

## Original Research Article

# Cytotoxic activity and apoptotic induction of some edible Thai local plant extracts against colon and liver cancer cell lines

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### Abstract

**Purpose:** To evaluate eight edible Thai local plant extracts (*Camellia sinensis*, *Careya sphaerica*, *Cratoxylum formosum*, *Eleutherococcus trifolius*, *Ficus auriculata*, *Persicaria odorata*, *Schima wallichii*, and *Vaccinium sprengelii*) against colon and liver cancer cell lines.

**Methods:** The 80 % ethanol plant extracts were screened for cytotoxic activity against human colon adenocarcinoma (HT-29) and human hepatocellular carcinoma (HepG2) cells by MTT assay. The 50 % cytotoxic activity concentration (CC<sub>50</sub>) was then determined. Apoptotic cell death was observed by inverted microscopy and DNA fragmentation using agarose gel electrophoresis.

**Results:** *P. odorata* and *S. wallichii* extracts showed strong cytotoxic activity, with the latter exhibiting more potent cytotoxic activity than the former. The CC<sub>50</sub> value of *S. wallichii* extract was 453 and 367 µg/mL against HT-29 and HepG2 cells, respectively. In contrast, *P. odorata* extract showed CC<sub>50</sub> value of 775 µg/mL against HT-29 and 1665 µg/mL against HepG2 cells. Microscopic observations indicate that the degree of morphological changes was concentration-dependent. The cell lines treated with both plant extracts displayed apoptosis.

**Conclusion:** The two plant extracts have high potentials for medicinal use in colon and liver cancer management. However, further studies are needed to isolate the active compounds responsible for the cytotoxic activities.

**Keywords:** Edible Thai local plants, Cytotoxic activity, Apoptotic induction, Colon cancer, Liver cancer

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## INTRODUCTION

The numbers of cancer patients have increased every year and cancer is the second cause of death worldwide. This non-communicable disease is characterized by uncontrollable

proliferation of abnormal cells. It is accompanied by uncontrolled development of cells, which have a tendency to multiply, and in some cases, to spread into surrounding tissues. Among various types of cancer, colorectal cancer and hepatocellular carcinoma are reported to be the

most common forms [1]. Until recently, cancer patients have been treated with combinations of several therapies: chemotherapy, cytotoxic drugs, radiation, and surgical resection. However, these combined treatments, in particular, chemotherapeutic drugs produce significant side effects from which patients often suffer more than the from the cancer itself.

To alleviate these side effects, novel or alternative measures have been sought for drugs as treatments for cancer patients. One possible approach is the use of natural product therapy, a widely accepted alternative for cancer treatment. Because this therapy is based on plants or plant extracts, it usually has less side effects, and lower costs for treatment. Recent studies indicated that some medicinal plants may have anticancer effects. Hajiaghaalipour *et al* [2] found that *Camellia sinensis* extract exhibited anti-proliferative effects on HT-29 cells. Devika and Mohandas [3] reported that extracts of *Foeniculum vulgare* induced apoptosis in cervical cancer cells and had anti-proliferative effects through DNA fragmentation. Manapradit *et al* [4] showed that butanolic leaf extracts from *Barleria strigosa* exhibited the highest cytotoxicity against the P-388 cell line with a CC<sub>50</sub> of 127.42 µg mL.

Many plants growing in Thailand, including *Camellia sinensis*, *Careya sphaerica*, *Cratoxylum formosum*, *Eleutherococcus trifolius*, *Ficus auriculata*, *Persicaria odorata*, *Schima wallichii*, and *Vaccinium sprengelii* have been reported to have high polyphenol content and antioxidant activities [5].

Therefore, they were selected to investigate their cytotoxic and apoptotic abilities in colon and liver cancer cell lines.

## EXPERIMENTAL

### Plant materials and reagents

All plant materials were harvested from May to August in 2014 (Table 1). *C. sphaerica* and *C. formosum* were collected from Yasothon Province in the area of Khumkeunkaew District, while the other six plants were collected from Chiang Mai Province in the Fang District. The species of plants were identified by the Royal Project Foundation of Thailand. The voucher specimens of the plants were kept in the herbarium of Faculty of Agro-Industry at King Mongkut's Institute of Technology Ladkrabang (KMILT). The reagents, Rosewell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), trypsin, and MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Gibco, (El Paso, TX, USA). The blood DNA extraction kit and DNA markers were purchased from Vivantis, (Oceanside, CA, USA). Mitomycin C was purchased from Kyowa Hakko Kirin, (Tokyo, Japan).

### Preparation of plant extracts

Samples were dried at 50 °C in a tray dryer until the moisture content was below 10 %. After cutting the dried sample into approximately 1 cm lengths, it was ground to a powder. The sample powder (25 g) was blended with 125 mL of 80 % aqueous ethanol and extracted twice. The samples were first shaken at room temperature for 8 h and then filtered; the residues were extracted again using a ratio of 1:3 (dried sample : solvent) for an additional 8 h. The extracts were combined and filtered using a 0.45 µm filter and then concentrated in a rotary evaporator.

**Table 1:** Botanical name part used, and collection number of the eight edible Thai local plants

Botanical name	Thai local name	Part used	Voucher no.
<i>Camellia sinensis</i> (L.) Kuntze var. <i>assamica</i> (J.Masters) Kitam.	Miang pa, Miang, Miang Doi	Leaf	JUN2010
<i>Careya sphaerica</i> Roxb.	Kradon, Kradonbok	Leaf	MAY2011
<i>Cratoxylum formosum</i> (JACK) Dyer spp.	Tio khao, Tio	Leaf	MAY2010
<i>Eleutherococcus trifolius</i> (L.) S.Y. Hu	Phak paem, Paem	Stem and leaf	MAY2013
<i>Ficus auriculata</i> Lour.	Duea wa	Leaf	JUN2013
<i>Persicaria odorata</i> (Lour.) Soja'k	Phak phai, Phak paew	Stem and leaf	AUS2010
<i>Schima wallichii</i> (DC.) Korth	Talo, Mung Tan	Leaf	AUS2010
<i>Vaccinium sprengelii</i> (G.Don) Sleumer	Som Pi, Maohin, Maohuawaen, Sompae, Somsad	Leaf	JUN2010

Dry crude extracts of 0.2 g were dissolved in 1 mL of 100 % dimethyl sulfoxide (DMSO) and 9 mL of phosphate-buffered saline (PBS) was added to give a final concentration of 20 mg/mL. The samples were kept at - 20 °C until use.

### Cell culture

Colon cancer (human colon adenocarcinoma, HT-29) and liver cancer (human hepatocellular carcinoma, HepG2) were obtained from the National Cancer Institute in Thailand. Cells were grown in medium (RPMI 1640 containing 8 % FBS) and gentamycin at 50 µg/mL). The cells were cultured at 37 °C in humidified air with 5 % CO<sub>2</sub>.

### MTT proliferation assay

The MTT assay has been widely used as an indirect measure to determine the viability of cells. It is a rapid and highly accurate colorimetric test that measures the decrease in the conversion of the MTT reagent (yellow colour) by mitochondrial succinate dehydrogenase. The MTT passes into the cell mitochondria membrane, and viable cells transform the yellow-coloured MTT to a purple-coloured formazan crystal. The MTT assay followed the method of Mosmann [6] with some modifications. HT-29 and HepG2 cell lines were grown overnight in 96-well tissue culture plates with 1x10<sup>5</sup> cells per well. Then, the extract at a final concentration of 2,000 µg/mL was added for cytotoxic screening. The cells treated with mitomycin C (cytotoxic drug) at 50 µg/mL were used as a positive control while negative control cells were treated with 0.2 % DMSO. Untreated cell cultures were used as a control and blank wells contained 100 µL of medium without cells. The cells were cultured at 37 °C for 21 h under 5 % CO<sub>2</sub> atmosphere. Then, 50 µL of MTT (2 mg/mL) was added to each well for further incubation for 3-4 h. MTT crystals were solubilized with 100 µL of a mixture of DMSO and absolute ethanol (ratio 1:1). The absorbance of each well was measured at 570 nm using a microplate reader. The percentage of cytotoxicity was determined using Eq 1.

$$\text{Cell cytotoxicity (\%)} = \left( 100 - \frac{As - Ab}{Ac - Ab} \right) \times 100 \dots (1)$$

where As, Ab and Ac were the absorbances of the treated sample, blank, and control samples, respectively.

Plant extracts were selected and tested for the 50 % cytotoxic concentration (CC<sub>50</sub>). The final concentrations were 250, 500, 1,000, 2,000, and 4,000 µg/mL. The CC<sub>50</sub> values for growth

inhibition were computed using GraphPad Prism 5 (La Jolla, CA, USA). All experiments were performed in triplicate.

### Morphological analysis

HT-29 and HepG2 cells were treated with extracts at concentrations of 500 and 4,000 µg/mL for 24 h. Upon completion of incubation, morphological changes of cells were monitored under an inverted light microscope at 200x magnification.

### Apoptotic DNA ladder assay

DNA fragmentation analysis was carried out using agarose gel electrophoresis as described by Herrman *et al* [7] with some modifications. The HT-29 and HepG2 cells were treated with each extract and then incubated for 48 h. The treated cells were harvested by trypsinization, washed twice with PBS, and then used for DNA isolation. DNA extraction was performed using a blood DNA extraction kit as indicated in the manufacturer's guidelines. Electrophoresis was conducted at 100 V for 30 min. The agarose gel was stained with ethidium bromide for 10 min and then rinsed in distilled water for another 10 min. The DNA bands were photographed under UV illumination. A 1 kb DNA marker was used to estimate the size of the DNA fragments.

### Statistical analysis

Each experiment was run in triplicate. The results were reported as the mean ± SD. Analysis of variance was performed by ANOVA tests and significant differences between means of cytotoxicity were *p* < 0.05 using IBM SPSS software, version 24 (IBM Singapore Pte. Ltd., Changi, Singapore).

## RESULTS

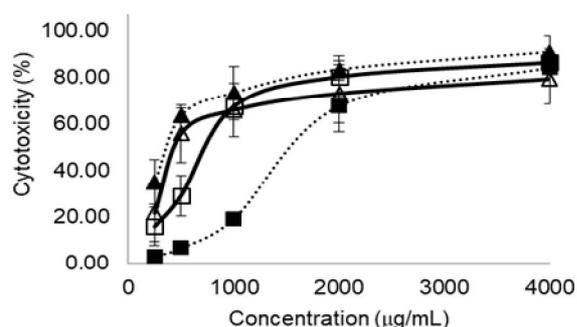
### Cell proliferation

All the plant extracts exhibited different anti-proliferative activity against HT-29 and HepG2 cells (Table 2). The plant extracts showed higher cytotoxicity for HT-29 cells than the positive control or the cytotoxic drug, mitomycin C, at 50 µg/mL. *P. odorata* and *S. wallichii* extracts showed strong anticancer activity, particularly against HT-29 cells with cytotoxicity levels of more than 50 % cell death. In the case of the HepG2 cells, no plant extracts showed stronger anticancer activity than the positive control. However, extracts from three species inhibited HepG2 cell growth relatively effectively: *V. sprengelii*, *P. odorata* and *S. wallichii*.

**Table 2:** Cytotoxic activity on HT-29 and HepG2 cell lines of plant extracts at 2,000 µg/mL and of mitomycin C at 50 µg/mL

Extract	Cytotoxic activity (%)	
	HT-29	HepG2
<i>Camellia sinensis</i>	45.15 <sup>b</sup> ±13.22	44.38 <sup>a-d</sup> ±15.45
<i>Careya sphaerica</i>	37.52 <sup>b</sup> ±3.83	31.24 <sup>a-c</sup> ±15.35
<i>Cratoxylum formosum</i>	35.25 <sup>b</sup> ±5.95	17.13 <sup>a</sup> ±0.58
<i>Eleutherococcus trifoliatus</i>	35.51 <sup>b</sup> ±2.08	45.06 <sup>a-d</sup> ±15.73
<i>Ficus auriculata</i>	31.02 <sup>b</sup> ±2.11	30.56 <sup>ab</sup> ±7.04
<i>Persicaria odorata</i>	66.86 <sup>c</sup> ±12.95	68.94 <sup>d</sup> ±17.70
<i>Schima wallichii</i>	65.42 <sup>c</sup> ±2.64	64.95 <sup>cd</sup> ±12.04
<i>Vaccinium sprengelii</i>	42.29 <sup>b</sup> ±12.53	62.20 <sup>cd</sup> ±7.76
Mitomycin C	17.32 <sup>a</sup> ±3.75	81.35 <sup>e</sup> ±10.18

<sup>a-e</sup>Significantly different at  $p < 0.05$



**Figure 1:** The 50 % cytotoxic concentration (CC<sub>50</sub>); ■ *P. odorata*, ▲ *S. wallichii* in HepG2 and □ *P. odorata*, △ *S. wallichii* in HT-29 cell lines

The MTT assay data indicated that the extracts from *S. wallichii* and *P. odorata* possessed strong anti-proliferative activity in both cell lines. Therefore, they were selected for further studies to determine the CC<sub>50</sub>. The level of cytotoxicity generally increased gradually with increasing concentrations of the extracts (Figure 1). The *S. wallichii* extract had greater cytotoxic activity than the *P. odorata* extract in both cell lines. The CC<sub>50</sub> of the *S. wallichii* extract on HT-29 cells was 453 µg/mL, and 367 µg/mL for HepG2 cells. The CC<sub>50</sub> of the *P. odorata* extract on HT-29 cells was 775 µg/mL and 1,665 µg/mL for HepG2 cells. The CC<sub>50</sub> of the *S. wallichii* extract was 1.7 and 4.5 times greater than that of *P. odorata* extract against HT-29 and HepG2 cells, respectively. The results indicate that the *S. wallichii* extract had significantly greater cytotoxicity than the *P. odorata* extract against both cell lines.

### Morphological changes

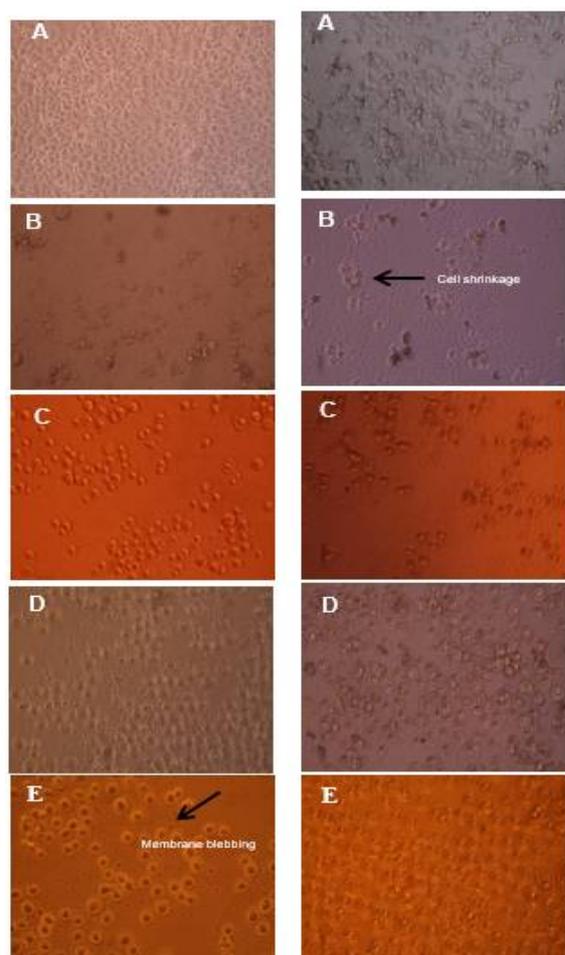
The effects of *S. wallichii* and *P. odorata* extracts on cell morphology of HT-29 and HepG2 cells were observed using inverted light microscopy. No morphological changes were observed in control cells; however, both extract treatments resulted in morphological changes in a

concentration-dependent manner (Figure 2). The two extracts (500 µg/mL each) induced changes in morphologies of HT-29 and HepG2 cells indicating an early stage of apoptosis, including cell shrinkage. The cytoplasm was denser and the shape more tightly packed in the treated cells. As the concentration of extracts increased to 4,000 µg/mL, the loss of cell adhesion, reduced cell density, and membrane blebbing occurred.

### Apoptotic DNA ladder

DNA fragmentation is an important characteristic of apoptosis. Prior to the fragmentation of the nucleus, condensation and degradation of chromatin/DNA were observed. Cheung *et al* [8] reported that the first marker in the apoptosis process was DNA laddering, which is a sign of cell death. Following chromatin condensation, caspase activated DNase (CAD) degrades DNA and caspase-3-treated cell lysates are observed [9]. Figure 3 (A and B) shows that the extracts induced DNA fragmentation in HT-29 and HepG2 cells. Cells treated with *S. wallichii* and *P. odorata* extracts showed a smear pattern when compared with the DNA ladder; in contrast, the smear pattern of damaged DNA was not observed in control cell samples. Apoptosis was therefore induced by the extracts in HT-29 and HepG2 cells.

Both extracts showed a concentration-dependent pattern of DNA fragmentation. In the case of HT-29 cells, *S. wallichii* extract was more effective than *P. odorata* extract (Figure 3A). Conversely, with HepG2 cells the *P. odorata* extract was more effective than the *S. wallichii* extract (Figure 3B). As a positive control, mitomycin C (100 µg/mL) caused more DNA fragmentation in HepG2 cells compared to HT-29 cells than the two extracts.

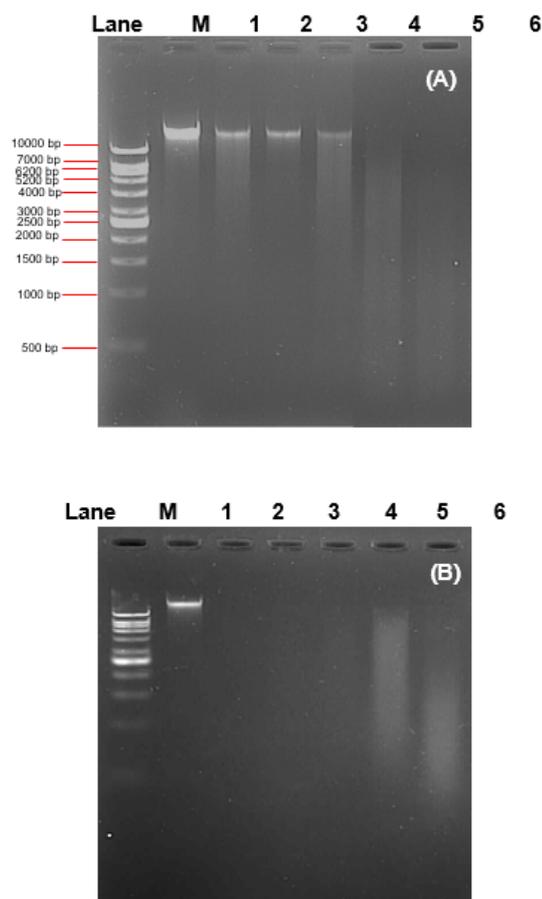


**Figure 2:** Photomicrographs (200x) of *P. odorata* (PP) and *S. wallichii* (TL) extract-treated HT-29 cells (left) and HepG2 cells (right); A: cells without treatment (control), B-C: PP at 500 and 4,000 µg/mL, D-E: TL at 500 and 4,000 µg/mL. Arrows indicate cell shrinkage and membrane blebbing

## DISCUSSION

The potent biological activities of plant extracts are associated with their phytochemical constituents. *P. odorata*, *S. wallichii*, and *V. sprengelii* have been reported to have high polyphenol content [5]. Polyphenols induce pro-apoptotic properties. Ramos [9] described the pro-apoptotic effects of dietary polyphenols on various human cancer cell lines including colon, prostate, lung, breast cancer and leukaemia.

The major phytochemicals of *S. wallichii* include saponins and tannins [10]. Saponins, triterpenoid glycosides, show different biological activities and have the potential for pharmaceutical applications [11]. They reportedly prevent the proliferation of cancer cells [12]. Hu *et al* [13] identified a compound, Nigella A, which was extracted from *Nigella glandulifera* (Nepenthaceae) and reported to be the major triterpene saponins.



**Figure 3:** Agarose gel electrophoresis of DNA fragmentation patterns in HT-29 cells (A) and HepG2 cells (B) treated with *P. odorata* (PP) and *S. wallichii* (TL) extracts for 48 h. M. marker, 1 kb; 1, control; 2, positive control (100 µg/mL); 3-6, cells treated with PP (500 µg/mL), PP (1,000 µg/mL), TL (500 µg/mL), or TL (1,000 µg/mL), respectively

This compound inhibited the growth of human lung carcinoma A-549 cells. Saponin separated from the leaves of *Panax notoginseng* (Araliaceae) demonstrated cytotoxic effects against four cancer cells; KP4 cells (human pancreatic cancer), NCI-H727 cells (human lung cancer), HepG2 cells (human hepatocellular cancer) and SGC-7901 cells (human gastric adenocarcinoma) [14].

Tannins also exhibit biological activities such as anticancer activity. Hydrolysable tannin isolated from *Cuphea hyssopifolia* (Lythraceae) showed antitumor activity on HL-60 cells [15]. Gallotannin inhibited cell growth and induced apoptosis in T-84 cells (human colon cancer) [16].

Flavonoids were reported to be the main phytochemical responsible for anticancer activity in *P. odorata* [17]. The biological activities of flavonoids include anti-inflammatory, anti-spasmodic, and anti-allergic activities as well as

protective activities for hepatic and vascular disorders [18]. Flavonoids also show anticancer activity in various cancer cells. For example, (+)-catechin and (-)-epicatechin play a role in protecting liver cancer cells from DNA damage against N-nitrosodimethylamine, N-nitrosopyrrolidine and benzo (a) pyrene [19].

The  $CC_{50}$  showed that the *S. wallichii* extract was more effective against HepG2 cells than HT-29 cells (Figure 1), which indicated that the phytochemical in *S. wallichii* extracts was more sensitive and specific for HepG2 than HT-29 cells. In addition, the anticancer activity of phytochemicals mainly depends on their multi-target mechanisms of action, including antimutagenic, antioxidant and, anti-proliferative activities [20].

The cytotoxic activity assay of crude extracts showed potential against cancer cell lines, which were in accordance with Momtazi-Borojeni *et al* [21], that crude methanol extract of *Avicennia marina* potentially inhibited the viability of MDA-MB 231 cells (human breast cancer cell) with a  $CC_{50}$  value of 250  $\mu\text{g/mL}$ . For the rhabdomyosarcoma (RD) tumour cell line and murine fibroblast (L20B) cell line, the crude methanol extract of *Nicotiana tabacum* showed  $CC_{50}$  values of 2,100  $\mu\text{g/mL}$  for RD and 2,150  $\mu\text{g/mL}$  for L20B cells after 72 h [22].

The morphological changes increased as the extract concentration increased, and were characteristics of apoptotic cell death. Apoptosis can occur via two routes, either extrinsic (activation of death receptors) or intrinsic (mitochondrial-mediated) pathways. In this study, increasing the concentration of the extracts was expected to induce the intrinsic pathway. The intrinsic signaling pathway activation occurs first, following by mitochondrial outer membrane permeabilization resulting in the release of pro-death factors into the cytosol [23].

*P. odorata* extract more effectively caused DNA fragmentation than the *S. wallichii* extract at both concentrations. The phytochemicals in the *P. odorata* extract may activate the caspase family of proteases better than the *S. wallichii* extract, and eventually lead to the degradation of chromosomal DNA in HepG2 cells.

*P. odorata* extract therefore has a higher potential for DNA fragmentation than *S. wallichii* extract in HepG2 cells. Increasing the concentration of *P. odorata* and *S. wallichii* extracts resulted in the typical DNA laddering in agarose gels, due to activation of caspase

enzyme activity and oxidative stress in cells as observed in both cell lines.

The results using *S. wallichii* extract were similar to those of Halimah *et al* [24] who showed that the major compound (kaempferol-3-O-rhamnoside) in the ethylacetate fraction of *S. wallichii* inhibited MCF-7 cell growth via activation of caspase-9 and caspase-3, inducing apoptosis.

The effect of *P. odorata* extract on HepG2 cells regarding the fragmentation of DNA was opposite to the  $CC_{50}$  observed from MTT results (Figure 1). *S. wallichii* extract was more effective than the *P. odorata* extract. The MTT assay is an indirect measurement of mitochondria effects. The plant extracts inhibited succinate dehydrogenase in the mitochondria, which could hinder reactions with MTT reagent to form formazan crystals. However, DNA fragmentation is associated with caspase-3-mediated cleavage releasing caspase activated DNase (CAD) and resulting in degradation of DNA into oligonucleosomal fragments [25].

## CONCLUSION

The results of this study demonstrate that all the plant extracts examined in this study exert cytotoxic activity against colon cancer cells. Of the eight plant species studied, *P. odorata* and *S. wallichii* extracts possess the highest anti-proliferative activity against colon and liver cell lines, and they also induce apoptosis by DNA fragmentation, thus demonstrating their potentials as anticancer chemotherapeutic agents. Further studies, however, are needed to isolate their active compounds.

## DECLARATIONS

### Acknowledgement

The authors would also like to thank the Royal Project Foundation for providing plant samples for this study.

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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