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Original article

Cytotoxicity and apoptotic mechanisms of different solvent extracts from *Ipomoea pes-caprae* on human nasopharyngeal cells

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Background: *Ipomoea pes-caprae* serves as a valuable medicinal plant with the pharmacological activities like antioxidant, analgesic, anti-inflammatory, antispasmodic, and anticancer activities. Although some scientific literatures have demonstrated its anticancer activities, the exact mechanism is not fully elucidated.
Objectives: To study the cytotoxicity and apoptotic mechanisms of different solvent extracts from *Ipomoea pes-caprae* on human nasopharyngeal (KB) cells.
Methods: The dried plant was macerated in hexane (Hex) or ethanol: water (EtOH). The supercritical fluid extraction process used carbon dioxide (SCO_2) as the extracting solvent. Cytotoxic activity was determined by MTT assay. The apoptotic mechanism and its effect on mitochondrial membrane potential were measured by DNA agarose gel electrophoresis assay, nuclear staining with DAPI, JC-1 mitochondria staining and caspase-3 activity analysis.
Results: EtOH extraction did not exhibit cytotoxic effect on peripheral blood mononuclear and KB cells. The IC_{50} values of Hex, SCO_2 extract and doxorubicin were found at 200 ± 12.3, 70 ± 4.2 and 2.0 ± 0.08 μg/mL, respectively. Morphological changes including cell shrinkage, DNA fragmentation and condensation of chromosomes were observed. Further, Hex, SCO_2 extract induced loss of mitochondrial membrane potential and induction of caspase-3 activity.
Conclusions: The results strongly supported the ability of these extracts to induce the KB cell apoptosis through the mitochondrial and caspase-3 pathway. The presence of various bioactive compounds in the *Ipomoea pes-caprae* may be a valid strategy for chemoprevention and chemosensitization.

Keywords: *Ipomoea pes-caprae*, apoptosis, caspase-3, KB cells.

The medicinal plants employ a sort of pharmacological action and scientific researches have been carried out to confirm their therapeutic purposes. *Ipomoea pes-caprae* (L.) R. Br., Convolvulaceae, discovered by traditional societies provide unlimited chances for new drug discoveries because of the chemical diversity that can be used to treat various types of inflammation including jellyfish sting and dermatitis. Previous investigations have confirmed some pharmacological properties of this plant. The extract of petroleum ether inhibited the contraction of guinea-pig ileum stimulated by four different spasmogens in a dose-dependent manner. [1] The isolation of β-damascenone and isoprenoids E-phytol showed antispasmodic activity, which was equipotent to papaverine, a known spasmolytic agent. [2] The extract contained active compounds such as eugenol, (-)-mellein and 4-vinylguaiacol which exhibited anti-inflammation via the inhibition of prostaglandin activity. [3] The hydroethanolic extract showed antinociceptive and anti-inflammatory effects using the writhing test and carrageenan-induced pleurisy models in mice with lower toxicity. [4] In a related study, this extract showed antinociceptive and anti-inflammatory effect against P. physalis venom (a jellyfish like) via the mechanism involved with histamine (H1), vanilloid (TRPV1), kinins (B2 receptor) and proteases (PAR-2) receptors activation. [5] The ethanolic extracts from aerial parts exhibited significant anti-inflammatory activities in rats using the cotton pellet-induced granuloma model. The percentage inhibition of this extract (400 mg/kg) was

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compared with diclofenac sodium (5 mg/kg).[6] These literature data confirm the traditional use of this plant toward dermatitis caused by venomous jellyfishes.

Cytotoxicity activity has shown that the hexane-soluble extract yielded six lipophilic glycosides, namely, pescaproside A, pescapreins I-IV and stoloniferin III exhibited weak cytotoxicity against nasopharyngeal (KB), colon (HCT 15), squamous cell cervical (SQC-1 UISO) and ovarian (OVCAR) carcinomas.[7] Pentasaccharide resin glycosides isolated from the aerial parts were not toxic against multidrug resistance in MCF-7/ADR cells but the combination of pentasaccharide (5 μg/mL) with doxorubicin increased the cytotoxicity of doxorubicin by 1.5-3.7-fold.[8] In addition, the resin glycoside, murucoidin V, exerted a potentiation effect pump inhibitors of the P-gp transporter in vinblastine-resistant human breast carcinoma cells (MCF-7/Vin). These results suggest that resin glycosides could be used to overcome the multidrug resistance for future cancer therapy.[9] The *in vivo* antitumor potential against mice melanoma (B16F10) cancer cells was found in following order methanolic extract > aqueous extract >swaras> petroleum ether extract which may be attributed to the presence of alkaloids, flavonoids, tannins, terpenoids, and glycosides in the crude extract.[10] These compounds with scaffold phytochemistry have a variety of therapeutic properties ranging from antioxidant, anti-inflammation, collagenase inhibitory, immune-stimulatory and anticancer activities.[11] With *Ipomoea pes-caprae* being a promising candidate for anticancer drug development, research is much needed to be done on based on the plant extracts that may yield many bio-active compounds with minimal toxicity. Consequently, the objective of this study was to investigate the cytotoxicity and apoptotic mechanism of the different solvent extracts from *Ipomoea pes-caprae* on human nasopharyngeal epidermoid carcinoma (KB cells), *in vitro* study.

Materials and methods

Plant material and preparation of extracts

The aerial parts of *Ipomoea pes-caprae* were collected from Bangsaen costal area in Chonburi Province, Thailand during January - April 2015. All plants were washed with running tap water, oven-dried at 45°C for 48 h and subsequently grounded. In the maceration method, 500 g of the dried plant was macerated in 500 mL hexane (Hex) or ethanol: water (50:50, v/v) (EtOH) for 7 days and the solvent were removed via a rotary evaporator at 45°C. In supercritical fluid extraction method, carbon dioxide (SCO_2) was the supercritical fluid as the extracting solvent. The pressure and temperature were performed in 74 bar at 31°C. All of the extract samples were dissolved in 0.5% EtOH.

Gas chromatography - mass spectrometry (GC-MS)

The gas chromatography–mass spectrometry (GC-MS) analyses were made in FOCUS DSQ Single Quadrupole GC-MS (Thermo Electron Corporation, USA) in ZB-5ms glass capillary column (Dimension: 30 m, ID: 0.25 mm, Film: 0.25 μm). The column temperature program was maintained from 60°C up to the end temperature of 220°C at the rate of 10°C/min. The sample (1 μL) was injected and the flow rate of the helium carrier gas was set to 1 mL/min in a splitless mode with a total run time of 30 min. The mass spectrometry detector was operated with vacuum pressure of 60 mTorr, transfer line temperature of 275°C, ion trap temperature of 240°C and ionization energy of 70 ev. The chromatograms of the sample were identified by comparing their mass spectra with the spectral database of known components in the GC-MS library, National Institute Standard and Technology (NIST). The name and molecular weight of the compounds were determined and the percentage composition displayed the relative amount of each fragment from the compounds.

Human peripheral blood mononuclear cells (PBMC) preparations

PBMC were isolated from whole blood (20 mL) obtained from healthy human volunteers. This research project was approved by the Ethics Committee of Burapha University, according to the document number 84/2556. Blood samples were collected into heparinized tubes and processed by Ficoll-Hypaque gradient centrifugation. The gradient interface containing the mononuclear cell fraction was washed with PBS, counted in trypan blue, and resuspended in RPMI 1640 complete medium.

Human cancer cell line

KB cells, human carcinoma of nasopharynx cells, were provided from the National Center Institute (Bangkok, Thailand). They were grown in Dulbecco's modified eagle medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, and 1%

penicillin/streptomycin. The cell lines were cultured routinely in a humidified atmosphere with 5% CO_2, 95% air at 37°C. Experiments were performed when cells were cultured to exponential growth with a viability >95%, as determined by trypan blue exclusion.

Cell viability assay

The cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, KB or PBMC cells were seeded on a 96-well plate ($1x10^5$ cells/well) for 24 h until they reached at 70% of confluency. On the second day, different concentrations of test samples, EtOH (0.5%) and doxorubicin (Dox) were added to the wells for 48 h. Thereafter, a 10 µL of the MTT (5g/L) was added to each well for 4 h at 37°C and 5% CO_2. The medium was removed, and the purple formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO). The absorbance at 570 nm was read with a microplate reader (Cecil Bioquest 2000 Series). Results were expressed as a percentage of the control (100%), and the cytotoxicity was expressed as concentration of 50% of cytotoxicity (IC_{50}).

Agarose gel electrophoresis of fragmented DNA

The GF-1 tissue DNA extraction kit was used for DNA purification. At the end of incubation period, cells were washed with PBS, and the cell pellets were then lysed with digestion buffer containing proteinase K (400 µg/mL) for 10 min at 60°C, and subsequently with RNase A (10 µg/mL) for 10 min at 37°C. The DNA was precipitated by ice-cold absolute ethanol. High-purity genomic DNA has an absorbance of 260/280 ratio between 1.7, and 1.9. DNA samples (20 ng) were electrophoretically separated at 125 V on 1% agarose gel containing SYBER Gold in Tris-borate/EDTA electrophoresis buffer (TBE). DNA was visualized under a transilluminator (Clare Chemical Research).

Nuclear staining by 4',6-diamidino-2-phenylindole (DAPI)

KB cells were grown in 8-well slide chamber and later treated with test sample at IC_{50} concentration for 48 h. For nuclear staining with DAPI, the glutaraldehyde (2.5%)-fixed cells were washed with PBS, and stained with 5 µg/mL DAPI solution for 10 min. Then, the cells were washed twice with PBS, and mounted by placing a small drop of glycerol-PBS solution. The nuclear morphology was visualized and photographed by fluorescence microscopy (BX51TR, Olympus, Tokyo, Japan) at 100 oil objectives with a DAPI filter. For each of triplicate samples, 200 nuclei were counted with at least 5 fields of view for each slide.

Mitochondrial membrane potential ($\Delta\Psi m$) analysis

After treatment with IC_{50} concentrations of test samples, both adherent and detached cells were collected and incubated with 5 µg/mL of 5,5′, 6,6′ -tetrachloro-1,1′ ,3,3′ -tetraethyl benzimidazol-carbocyanine iodide (JC-1) at 37°C for 15 min. Cells then washed twice with PBS and $\Delta\Psi m$ was monitored by determining the relative amounts of dual wavelength emissions for both green (JC-1 monomers) versus red (JC-1 aggregates) using flow cytometry (Becton Dickinson, BD LSR II) under Argon-ion 488 nm laser excitation. Mitochondrial depolarization was determined by a decrease in the red/green fluorescence intensity using the BD FACSDiva Software.

Analysis of caspase-3 activity

The activities of caspase-3 were determined by a colorimetric assay kit, according to the manufacturer's instructions. Cells were cultured in a 6-well plate and incubated with IC_{50} concentrations of test samples for 48 h. The cell pellet was suspended in lysis buffer for 30 min on ice and supernatants were collected for the assay. Proteolytic reaction was performed in supernatant proteins (100 µg/sample) and caspase-3 substrate (DEVD-pNA) at 37°C in the dark for 2 h. Then, the formation of p-nitroanilide (pNA) was read at 405 nm using an ELISA reader. The data were represented as 'fold of control'.

Statistical analysis

All results were presented as the mean values ± standard error of the mean (SEM) in at least 3 independent experiments. The significance of difference between groups was calculated using the Student's *t* - test, and $P < 0.05$ were considered statistically significant.

Results

The Hex extract of *Ipomoea pes-caprae* had the highest yield (2.9%) (w/w), followed by EtOH (1.9%) and SCO_2 (1.3%). In GC-MS chromatogram analysis, the name, molecular weight, and structure

of the phytochemical constituents with the NIST library were identified in Table 1. The eugenol was one of the chemical compounds that can be determined from *Ipomoea pes-caprae* in the GC-MS analysis from the time range of 11.08 - 11.10 min. The SCO_2 condition yielded the highest for amount of eugenol compound (3.19%) followed by Hex (2.2%) and EtOH (0.5%).

The *in vitro* cytotoxicity conducted as the first step in screening of potential anticancer activity from *Ipomoea pes-caprae*. The EtOH, Hex and SCO_2 extracts exhibited weak cytotoxicity against PBMC and the viability of PBMC remained above 80 % at a concentration of 1000 μg/mL (data not shown). The treatment of KB cells with EtOH extraction did not result in a significant cytotoxic effect. On the contrary, Hex, SCO_2 extract and Dox exhibited a marked growth inhibitory effect on KB cells in a dose-dependent manner with IC_{50} (50% inhibition concentration) of 200 ± 12.3, 70 ± 4.2 and 2.0 ± 0.08 μg/mL, respectively (Figure 1). Morphology of KB-treated cells was changed significantly into the round, membrane blebbing and appearances of apoptotic cells; while the 0.5% EtOH-treated control cells remained to polygonal adherent cells.

The Hex and SCO_2 but not EtOH extracts induced small DNA fragments that moved through the gel faster than those of the control group. The longest pieces of DNA will remain near the wells in control and EtOH extract group (Figure 2). To assess nuclear morphology by fluorescence microscopy, the nuclei of KB cells treated with Hex and SCO_2 extracts displayed a fragmentation of the nucleus compared to controlled nuclei that exhibited diffuse staining. In the control group (0.5% EtOH), the quantitative estimation of normal cells was 100%, whereas the nuclear fragmentation in KB cells treated with EtOH, Hex, SCO_2 and Dox were 9 ± 0.65, 61 ± 4.26, 65 ± 3.58, and 70 ± 8.66%, respectively (Figure 3; DAPI). The morphological changes detected under the microscope showed the KB cell disintegrates into apoptotic bodies, cell shrinkage, rounding, poor adherence, and membrane blebing (Figure 3; Bright field).

Table 1. Compounds identified in the extract of *Ipomoea pes-caprae* in GC-MS.

Retention time	Name of compound	Molecular weight	Molecular formular	Amount (% relative)		
				EtOH	Hex	SCO_2
11.08-11.10	eugenol	164.20		0.50	2.22	3.19
11.85	alpha cubebene	204.357	C15H24	-	14.38	6.36
12.17	isoledene	204.357	C15H24	-	10.10	5.98
13.08	beta caryophyllene	204.36	C15H24	-	4.11	1.79
13.37	aristolene	204.357	C15H24	-	0.45	0.21
14.10	azulene	128.17	C10H8	-	0.66	0.30
14.84	germacrene D	204.357	C15H24	-	9.90	7.82
15.94-16.00	beta cadinene	204.351	C15H24	-	11.88	8.89
17.25	gamma gurjunene	204.357	C15H24	-	0.12	0.26
17.98	aromadendrene	204.357	C15H24	-	0.13	0.14
20.51-20.54	alpha curcumene	202.341	C15H22	-	0.19	1.22
24.89	unknown			4.67	0.23	-
26.31	4-tetradecyne	194.362	C14H26	-	2.02	3.56
26.99	7-heptadecyne	236.436	C17H32	-	0.45	-
27.43	unknown			-	0.71	27.52
27.65	pentacosane	352.691	C25H52	-	-	-
27.85	pentatricontane	492.96	C35H72	-	7.98	-
27.89	ethyl linoleate	308.506	C20H36O2	-	-	-
28.08	ethyl linoleolate			58.01	-	0.85
28.40	unknown			-	0.73	0.71
28.61	docosane	310.60	C22H46	1.91	3.59	22.97
29.22	eicosanoic acid	312.53	C20H40O2	20.52	-	7.07
29.60	methyl palmitate	270.457	C17H34O2	0.63	28.96	14.30

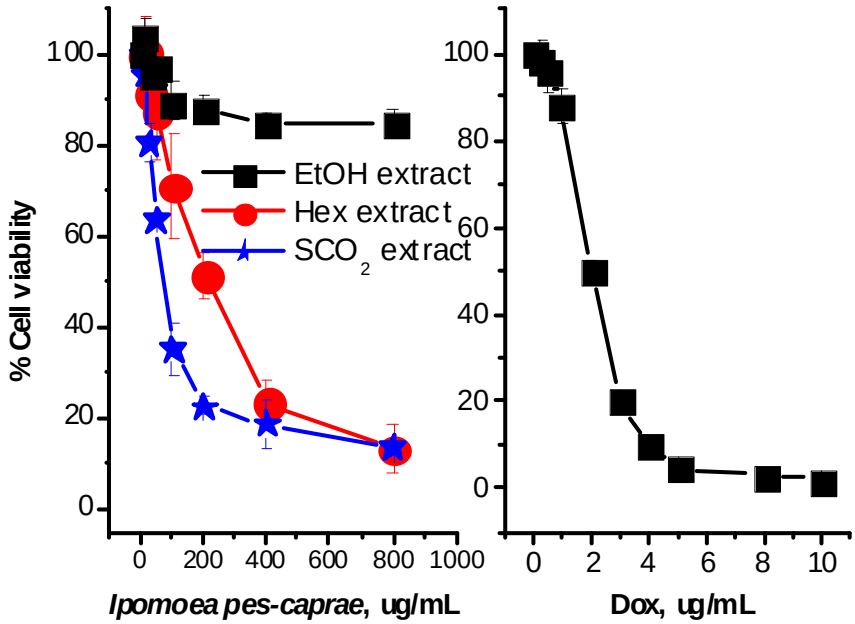

Figure 1. Percent cell viability of KB cells after exposure to extract of *Ipomoea pes-caprae* and Dox for 48 h. Cell viability was determined by MTT assay. Each point is mean ± SEM of three experiments.

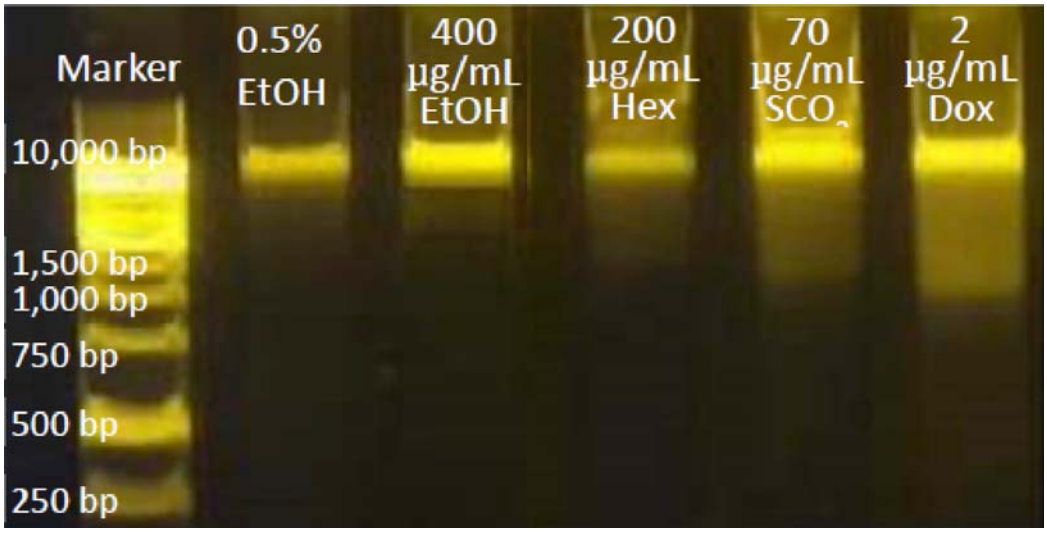

Figure 2. Agarose gel electrophoresis of DNA from *Ipomoea pes-caprae*-treated KB cells. Purified genomic DNA was analyzed by electrophoresis on 1.0% agarose gel, stained with SYBER Gold and visualized under ultraviolet. A photograph was representative of three independent experiments.

The ΔΨm is a key indicator of cell apoptosis related to mitochondria-derived ATP. For monitoring changes in ΔΨm, the membrane-permeant JC-1 dye exhibits potential-dependent accumulation in mitochondria specified by a fluorescence emission shift from green (JC-1 monomers) to red (JC-1 aggregates). The loss of ΔΨm has been regarded as a decrease in red and increase in green fluorescence intensity. The percentage of cells with reduced JC-1 red fluorescence followed by treatment with 0.5% EtOH, EtOH, Hex, SCO_2 and Dox was 97.2, 92.8, 86.4, 79.9, 73.4%, respectively. On the one hand, the percentage of cells with augmented JC-1 green fluorescence was at 2.8, 7.2, 13.6, 20.1, 26.6%, respectively. Representative histograms from 3 individual experiments presented in (Figure 4). Mitochondrial depolarization can be estimated by a decrease in the red-to-green fluorescence intensity ratio as compared to the 0.5% EtOH control. The observed difference was statistically significant ($P < 0.05$) between the control and the extract of *Ipomoea pes-caprae* (Hex & SCO_2).

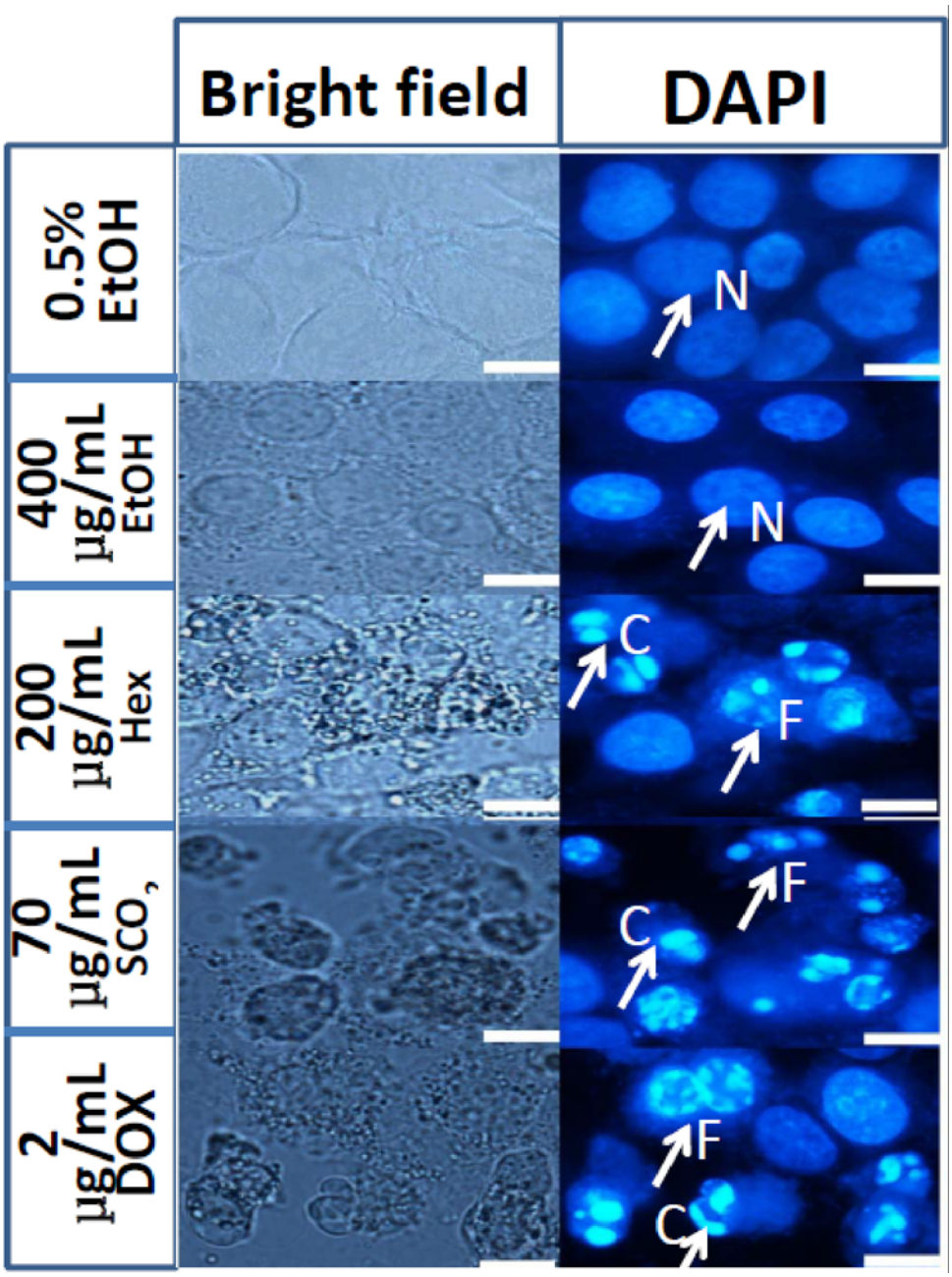

Figure 3. Matching of surface morphology (bright field) and nuclear features (DAPI staining) at the same view point. KB cells were treated with extract of *Ipomoea pes-caprae* and Dox for 48 h and stained with DAPI. C: chromatin condensation; F: nuclear fragmentation; and N: normal nuclei. Scale bar is 10 µM. A photograph was representative of three independent experiments.

The relative caspase-3 activity significantly increased by 2.32 ± 0.32, 2.68 ± 0.25 and 3.33 ± 0.54 folds ($P < 0.05$), respectively, versus control. In the presence of caspase-3 inhibitor, the caspase-3 activity significantly decreased to 1.33 ± 0.1, 1.39 ± 0.45 and 1.83 ± 0.41 ($P < 0.05$), respectively, when compared with non-caspase-3 inhibitor group (Figure 5).

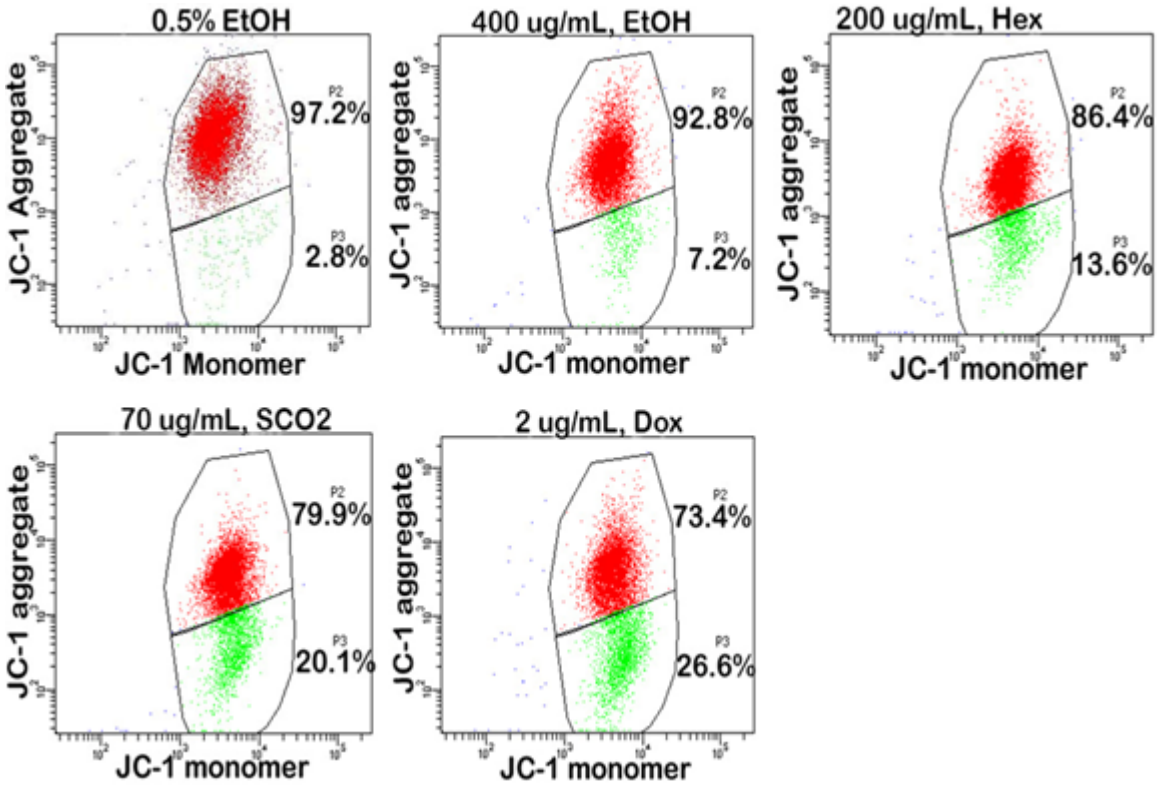

Figure 4. Mitochondrial membrane potential was measured by flow cytometry. KB cells were incubated with extract of *Ipomoea pes-caprae* and Dox for 48 h and stained with JC-1. The JC-1 fluorescence intensity was visualized using FACS analysis with two dimensional scatter-plots in channel green (J-monomers) versus red (J-aggregates) fluorescence. Experiments were performed triplicate and one representative plot was shown.

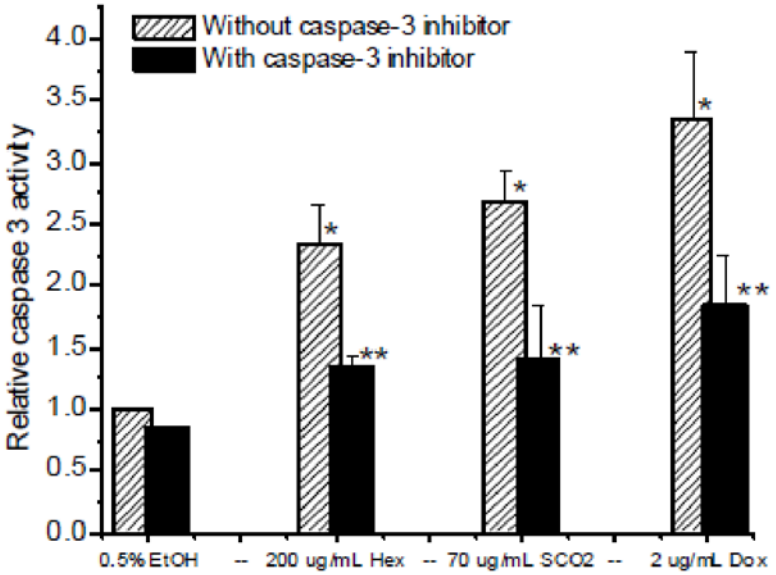

Figure 5. Caspase-3 enzymatic activity in KB cells treated with the indicated concentrations. The control group was set to 1, and the values of other groups were reliable against it. Data were expressed as mean ± SEM of three determinations, **P* < 0.05 compared with control, ***P* < 0.05 compared with non caspase-3 inhibitor group.

Discussion

Ipomoea pes-caprae, is a valuable folk-medicinal plant, distributed beach plants throughout tropical and subtropical areas. The present study which evaluated the anticancer from aerial parts of *Ipomoea pes-caprae* and their efforts were also made to provide new insights of the probable apoptotic mechanism. For general screening of bioactivity, the different solvents (with different polarity) were used in the present study. The active compounds from EtOH, Hex and SCO_2 extracts included the polar, non-polar and both substances, respectively. The highest yield was found with Hex and EtOH; whereas the minimum yield was found with SCO_2 extract indicating that the *Ipomoea pes-caprae* contained mostly of lipophilic compounds such as waxes, chlorophyll, and organic compounds. In literature review, *Ipomoea pes-caprae* contains chemical diversity such as naphthalenone, (-)-mellein, eugenol, 4-vinyl-guaiacol and resin glycosides depending on optimizing maceration and extraction protocols. [7, 12] The eugenol was chosen as chemical markers in this study by GC-MS because of its well-known anti-inflammatory and anticancer properties. [13, 14] At the same duration of extraction time, SCO_2 extract has been identified as the highest eugenol extract than Hex and EtOH. Moreover, supercritical carbon dioxide extractions can be regarded as a suitable alternative for using non-toxic solvent and thus providing better extract of the natural compounds than the conventional solvents. [15]

The SCO_2 extract showed the moderate cytotoxic activity followed by Hex againts KB cell line without causing excessive damage to normal PBMC cells. These results did not meet the criteria according to the U.S. NCI plant screening program. The crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value is less than 20 μg /mL. [16] Thus, the combination of these extracts with an anticancer agent may be considered as potential possibilities for cancer treatments. Previous reports showed that the pentasaccharide resin glycosides isolated from *Ipomoea pes-caprae* were not only cytotoxic against multidrug resistance in MCF-7/ADR cells. In combination of pentasaccharide with doxorubicin, the cytotoxicity of doxorubicin increased by 1.5-3.7-fold. [8] Moreover, the traditional utilization by local people has proclaimed *Ipomoea pes-caprae* as safe, and the plant sources are easily available. Additionally, it can be assumed that multiple phytochemicals rather than a single compound may have contributed to the cytotoxic effects in KB cells. Further studies still needed to fractionate the bioactive components and then to treat on more cancer cell lines.

In order to understand the molecular mechanisms related to the observed cell death, a number of techniques such parameters as DNA fragmentation on agarose gel electrophoresis, cleavage of nuclear DNA by DAPI staining, loss of $\Delta\Psi m$ by JC-1 staining and caspase-3 activity analysis were investigated. The nucleosomal DNA ladder in agarose gels was one of the biochemical hallmarks of apoptosis. [17] Here, these results showed that the DNA fragmentation in a smear pattern was observed. In the *in vitro* study, cells have no macrophages to engulf apoptotic cells, so DNA fragmentation due to both apoptosis and necrosis was considered. The cells which exhibited late apoptosis or necrotic death produced random DNA fragmentation of multiple sizes after gel electrophoresis. [18] Consistent with the results of genomic DNA electrophoresis, the observation by microscopy also demonstrated chromatin condensation and nuclear fragmentation of treated cells. Moreover, the losses of $\Delta\Psi m$ and caspase-3 activation were observed. The collapse of $\Delta\Psi m$ occurred through formation of pores in the mitochondrial membrane with release of cytochrome C, and proapoptotic proteins that activated caspase-3. [19] Caspases, a family of cysteine proteases, are the key effector molecules led to the induction of apoptosis. The executioner caspase-3 is the final pathway of apoptosis, which activates the endonuclease (caspase-activated DNase) in the process of DNA fragmentation and cleaves various intracellular targets, including cytoskeletal protein. [19] The role of mitochondrial membrane potential and caspases-3 activation in *Ipomoea pes-caprae*-treated KB cells has not been evaluated before. These findings indicated that the Hex and SCO_2 extract-induced apoptosis in KB cells, at least in part through the mitochondrial and caspase-3 pathway.

It has long been recognized that cancer is closely linked to inflammation. Thus, the presence of chronic inflammation appears to facilitate the initiation and progression of cancers by providing a proper microenvironment for the exponential growth of cancerous cells. Thus, there are numerous literatures approving of anti-inflammatory agents for increasing apoptosis and increasing sensitivity to other chemotherapeutic agents. [20] A recent study by

Yu BW, et al. demonstrated that *Ipomoea pes-caprae* induced synergistic cytotoxic effects *in vitro* when combined with doxorubicin making them potential candidates for a decrease of the toxicity of conventional chemotherapeutic agents. [8] The presence of various bioactive compounds in the *Ipomoea pes-caprae* may be a valid strategy for chemoprevention and chemosensitization. In addition, new combinations will be able to warrant further in-depth investigations of each of the bioactive compounds present in *Ipomoea pes-caprae*.

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Conflict of interest

None of the authors has any potential conflict of interest to disclose.

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