



International Journal of
Cancer Research

ISSN 1811-9727



Academic
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Cytotoxic and Anti-Angiogenic Properties of the Stem Bark Extract of *Sandoricum koetjape*

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Abstract: Cytotoxic and anti-angiogenic properties of n-hexane extract of *Sandoricum koetjape* stem bark were investigated *in vitro*. 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell proliferation assay was used to study the cytotoxic properties on Human Umbilical Vein Endothelial Cell (HUVEC) and both colon cancer and normal cell lines; HCT-116, HT-29 and CCD-18CO. Rat aorta ring assay was used to study the anti-angiogenic properties of the extract. At 100 $\mu\text{g mL}^{-1}$, the extract showed 94 \pm 5.5% inhibition of the outgrowth of the blood vessels from the rat aorta rings. The extract also showed a dose dependent growth inhibition of all tested cell lines, IC₅₀ values against HCT-116, HUVEC, CCD-18CO and HT-29 were 14, 23, 50 and 52 $\mu\text{g mL}^{-1}$, respectively. At 50 $\mu\text{g mL}^{-1}$, the extract had potently induced apoptotic cell death of HCT-116 colon cancer cell line by inducing caspases 3 and 7 activity. These results showed that n-hexane extract of *S. koetjape* possess both anti-angiogenic and apoptotic properties on colon cancer cell lines making it a good candidate for further studies.

Key words: *Sandoricum koetjape*, anti-angiogenesis, cytotoxic and apoptotic properties, colon cancer

INTRODUCTION

Natural products derived from plant resources have been extensively used in folk medicine for the treatment of different human illnesses including cancer. Phytochemicals or their synthetic analogues are among the most used anticancer agents such as taxol and Vincristine. Naturally occurring substances play a pivotal role in drug discovery and development, it's estimated that about 20-25% of new drugs are derived from natural products or their synthetic analogues (Newman *et al.*, 2003).

Sandoricum koetjape from the family Meliaceae is a medium sized tree native to South East Asia including Malaysia and Philippine Islands, the local name in Malaysia is Sentul. Traditionally a decoction of the bark is used by Malays as a tonic after childbirth (Burkhill, 1966; Rasadah *et al.*, 2004). Recently, four triterpenes were isolated from the stem bark extract

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of *S. koetjape* Merr; 3-oxo-12-oleanen-oic acid, katiconic acid, B-sitosterol and koetjapic acid, the first two compounds were reported to have anti-inflammatory activity *in vivo* (Rasadah *et al.*, 2004). Sandoricin and 6-hydroxysandoricin, two limonoids were isolated from the seeds of *S. koetjape*, both were reported to have anti-feedant activity (Powell *et al.*, 1991). Three other modified limonoids were isolated from the leaves; namely Sandrapins A, B and C (Ismail *et al.*, 2003a). Other studies have indicated that the stem contains anticancer agents as katiconic acid and 3-oxo-olean-12-en-29-oic acid with significant activity when tested on P-388 cells (Kaneda *et al.*, 1992). Also, Ismail *et al.* (2003b) have reported anti-carcinogenic activity of both koetjapic acid and 3-oxo-olean-12-en-29-oic acid.

Angiogenesis is a process by which new blood vessels are formed from pre-existing vessels. Angiogenesis plays a crucial role in the growth and metastasis of solid tumors as well as several other chronic inflammatory diseases including rheumatoid arthritis, proliferative diabetic retinopathy and psoriasis (Folkman, 1995; Pepper, 1997). Anti-angiogenic drugs work by inhibiting the synthesis of new blood vessels supplying tumor cells with the required nutrients and oxygen aiming to delay both primary and metastatic tumor growth while overcoming the inherent cytotoxicities of classical chemotherapies (Dhanabal *et al.*, 2005). Earlier studies of Avastin; a monoclonal anti-body for Vascular Endothelial Growth Factor (VEGF); and fluorouracil-based combination therapy showed a significant improvement in survival of patients with metastatic colorectal carcinoma (Hurwitz *et al.*, 2004).

There are few reports on the bioactivity and pharmacological properties of *S. koetjape* with fewer reports on cytotoxic activity and no reports could be found about anti-angiogenic activity of *S. koetjape*, thus this research was done in order to study the *in vitro* cytotoxic and anti-angiogenic properties of the stem bark extract of *S. koetjape*. This study reported both *in vitro* cytotoxic and anti-angiogenic properties of *S. koetjape* indicating that *S. koetjape* is a good candidate for further *in vivo* studies targeting colorectal carcinoma.

MATERIALS AND METHODS

Plant Material

The stem bark of *S. koetjape* was collected from the main campus of University Sains Malaysia, Penang, Malaysia during middle of July 2008. The plant material was authenticated by the herbarium unit, School of Biology, USM. A voucher specimen of the plant (the leaves and the flowers) was deposited at both the herbarium unit; School of Biology and Bilik Herba; School of Pharmaceutical Sciences, USM (11015).

Preparation *S. koetjape* Extracts

Collected fresh stem bark was chopped and dried in oven at 50°C for overnight. The plant material was sequentially extracted with n-hexane and methanol. N-hexane extract (AE-17) was prepared by adding 500 mL n-hexane to 100 g of the pulverized plant material, extraction was carried out at room temperature with intermittent shaking for 24 h. The extract was filtered and concentrated under vacuum at 45°C by rotary evaporator (Buchi, USA) and was further dried at 45°C in oven for overnight. The plant residue was re-extracted with methanol for further 24 h the extract (AE-18) was processed as mentioned above. Stock solutions of both extracts were prepared at 20 mg mL⁻¹ in 100% dimethylsulfoxide (DMSO). Dimethylsulfoxide stock solutions as well as DMSO were serially diluted with cell culture medium, so the highest DMSO concentration exposed to the cells was 0.5% v/v.

Cell Lines and Cell Culture Reagents

Human colorectal adenocarcinoma cell line HT-29; Catalogue number (HTB-38), human colorectal carcinoma cell line HCT-116; Catalogue number (CCL-247) and human normal colon cell line CCD-18CO; Catalogue number (CRL-1459) were purchased from ATCC, USA. Human umbilical vein endothelial cell line HUVEC; Catalogue number (8000) was purchased from ScienCell, USA. M199 cell culture medium, RPMI 1640 cell culture medium, Foetal Bovine Serum (FBS) and Penicillin Streptomycin (PS) solution were from Gibco, USA. Endothelial Cell Medium (ECM) was from ScienCell, USA. XTT cell proliferation assay kit was from Sigma-Aldrich, Germany. All reagents for rat aorta ring assay were from Sigma-Aldrich, Germany. Caspase Glo 3/7 assay kit was from Promega, USA.

The HUVEC was maintained in ECM supplemented with 5% FBS, 1% P/S and 1% Endothelial Cell Growth Supplements (ECGS). Both HT-29 and HCT-116 cell lines were maintained in RPMI 1640 cell culture medium supplemented with 10% FBS and 1% PS. CCD-18CO cell line was maintained in DMEM cell culture medium supplemented with 10% FBS and 1% PS solution. Cell culture work was performed in class II biosafety cabinet (ESCO, USA) under sterile conditions the cells were incubated in a humidified incubator with 5% CO₂ at 37°C.

Experimental Animals

The 12-14 weeks old Sprague Dawley male rats were obtained from the animal house facility of USM and were kept for one week in transient animal house (School of Pharmaceutical Sciences) before doing the experiment. The animals were kept in well ventilated cages at 12 h light cycle with food and water provided all the time, the bedding was changed every other day. The animals were humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. A midline incision was made into the abdominal and thoracic cavities including splitting of the sternum and thoracic aortas were collected. All experimental work was done according to the guidelines of USM ethical committee and had their approval Reference number USM/PPSF/50 (084) Jld.2.

Rat Aorta Ring Assay

The assay was performed according to the standard protocol of Brown *et al.* (1996) with minor modifications. In brief, thoracic aortas were excised from Sprague Dawley male rats aged 12-14 weeks, rinsed with serum free medium, cleaned of the fibroadipose tissue and were cross sectioned into thin rings of about 1 mm thickness. One ring was seeded in each well of tissue culture treated 48-well plates previously loaded with 300 µL of fresh M199 medium; supplemented with fibrinogen at 3 mg mL⁻¹ and aprotinin at 5 µg mL⁻¹, then 10 µL of thrombin; prepared at 50 NIH U mL⁻¹ in 0.15 M NaCl; bovine serum albumin; was added to each well and was allowed to solidify at 37°C in 5% CO₂ incubator for 60-90 min. The upper layer consisting of 300 of fresh medium; supplemented with 20% fetal bovine serum, L-glutamine at 1%, aminocaproic acid at 0.1%, amphotericin B at 1%, gentamicin at 0.6%. The extracts were added at 100 µg mL⁻¹ and the tissues were incubated at 37°C in 5% CO₂ in a humidified incubator, on day four the upper layer was replaced with fresh medium prepared as previously mentioned with the plant extract added at 100 µg mL⁻¹. Dimethylsulfoxide was used as a negative control and suramin at 100 µg mL⁻¹ was used as a positive control. The magnitude of blood vessel outgrowth was quantified according to the technique developed by Nicosia *et al.* (1997). Briefly, the distance of blood vessels outgrowing from the primary tissue ex-plant was measured on day five under the 4x magnification power of inverted light microscope supplied with Leica Quin computerized imaging system. The experiment was

performed in triplicates each replicate containing six rings and the results were presented as a mean percent inhibition to the negative control ($n = 18$) \pm Standard Deviation (SD). The following formula was used to calculate the percent of inhibition:

$$\text{Percent of blood vessel inhibition} = (1 - (A_0/A)) \times 100$$

Where:

A_0 = Distance of blood vessel growth in the samples

A = Distance of blood vessel growth in the control

Preliminary Cytotoxicity Studies

The XTT cell proliferation assay was performed to study the cytotoxic properties of the extracts. The assay was performed in 96-well plate format according to directions of the supplier (Sigma-Aldrich). The cells were seeded at $(1.5-2) \times 10^4$ cell/well in 180 μL of fresh culture medium and were allowed to attach for overnight at 37°C in 5% CO_2 . The cells were treated with the extracts at $100 \mu\text{g mL}^{-1}$ for 48 h. The XTT solution; prepared at 0.5 mg mL^{-1} in sterile PBS; was added to each well at 10% v/v and was incubated at 37°C in 5% CO_2 for 5 h. Absorbance of the water-soluble formazan salt was measured by Multiskan Ascent microplate reader (Thermolab Systems 354, Finland) at primary wave length of 450 nm and a reference wave length of 650 nm. Each plate contained samples, positive control, negative control and blank. Dimethylsulfoxide at less than 0.5% v/v was used as a negative control, betulinic acid was used as a positive control for colon cancer cells and Vincristine was used as a positive control for HUVEC. The assay was performed in quadruplicates and the results were presented as a mean percent inhibition to the negative control \pm SD. The following formula was used to calculate the percent of inhibition:

$$\text{Percent of inhibition} = (1 - (OD_0/OD)) \times 100$$

Where:

OD_0 = Optical density of the samples

OD = Optical density of the negative control

Dose Response Cytotoxic Properties of n-Hexane Extract

To study the dose-response relationship of n-hexane extract, XTT assay was performed as mentioned above. Serial dilutions of the extract stock solution were prepared in corresponding cell culture medium. Cells were treated for 48 h and quantification of the results was done as mentioned above. The dose response curves were obtained by blotting the percent inhibition versus the concentrations, the regression equations were used to calculate the median inhibitory concentrations (IC_{50} values).

Caspase GLO 3/7 Apoptosis Assay

Caspase Glo 3/7 assay was performed according to directions of the supplier, Promega, USA. Briefly, HCT-116 cells were seeded in white 96-well plate format at 4×10^4 well $^{-1}$ in 200 μL of RPMI 1640 cell culture medium and were allowed to attach for overnight. Next day the medium was replaced with fresh one containing AE-17 at 50 and 25 $\mu\text{g mL}^{-1}$ and betulinic acid at 25 $\mu\text{g mL}^{-1}$, DMSO was used as a negative control and cell-free medium was used as a blank. Cells were treated for 4.5 h at 37°C in 5% CO_2 ; after treatment culture medium was replaced with 200 μL of 1:1 v/v mixture of RPMI 1640: Glo 3/7 reaction buffer and was

incubated for 30 min at 37°C in 5% CO₂. Luminescence was measured by HIDEX Plate CHAMELEON microplate reader (Multitechnology Plate Reader type, 425-100 Finland). The assay was performed in triplicates and the results were presented as mean of Relative Light Units (RLU) ± SD according to the following equation:

$$\text{RLU} = \text{Luminescence (samples)} - \text{Luminescence (blank)}$$

Statistical Analysis

All data is presented as Mean±SD. Statistical analysis of the data was carried out by using Student-t test and One way ANOVA followed by Tukey Post-hoc test. The differences between the means are considered significant at $\alpha = 0.05$. IC₅₀ values were calculated from the regression equations.

RESULTS

Extraction

Extraction results were presented as wt./wt. percent yield. A 5% wt./wt. yield was obtained for both n-hexane and methanol extracts.

Rat Aorta Ring Assay

The distance of blood vessels outgrowth was measured on day five, the average (n=18) was calculated and the results were presented as a mean percent inhibition to the negative control±SD. At 100 µg mL⁻¹, AE-17 extract of *S. koetjape* showed 94±5.5% and AE-18 showed 52±1.5% inhibition of the blood vessel outgrowth. Suramin at 100 µg mL⁻¹ showed 100% inhibition of the blood vessels outgrowth (Fig. 1A-D).

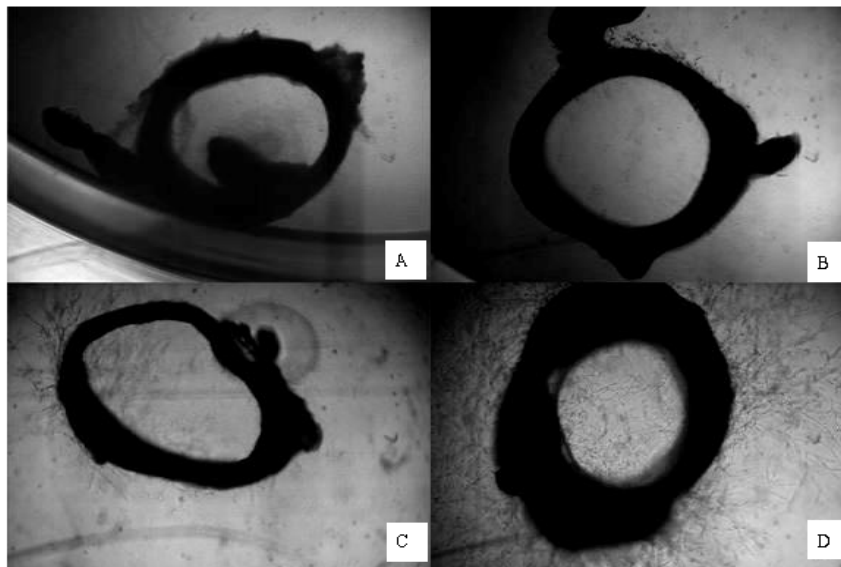


Fig. 1: Picture showing rat aorta ring assay results. Suramine showed 100% inhibition (A), AE-17 showed 94±5.5% inhibition of the blood vessel outgrowth (B), AE-18 showed 52% inhibition (C), DMSO treated rings (negative control) showed a full growth (D)

Preliminary Cytotoxicity Studies

A preliminary cytotoxicity study at $100 \mu\text{g mL}^{-1}$ was performed by XTT cell proliferation assay, the assay was performed in quadrates and the results were presented as mean percent inhibition to the negative control. N-hexane extract showed significant cytotoxic effects, the percent of inhibition on HCT-116, HT-29, HUVEC and CCD-18CO was; 93 ± 0.1 , 100 ± 0.6 , 99 ± 0.6 and $100 \pm 0.2\%$, respectively. The percent of inhibition induced by the methanolic extract on HCT-116, HT-29, HUVEC and CCD-18CO was 46 ± 6 , 1 ± 3 , 18 ± 6 and $10 \pm 3\%$, respectively.

Dose Response Cytotoxic Properties of N-Hexane Extract

The dose response curves of n-hexane extract of *S. koetjape* were obtained by blotting the percent inhibition versus concentrations. Figure 2-5 show the dose response curves of XTT cell proliferation assay results. N-hexane extract (AE-17) showed a dose dependent growth inhibition of HCT-116 (Fig. 2), HT-29 (Fig. 3), CCD-18CO (Fig. 4) and HUVEC (Fig. 5). IC_{50} values were calculated from the regression equations shown in Fig. 2-5; IC_{50} values were 14, 52, 50 and $23 \mu\text{g mL}^{-1}$, respectively.

Betulinic acid was used as a positive control for both colon cancer and the normal cell lines. Betulinic acid also showed a dose dependent growth inhibition (Table 1). IC_{50} values against HCT-116, HT-29 and CCD-18CO were calculated from the following non linear regression equations; $Y = 26.258\text{Ln}(x)-3.8471$, $Y = 26.391\text{Ln}(x)-5.9519$ and $Y = 27.602\text{Ln}(x)-6.2595$, respectively. IC_{50} values on HCT-116, HT-29 and CCD-18CO were 7.8, 8.3 and $7.7 \mu\text{g mL}^{-1}$, respectively. Vincristine was used as a positive control for HUVEC, at

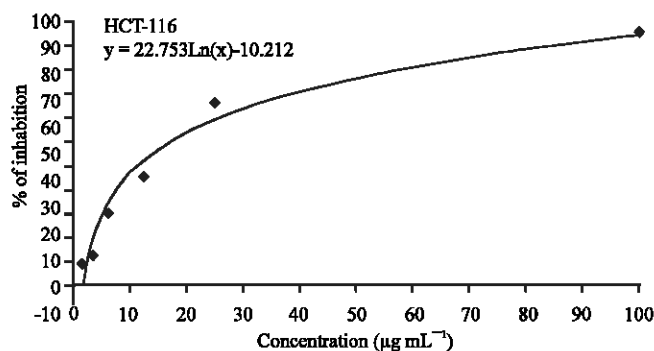


Fig. 2: Cytotoxicity results of AE-17 on HCT-116 colorectal carcinoma cell line

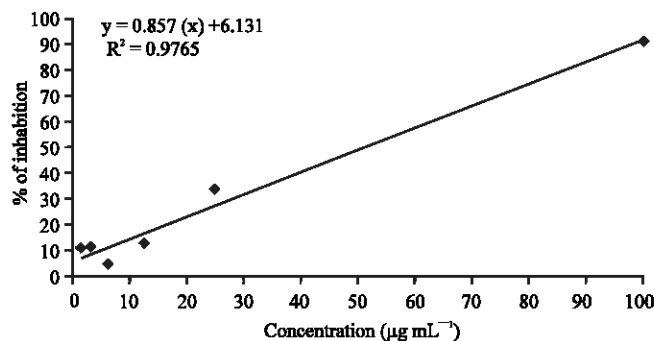


Fig. 3: Cytotoxicity results of AE-17 on HT-29 colorectal adenocarcinoma cell line

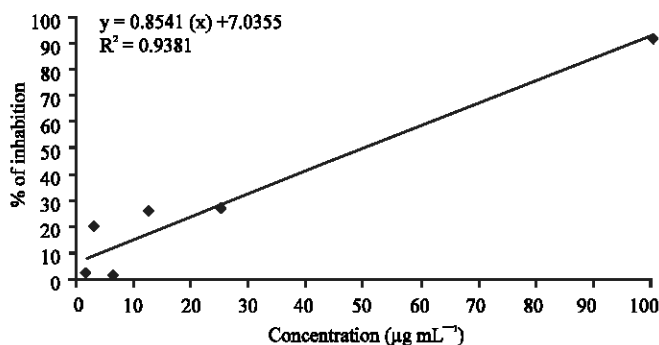


Fig. 4: Cytotoxicity results of AE-17 on CCD-18CO normal colon cell line

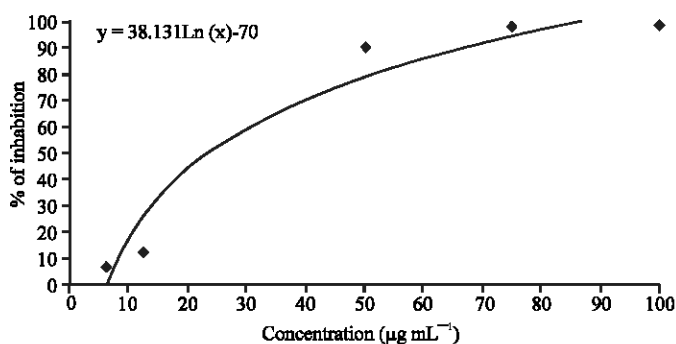


Fig. 5: Cytotoxicity results of AE-17 on HUVEC

Table 1: Cytotoxicity results of betulinic acid on HT-29, HCT-116 and CCD-18CO cell lines

Concentration ($\mu\text{g mL}^{-1}$)	HT-29	HCT-116	CCD-18CO
1	6 \pm 1	6 \pm 1	10 \pm 2
2	8 \pm 1	9 \pm 2	8 \pm 2
4	7 \pm 2	10 \pm 3	1 \pm 1
16	89 \pm 2	95 \pm 1	94 \pm 1
32	91 \pm 1	94 \pm 1	98 \pm 1
64	93 \pm 1	91 \pm 2	97 \pm 2

15 ng mL^{-1} vincristine has inhibited the growth of HUVEC by 55%. One-way ANOVA analysis of the results followed by Tukey test showed a significant difference between IC_{50} values of the following pairs HUVEC and HCT-116, HUVEC and HT-29, HUVEC and CCD-18CO, HCT-116 and HT-29, HCT-116 and CCD-18CO, $p < 0.05$, whereas no significant difference was found between HT-29 and CCD-18CO, $p > 0.05$.

Caspase GLO 3/7 Apoptosis Assay

Caspase 3/7 activity was presented as mean of Relative Light Units (RLU) \pm SD. Caspase 3/7 activity induced by AE-17 at 50, 25 $\mu\text{g mL}^{-1}$ and by betulinic acid at 25 $\mu\text{g mL}^{-1}$ was (7331210 \pm 120968) RLU, (2147876 \pm 20000) RLU and (2817876 \pm 80000) RLU, respectively. Caspase 3/7 activity induced by DMSO was (1907876 \pm 20000) RLU. When compared to DMSO, A student-t test showed significant difference of caspase 3/7 activity between AE-17 at 50 $\mu\text{g mL}^{-1}$ and DMSO, $p < 0.05$. While at 25 $\mu\text{g mL}^{-1}$, no significant difference of caspase 3/7 activity could be found, ($p > 0.05$, Fig. 6).

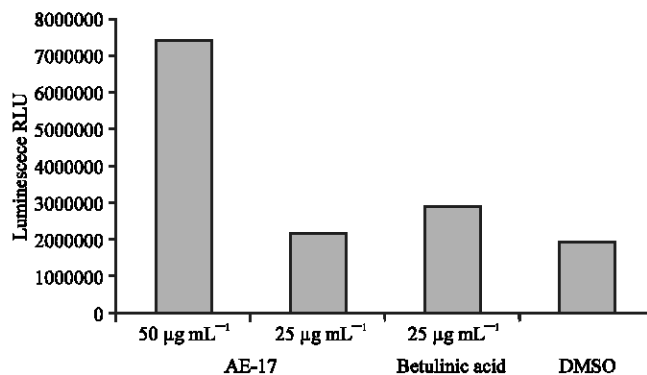


Fig. 6: Caspase Glo 3/7 apoptosis assay on HCT-116 cell line

DISCUSSION

The aim of this study was to investigate both anti-angiogenic and cytotoxic properties of the stem bark extracts of *S. koetjape*. Two extracts were prepared by n-hexane: methanol. N-hexane was selected in order to obtain the highly hydrophobic compounds whereas methanol was selected to obtain the hydrophilic and intermediate hydrophobic compounds. N-hexane extract showed significant inhibition of both blood vessels outgrowth from the rat aorta rings and cell growth by XTT assay. Compared with n-hexane extract, methanol extract didn't show significant inhibition, so the dose-response and apoptosis assays were done only for n-hexane extract. Cytotoxicity results of n-hexane extract on HCT-116, HT-29, HUVEC and CCD-18CO showed dose-dependent inhibition of growth with almost 100% inhibition at 100 µg mL⁻¹. IC₅₀ values on HCT-116, HT-29, HUVEC and CCD-18CO were 14, 52, 23 and 50 µg mL⁻¹, respectively. IC₅₀ values suggest that the colorectal carcinoma cell line HCT-116 and the endothelial cell line HUVEC were the most sensitive while HT-29 and CCD-18CO were more resistant.

Activation of caspase-3 and 7 is commonly used as a biomarker for assessment of apoptosis. Caspases 3 and 7 are effector caspases located at the end of the caspase cascade; upon their activation they activate nucleases leading to DNA fragmentation and to the final stages of cellular death, (Nicholson, 1999; Cohen, 1997). The n-hexane extract had significantly induced apoptotic cell death of HCT-116 when used as a model cell line by inducing caspases 3 and 7 activities. Based on apoptosis assay, cytotoxicity results reported in this study can be explained by induction of apoptotic cell death.

Cytotoxicity results reported in this study are consistent with previous study of Kaneda *et al.* (1992) and Ismail *et al.* (2003a, b) where cytotoxic and anti-carcinogenic triterpenes were isolated from stem of *S. koetjape*, however anti-cancer activity reported in this study is lower than that of the previously published reports. This difference can be explained as a crude extract was used in this study whereas pure compounds were used in the previous studies. Also, the cytotoxicity assays, cell lines and potency shown in this study differ from that of the previous studies.

Numerous experimental, clinical and epidemiologic studies indicate that Non Steroidal Anti-inflammatory Drugs (NSAIDs), particularly the highly selective COX-2 inhibitors, show promise as anticancer drugs. NSAIDs have been shown experimentally to stimulate apoptosis and to inhibit angiogenesis, two mechanisms that help to suppress malignant transformation and tumor growth (Thun *et al.*, 2002). A previous study of Rasadah *et al.*

(2004) showed *in vivo* anti-inflammatory activity of 3-oxo-12-oleanen-oic acid and katiconic acid, two terpenoids isolated from *S. koetjape*. Combined together; rat aorta ring assay, cytotoxicity, apoptotic and anti-inflammatory properties make *S. koetjape* a good candidate targeting colorectal carcinoma as this combined activity is preferred in the treatment of cancer in general and it was reported to be effective in treatment of colorectal carcinoma in case of combination of Avastin and 5-fluorouracil (Hurwitz *et al.*, 2004).

Finally, this study provided additional evidence for the presence of effective anti-cancer agents in the stem bark of *S. koetjape*. However, further studies are required to explore the involved anti-angiogenesis and apoptotic mechanisms of action.

ACKNOWLEDGMENTS

This study was supported partially by the research chair funded by King Saud University on drug targeting and treatment of cancer with nanoparticles and by University Sains Malaysia, grant number (203-PFARMASI-671154). Special thanks to Mr. Shunmugan A/C Vellosamy; herbarium unit at School of Biology; for his role in authentication of the plant.

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