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ANTIOXIDANT AND ANTICANCER PROPERTIES OF SOLVENT PARTITIONED EXTRACTS OF *Phyla nodiflora* L.

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ABSTRACT

We previously found that the crude extracts of *Phyla nodiflora* L. are capable to inhibit cancer cells. In this study, we reassessed their anticancer properties after solvent partition extraction of the methanolic extracts. We also determined their total phenolic content (TPC) and total flavonoid content (TFC). Water, ethyl acetate and methanolic extracts showed higher TPC and TFC than chloroform or hexane extracts. Most of stem extracts exhibited higher antioxidant activity than the leaf extracts except for leaf methanolic extracts. Six of the extracts showed 50% growth inhibition at the concentration of 36-80 µg/ml. Both stem and leaf ethyl acetate extracts showed profound inhibition. They also caused DNA fragmentation in MCF-7 cells, indicating the occurrence of apoptosis. Our findings demonstrated that liquid-liquid partition has successfully separated methanolic extracts into several fractions with antioxidant and anticancer properties. This will facilitate the future investigation in identifying bioactive compounds for chemotherapeutic intervention.

Keywords: *Phyla nodiflora*, solvent partition, anti-proliferation, apoptosis

INTRODUCTION

Initiatives to discover natural products have been taken by many scientists since the early 1950 in treating cancers. According to **Ribeiro *et al.* (2015)**, about 25-30% of medicines available were derived from natural products being as the reservoir where various bioactive compounds can potentially be used as alternatives in treating various types of diseases.

The use of natural products is recognized by US National Cancer Institute and this has furthered the discovery of anticancer drugs from natural products **(Shivakumar *et al.*, 2012)**. Phytochemicals in natural products have been demonstrated to lower the risk of cancer **(Cassady *et al.*, 1990; Mondal *et al.*, 2012 and Pratheeshkumar *et al.*, 2012)**. They have been widely shown as promising chemopreventive agents which targeting multiple signal transduction pathways and also modulating expression of genes related to cell cycle, cell proliferation and programmed cell death **(Hsieh *et al.*, 2011 and Prasana *et al.*, 2011)**.

Evidence have demonstrated that evoking apoptotic programmed cell death in cancer cells enhances chances in treating cancer diseases as it eliminates cancer cells without causing excessive damage to normal cells **(Taraphdar *et al.*, 2001; Tan *et al.*, 2005 and Tor *et al.*, 2014)**. Besides that, due to the drawbacks of the risk of long term toxicity and chemoresistance caused by synthetic drugs, apoptosis-inducing natural products have become an attractive alternative approach for cancer chemoprevention and chemotherapy.

Phyla nodiflora L. is commonly known as *Lippia* or frog fruit and it belongs to *Verbenaceae* family. It contains a variety of constituents such as triterpenoids, flavonoids, phenols, steroids and many others which might responsible for its reported antioxidant, anti-microbial, anti-fungal, anticancer, anti-inflammatory activities **(Durairaj *et al.*, 2008; Faheem *et al.*, 2011; Kavitha *et al.*, 2012; Vanajothi *et al.*, 2012; Teoh *et al.*, 2013 and Sudha and Srinivasan, 2014)**. Traditionally, the aerial parts of this plant are used as antibacterial, parasiticide and diuretic treatment. The leaves and fruits of this plant are also consumed by women after delivery, children with digestive problem, joints and knee pain **(Kavitha *et al.*, 2012)**. Besides that, halleridone, hallerone, lippiacian and eupafolin have been isolated from *P. nodiflora* **(Ravikanth *et al.*, 2000; Yen *et al.*, 2012 and Lin *et al.*, 2014)**. However, the biological functions of these compounds are largely unknown or not well studied except the anti-melanogenesis property of eupafolin.

Previously, we have reported the apoptosis-inducing effect of *P. nodiflora* in MCF-7 **(Teoh *et al.*, 2013)**. To ease the identification and purification of potential lead compound(s), we decided to separate phytochemicals from methanolic extracts of *P. nodiflora* by solvent partition technique and reexamine their antioxidant as well as anticancer effect.

MATERIAL AND METHODS

Chemicals, solvents and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), α-tocopherol, ascorbic acid, gallic acid, catechin, aluminum chloride, sodium carbonate, sodium hydroxide, sodium nitrite and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used (methanol, ethyl acetate, hexane, chloroform and dimethyl sulfoxide) were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). RPMI-1640 medium, fetal bovine serum and trypsin-EDTA were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of plant material

Phyla nodiflora L. was collected from areas around Kota Kinabalu, Sabah. The plant was verified by a botanist and specimen voucher (VBPN01/2013) was deposited in the herbarium of the university. The leaf and stem methanolic extracts were prepared as described previously **(Teoh *et al.*, 2013)**.

Liquid-liquid solvent partition of methanolic extracts

Liquid-liquid partition of methanolic extracts was performed according to **Debnath and Hussain (2013)** method with some modifications. 10 g of dried extracts was dissolved with 100 ml distilled water. It was individually partitioned with hexane, chloroform, and ethyl acetate respectively with ratio 1:1. The partitioned extracts were dried using a rotary evaporator and stored in -80°C until further use.

Determination of total phenolic content

The total phenolic content in the extracts was determined using Folin-Ciocalteau assay as described previously **(Cheong *et al.*, 2013)**. Firstly, 0.1 ml of samples was diluted with 4.5 ml of distilled water. Then, 0.1 ml of Folin-Ciocalteau reagent and 0.3 ml of 2% sodium carbonate were added to the mixture. The mixture was incubated at room temperature for 2 hours in dark. The absorbance was measured at 760 nm by using a spectrophotometer (Thermo Scientific, USA). Total phenolic content was expressed as mg gallic acid equivalents per gram (GAE mg/g) calculated by a standard curve.

Determination of total flavonoid content

Total flavonoid content in plant extracts was measured as described previously **(Cheong *et al.*, 2013)** with some modifications. Different concentrations of plant extract (0.625-5 mg/ml) and standard catechin solutions (0.016-0.05 mg/ml) were prepared. Briefly, 0.25 ml of samples was diluted with 1.25 ml of distilled water. 75 µl of 5% sodium nitrite was added and allowed to react for 6 min at room temperature. Then, 150 µl of 10% aluminum chloride was added and the mixture was allowed to stand for 5 minutes before adding 250 µl of 1 M sodium hydroxide. Finally, the volume was made up to 2.5 ml with distilled water. The absorbance was measured at 510 nm by using a spectrophotometer (Thermo Scientific, USA). Total flavonoid content was expressed as mg catechin equivalents per gram dry weight (mgCE/g) calculated by a standard curve.

DPPH free radical scavenging activity

The free radical scavenging activity of the plant extracts was measured according to **Teoh *et al.* (2013)** with some modifications. Briefly, 0.1 ml of samples was added to 3.9 ml of DPPH reagent. The mixture was rigorously mixed and left in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a spectrophotometer (Thermo Scientific, USA). EC_{50} values were obtained using linear regression line.

Cell culture

MCF-7 (human breast carcinoma cell line) was grown in RPMI-1640 media (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (Nacalai Tesque, Japan). The cells were maintained at 37°C in a CO_2 incubator.

Proliferation assay

Proliferation assay was performed using MTT Cell Proliferation kit (Roche, Switzerland) according to manufacturer's instruction with some modifications **(Cheong *et al.*, 2013; Teoh *et al.*, 2013)**. The plant extracts with the concentration of 10 to 90 µg/ml was used for treatment. After that, cells were incubated for 72 hours. The absorbance was measured at 570 nm by using an ELISA reader (Molecular Devices, USA). The 50% inhibitory concentration (IC_{50}) is the plant extract concentration required to cause 50% growth inhibition and obtained using a linear regression line.

DNA fragmentation

Cells were treated with selected extracts based on IC_{50} values obtained from proliferation assay and incubated at 37°C for 3 days. Genomic DNA was extracted using DNA Apoptotic DNA-ladder kit (Roche, Switzerland) according to manufacturer's instruction. After adding lysis buffer, cell lysate was transferred to a 1.5 ml tube followed by incubation at room temperature for 10 minutes. Then, 100 µl of isopropanol was added and the mixture was transferred into a spin filter tube. This was followed by washing steps. Finally, DNA was eluted by adding elution buffer. The formation of DNA ladder was confirmed by analyzing in 1.5 % agarose gel at 80 V for 50 min.

Statistical analysis

Experimental results were represented as mean ± standard deviation of three replicates. Data was analysed using one-way ANOVA (IBM SPSS Statistic version 20) to obtain significant differences between samples.

RESULTS AND DISCUSSION

Bioactive compounds from natural products have been reported to have numerous biological activities including antioxidant, anti-aging, anti-carcinogenic and anti-apoptotic activities **(Li *et al.*, 2008; Mabveng *et al.*, 2011 and Tasyriq *et al.*, 2012)**. Phenolic compounds such as flavonoid are one of the potential antioxidants and free radical terminators that commonly found in plants **(Govindarajan *et al.*, 2005; Ozsay *et al*,. 2008 and Mishra *et al*,. 2012)**. They act as the main agents to donate hydrogen to free radicals thus help in inhibiting their harmful effect on living system **(Rice-Evans *et al.*, 1996; Surveswaran *et al.*, 2007 and Tabaraki and Ghadiri, 2013)**.

In this study, we measured the amount of TPC and TFC present in *P. nodiflora* extracts. All extracts showed an increase of TPC in concentration-dependent manner but their TPC is lower compared to controls. As shown in Table 1, the leaf and stem extracts obtained from methanol, ethyl acetate and water fractions contained higher amount of TPC than other fractions. In contrast, hexane and chloroform fractions of both leaf and stem were found to have less phenolic compounds. For the leaf extracts, distilled water extract (LDPN) showed the highest TPC (0.118±0.004 mg GAE/g) while the lowest TPC was obtained from leaf hexane extract (LHPN=0.020±0.001 mg GAE/g). For the stem extracts, the highest TPC was also found in distilled water extract (SDPN=0.096±0.003 mg GAE/g) while the chloroform extract (SCPN) had the lowest TFC (0.012±0.000 mg GAE/g). The TPC obtained from this study is comparable to previous study. The methanol extract of the aerial part of *P. nodiflora* was 0.411 mg/g pyrocatechol equivalent **(Priya & Ravindhran, 2015)**.

Similar to TPC, the leaf and stem extracts obtained from methanol, ethyl acetate and water fractions demonstrated higher TFC compared to other fractions (Table 1). Both hexane and chloroform extracts of leaf and stem were found to have less flavonoid compounds. Among the leaf extracts, the ethyl acetate extract (LEPN) had the highest TFC (465.21±31.60 mg CE/g) and the lowest TFC was found in the chloroform extract (LCPN=21.56±2.04 mg CE/g). However, the highest TFC of stem extracts was obtained from distilled water fraction (SDPN=280.68±24.71 mg CE/g) and hexane extract (SHPN) had the lowest TFC (40.41±3.26 mg CE/g). The TFC in stem methanol extract (SMPN) was 4 times higher than leaf methanol extract (LMPN). Compare to previous study, the TFC reported by Priya & Ravindhran (2015) for the methanol extract of the aerial part of *P. nodiflora* was 0.312 mg/g.

Table 1 Total phenolic content and total flavonoid content of solvent partitioned extracts of *P. nodiflora*.

Extracts		TPC (mg GAE/g)	TFC (mg CE/g)
Leaf	Methanol (LMPN)	0.065±0.001	37.21±0.82
	Ethyl acetate (LEPN)	0.081±0.001	465.21±31.60
	Distilled water (LDPN)	0.118±0.004	231.34±19.88
	Hexane (LHPN)	0.020±0.001	28.77±1.32
	Chloroform (LCPN)	0.037±0.001	21.56±2.04
Stem	Methanol (SMPN)	0.063±0.003	137.74±4.68
	Ethyl acetate (SEPN)	0.080±0.002	171.34±25.05
	Distilled water (SDPN)	0.096±0.003	280.68±24.71
	Hexane (SHPN)	0.015±0.000[a]	40.41±3.26
	Chloroform (SCPN)	0.012±0.000[a]	78.9±5.95

[a] standard deviation was obtained after round off to 3 decimal points.

Next, we determined the antioxidant activities of *P. nodiflora* extracts. We found that the DPPH scavenging activity was increased in a dose-dependent manner for all leaf and stem extracts (Fig. 1 and Fig. 2). For leaf extracts (Figure 1), when the concentration was at 5 mg/mL, the order of DPPH scavenging activities for each extract was LMPN > LDPN > LHPN > LCPN > LEPN. On the other hand, all stem extracts exhibited more than 90% DPPH scavenging activity at 5 mg/ml (Fig. 2). The EC_{50} value is defined as the concentration of an antioxidant that causes a 50% decrease in the DPPH absorbance **(Chen *et al*., 2013)**. Therefore, low value of EC_{50} means high antioxidant capacity of a plant extract. The differences in scavenging activity for each extract were summarized in Table 2. Half of the plant extracts (SEPN, SDPN, SHPN, LMPN and LDPN) are found to have comparable EC_{50} value as the two controls, tocopherol and ascorbic acid. This indicates that these extracts have profound antioxidant activity. Similar to our previous results **(Teoh *et al.* 2013)**, leaf methanol extract showed higher antioxidant activity compared to stem methanol extract. However, the scavenging activity reported by Sudha & Srinivasan (2014) for aerial part of this plant was much lower than we reported here. The reported IC_{50} for the four fractions were 24, 26, 83 and 66 µg/ml, respectively for methanol, acetyl acetate, butanol and water extracts.

Table 2 DPPH scavenging activity and EC_{50} values of solvent partitioned extracts of *P. nodiflora*.

Extracts		The maximal DPPH scavenging activity (mg/ml)	EC_{50} value (mg/ml)
Leaf	Methanol (LMPN)	93.7±1.06	0.34±0.01
	Ethyl acetate (LEPN)	34.22±2.70	7.34±0.60
	Distilled water (LDPN)	93.32±0.17	0.33±0.01
	Hexane (LHPN)	73.38±1.05	2.52±0.17
	Chloroform (LCPN)	65.8±1.56	3.42±0.08
Stem	Methanol (SMPN)	91.37±0.00[a]	2.02±0.11
	Ethyl acetate (SEPN)	94.35±0.50	0.35±0.01
	Distilled water (SDPN)	93.8±0.71	0.33±0.00[a]
	Hexane (SHPN)	94.63±0.17	0.33±0.00[a]
	Chloroform (SCPN)	90.36±1.1	1.31±0.07
Positive controls	Tocopherol	91.28±1.61	0.244±0.00[a]
	Ascorbic acid	94.73±0.80	0.247±0.02

[a] standard deviation was obtained after round off to 3 decimal points.

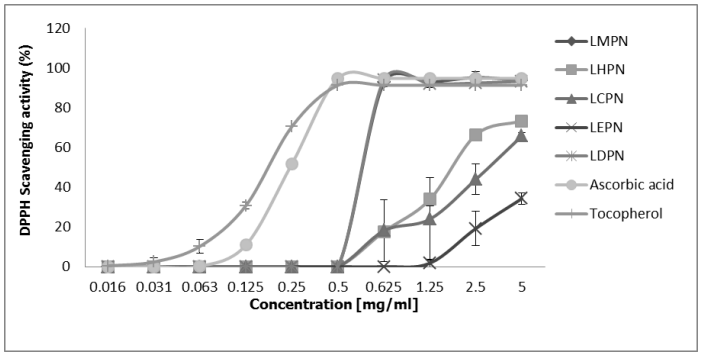

Figure 1 DPPH scavenging activity of *P. nodilfora* leaf extracts. Data obtained from three replicates.

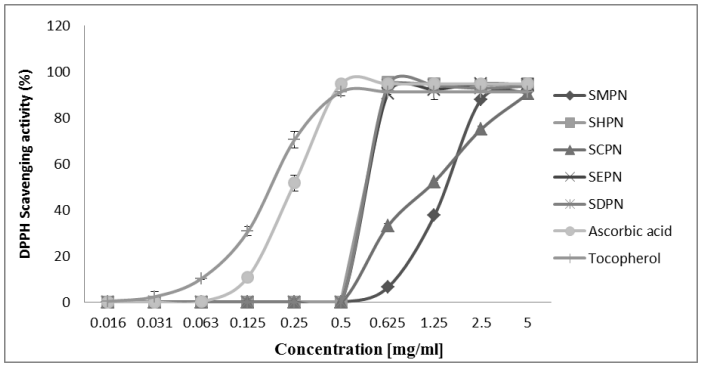

Figure 2 DPPH scavenging activity of *P. nodilfora* stem extracts. Data obtained from three replicates.

Taken together, our results showed that the amount of TFC is not necessary in proportional with the TPC especially in leaf extracts. In addition, we also noticed that there is no clear relationship between TFC and antioxidant activity for some extracts. For instance, LEPN extract showed the highest TFC but its DPPH scavenging activity was the lowest among the extracts while LMPN extract conversely had high scavenging activity although its TFC was quite low. This was also observed in LHPN, LCPN, SHPN and SCPN extracts. The differences in DPPH scavenging activity which shown in different extracts at various concentrations may be due to various biological active metabolites present in the extracts **(Ramasubbrurayan *et al.*, 2015)**. This could be due to some extracts require longer time to attain steady state to complete the redox reaction in DPPH scavenging activity **(Molyneux, 2004)**. In our experiment, DPPH assay was performed at a fixed reaction time which was 30 minutes. Besides that, different types of antioxidants such as nitrogen radical present in the extracts might exhibit different kinetics and the amount of antioxidants might also affect the chemical reaction of some other compounds **(Huang *et al.*, 2005)**. The discrimination in TFC, TPC and DPPH reported in present and previous studies is not uncommon, as the amount of phytochemicals present in a plant could be significantly affected by plant extraction methods, starting materials and assays used for quantification.

To confirm the anti-proliferative effect of these solvent partitioned extracts, we reexamined their effect on MCF-7 cells. From Figure 3 and Figure 4, we found that different extracts react differently on the proliferation of MCF-7 cells. Some extracts showed a reduction of cell proliferation when the concentrations of the extracts increased while the others showed no obvious inhibition. For example, both SEPN and SCPN extracts showed a decrease of >50% of cell proliferation whereas no significant decrease of cell proliferation was observed in cells treated with SDPN and SHPN (Fig. 3). Compared to the stem extracts, MCF-7 cells are more susceptible to leaf extracts as almost all extracts caused inhibitory effect on cell growth except for LMPN (Fig. 4). The profound inhibition could be observed in cells treated with SCPN and LEPN extracts where their IC_{50} values were 37.7±0.88 µg/ml and 36.9±0.54 µg/ml respectively (Table 2). This was subsequently followed by SEPN, LCPN and LDPN. The IC_{50} values obtained in this study are relatively lower compared to our previous results **(Teoh *et al.*, 2013)**. This indicates that solvent partition of methanolic extracts of *P. nodiflora* is capable of isolating or concentrating potential phytochemical compound(s) that play a role in growth inhibition.

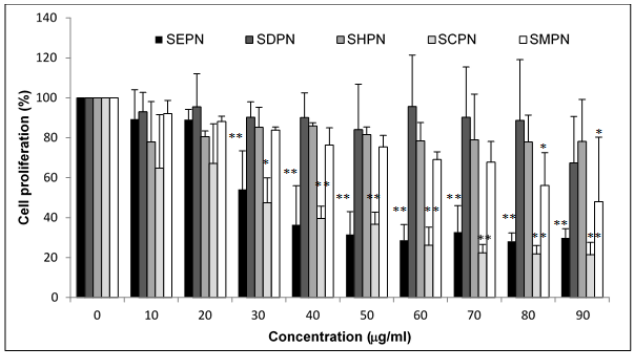

Figure 3 Anti-proliferative effect of *P. nodiflora* stem extracts on MCF-7. Cells were treated with plant extracts at various concentrations for 3 days. Data were obtained from the average of three independent experiments performed in duplicate. Asterisks denote differences with statistical significances compared with the untreated cells (*P < 0.05 and **P < 0.001). P values were obtained from one-way ANOVA.

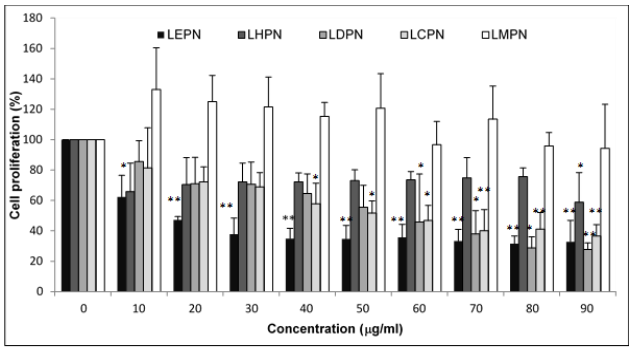

Figure 4 Anti-proliferative effect of *P. nodiflora* leaf extracts on MCF-7. Cells were treated with plant extracts at various concentrations for 3 days. Data were obtained from the average of three independent experiments performed in duplicate. Asterisks denote differences with statistical significances compared with the untreated cells (*P < 0.05 and **P < 0.001). P values were obtained from one-way ANOVA**.**

Table 2 The IC_{50} values of leaf and stem extracts of *Phyla nodiflora*.

***Phyla nodiflora* extracts**	**IC_{50} (µg/ml)**
Leaf methanol (LMPN)	-
LEPN	36.9±0.54
LDPN	57.1±0.69
LHPN	-
LCPN	56.4±2.28
SMPN	80.6±0.86
SEPN	47.9±0.73
SDPN	-
SHPN	-
SCPN	37.7±0.88

The apoptosis inducing effect of the selected solvent partitioned extracts was confirmed by DNA fragmentation assay. As shown in cells treat with camptothecin, distinct multiple of 180 bp apoptotic fragments was clearly seen in Fig. 5 (Lane 6). The formation of DNA laddering was also found in samples treated with LEPN and SEPN (Figure 5, lane 3 & 4). Similar to the ethyl acetate crude extracts that we published previously **(Teoh *et al.*, 2013)**; both LEPN and SEPN extracts obtained after solvent partition could induce apoptosis in MCF-7 cells. Besides our work, **Vanajothi *et al.* (2012)** has also shown that methanolic leaf extract of *L. nodiflora* is capable of inhibit lung cancer cell growth through apoptosis.

The present study has led us to speculate that the pro-apoptotic effect exerted by SEPN and LEPN could be due to the presence of high flavonoid compounds as previously reported although they show different antioxidant capacity **(Ashokkumar *et al.*, 2008 and Sudha and Srinivasan, 2014)**. Nonetheless, the role of other compound(s) could not be excluded because the recovery of phenols and flavonoids from different bioactive compounds is influenced by the types of solvents used and extraction techniques **(Sultana *et al.*, 2009 and Do *et al.*, 2014)**. Besides that, different extracts might execute anticancer effect through multiple cellular pathways. Therefore further investigations are required to reveal the mode of actions of each extract.

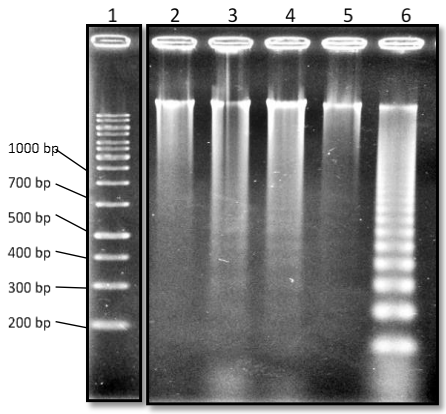

Figure 5 *P. nodiflora* induces internucleosomal DNA fragmentation. MCF-7 cells were treated with plant extracts based on the obtained IC_{50} values for 3 days. Lane 1: 1 Kb DNA ladder; lane 2: SMPN; lane 3: SEPN; Lane 4: LEPN; lane 5: untreated and lane 6: positive control.

CONCLUSION

This study demonstrated that solvent partitioned extraction is effective in concentrating or separating phytochemicals found in methanolic crude extracts. In comparison to our previous study, most of the solvent partitioned extracts such as SCPN, SEPN and LEPN are found to prevent cancer cell growth at lower concentration although its link to antioxidant is not mutual and conclusive for all extracts. This information will facilitate our further study in identifying and purifying potential bioactive compounds from *P. nodiflora*. Its apoptosis inducing property also serves as a stepping stone in exploring how its anti-proliferative effect is mediated via apoptosis pathway.

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