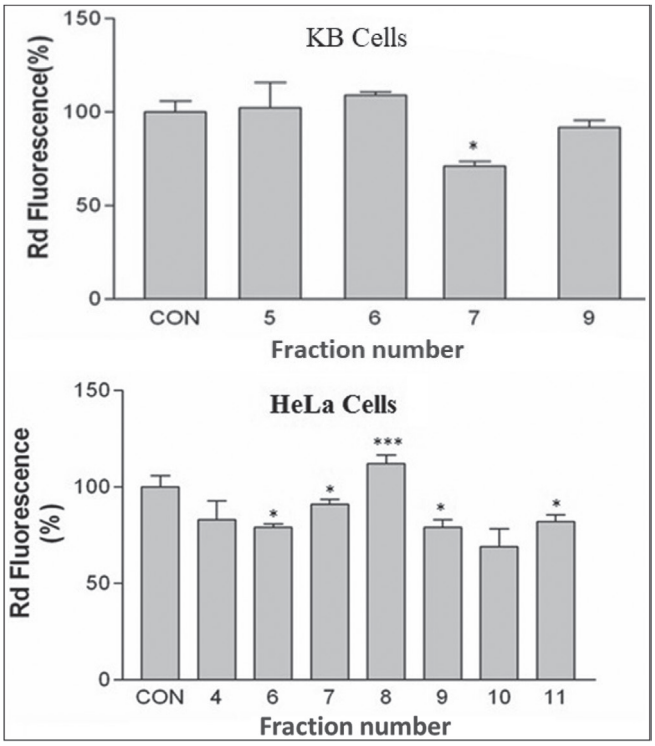

Figure 5: Effect of different fraction of dichloromethane (DCM) extracts on mitochondrial membrane potential (MMP) collapse in HeLa and KB cell lines. (Mean ± SEM, *n* = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control group

Discussion

Numerous active natural products against cancer cells have been found from plant origin. They may inhibit microtubule, topoisomerase, and heat shock protein 90, or affect the cell cycle and protein synthesis, for example.[22] Cytotoxic activity of different species of Iranian flora, including *Artemisia* species,[23,24] has been studied. The crude hydroethanolic extract of *A. turanica* did not exhibit strong toxicity against the HepG2 cell line.[17] This agrees with the results of the current study that showed the most potent cytotoxic phytochemicals of the plant species have been extracted by DCM.

A previous study has examined the cytotoxic and apoptotic effects of various extracts of *A. turanica* on two human leukemic cancer cell lines (K562 and HL-60). DCM extract of *A. turanica* showed the most potent antiproliferative effect among all tested extracts with IC_{50} values of 69 and 104 µg/mL on K562 and HL-60 cells, respectively. The sub-G1 peak in flow cytometry histogram confirmed induction of apoptosis by DCM extract.[18] In the similar studies, the cytotoxic effect of DCM extracts of *A. aucheri*[25,26] and *A. armeniaca*[14] against the various cancerous cell lines including SKNMC, MCF-7, A2780, HL-60, and K-562 has been reported. DCM extract of *A. diffusa* (IC_{50} values of 71 and 42 µg/mL in HeLa and HT-29 cells, respectively), the DCM fractions of *A. santolina* (IC value of 91 µg/mL in HT-29 cells), and *A. ciniformis* (IC_{50} values of 35, 94, and 29 µg/mL in AGS, HT-29, and MCF-7 cells, respectively) were all effective in inhibiting the proliferation of a panel of cell lines.[27]

The current study examined different fractions obtained from DCM extract of *A. turanica* with the aim of assessing cellular toxicity and induction of cellular apoptosis. At first DCM extract was separated into 13 fractions.

In the next step, the cytotoxicity of all fractions was determined by the MTT method. Fractions 5, 6, 7, and 9 had higher cell growth inhibition potency toward KB cells, and fractions 4, 6, 7, 8, 9, 10, and 11 induced significant toxicity in HeLa cells. Since the induction of cell death through apoptosis is one of the important goals in the design of chemotherapy drugs, the present study examined the apoptotic effects of the fractions by examining well-characterized apoptosis markers in human carcinoma cell lines.

Fractions 6, 7, 8, 9, and 11 reduced MMP in HeLa cells, and fraction 7 reduced MMP in KB cells. Caspase-3 and 9 activities increased in HeLa cell line after exposure to fractions 4, 6, 7, and 9. In addition, the enzymatic activity of caspase-8 increased after exposure to fractions 4 and 6 for the HeLa cells. In addition, the enzymatic activity of caspase-8 increased after exposure to fractions 4 and 6 for the HeLa cells. In addition, the enzymatic activity of caspase-8 increased after exposure to fractions 4 and 8 for the HeLa cells. ROS levels decreased after exposure to fractions 6, 7, 9, and 11 for the HeLa cell line and exposure to fraction 7 for KB cell line. Overall, these results demonstrated that fraction 7 induced apoptosis in KB cells through the intrinsic pathway. In HeLa cells, fractions 4 and 6 inhibit proliferation through both pathways of apoptosis, whereas 24-h exposure to fractions 7 and 9 activates only the mitochondrial pathway.

The results of preliminary TLC analysis of selected fractions (4–11) suggested that three fractions (4–6) contained large amounts of chlorophyll. The alkaloids could not be considered as the main constituents of any fraction. Also, there was no evidence about the presence of significant amounts of secondary metabolites including coumarins and anthracene derivatives in fractions 10 and 11. TLC analysis of the current study suggested the probable presence of terpenoids as the major components of fractions 7–11, and as the minor constituents of fractions 4–6. A previous study showed that the terpenoid extract of *A. turanica* probably contained a little amount of sesquiterpene lactones.[2,28] Davanone, which has no lactone moiety in its structure, is known as the major sesquiterpenoid in the essential oil of *A. turanica*.[4] The cytotoxic activity of davanone-type sesquiterpenoids[29] may help to justify the results of the current study. Further phytochemical study on the active fractions should be performed to isolate and identify the pure component(s) responsible for the cytotoxic activity of the selected fractions.

Conclusion

In general, the results showed that for HeLa cells, fractions 4, 6, 7, and 9 from the DCM extract of *A. turanica*, and for KB cells, fraction 7 extract, were able to activate apoptotic pathways. The probable presence of terpenoids might be related to the reported cytotoxic effects on cancerous cell lines after exposure to selected fractions. The species is suggested as the potential source of cytotoxic phytochemicals and might be useful candidates for further study on the treatment of cervical and oral epithelial cancers.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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