



International Journal of
Cancer Research

ISSN 1811-9727



Academic
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***In vitro* Cytotoxic and Apoptotic Properties of the Stem Bark Extract of *Sandoricum koetjape* on Breast Cancer Cells**

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Abstract: Both cytotoxic and apoptotic properties of the stem bark extract of *Sandoricum koetjape* were investigated *in vitro*. 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell proliferation assay was used to study the cytotoxic properties on three breast cancer cell lines and one normal cell line; MCF-7, MDA-MB-231, T47D and MCF-10A, respectively. Caspase Glo 3/7 assay was used to study the apoptotic activity on MCF-7 as a model cell line. The n-hexane extract showed a dose dependent growth inhibition of all tested cell lines with IC₅₀ values between 44 and 48 µg mL⁻¹. At 100 µg mL⁻¹, the extract induced apoptotic cell death of MCF-7 by inducing activity of the effector caspases 3 and 7. These results indicated that n-hexane extract of *S. koetjape* has cytotoxic and apoptotic properties on breast cancer cell lines making it a good candidate for further studies.

Key words: Cytotoxic and apoptotic properties, *Sandoricum koetjape*, breast cancer

INTRODUCTION

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). Few reports are available about biological and pharmacological properties of pure compounds isolated from different parts of *S. koetjape*. Sandoricin and 6-hydroxysandoricin two limonoids were isolated from the seeds of *S. koetjape* have been 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). Kaneda *et al.* (1992) have reported the presence of anticancer agents including kationic acid and 3-oxo-olean-12-en-29-oic acid in the stem of *S. koetjape*. Also, Ismail *et al.* (2003b) have

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reported anti-carcinogenic activity of both koetjapic acid and 3-oxo-olean-12-en-29-oic acid from the bark of *S. koetjape*. Recently, four triterpenes were isolated from the stem bark extracts of *S. koetjape merr*; 3-oxo-12-oleanen-oic acid, kationic acid, B-sitosterol and koetjapic acid, the first two compounds were reported to have anti-inflammatory activity *in vivo* (Rasadah *et al.*, 2004).

Breast cancer is the most common malignancy in American and Northwestern European women (Parkin *et al.*, 1993). It is estimated that one in eight American women and one in 12 women in the United Kingdom will develop breast cancer in their lifetime and the incidence rates in industrialized nations are on the rise. Approximately one-third of women with breast cancer develops metastases and ultimately dies from the disease (Simstein *et al.*, 2003). So, this study was designed to investigate the *in vitro* cytotoxic and apoptotic properties of the stem bark crude extracts of *S. koetjape*, a mechanism-based screening approach targeting both hormone responsive and non-responsive breast cancer cell lines was employed to achieve our goals. The results presented in this study suggest *S. koetjape* as a good candidate for further research targeting cancer treatment.

MATERIALS AND METHODS

Plant Material

The stem bark of *S. koetjape* was collected from the main campus of University Sains Malaysia (USM), 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 of *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. The 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 for 24 h with intermittent shaking. The extract was filtered and concentrated under vacuum at 45°C by rotary evaporator (Buchi, USA) and further dried overnight at 45°C and kept at -20°C until use. The plant residue was re-extracted with methanol for 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).

Cell Lines and Cell Culture

Human hormone sensitive and invasive breast cancer cell line MCF-7; Catalogue number (HTB-22), Human hormone resistant breast cancer cell line MDA-MB-231; Catalogue number (HTB-26), Human hormone sensitive early stage breast cancer cell line T47D; Catalogue number (HTB-133) and human normal breast cell line MCF-10A; Catalogue number (CRL-10317) were purchased from ATCC, USA.

Cell culture reagents were purchased from Gibco, USA. RPMI 1640 medium; Catalogue number (A10491-01), Dulbecco's Modified Eagle Medium (DMEM); Catalogue number (21063-045), Minimum Essential Medium (MEM); Catalogue number (41090036), Foetal Bovine Serum (FBS); Catalogue number (10100139), Penicillin/Streptomycin (PS) solution; Catalogue number (15140163), sodium pyruvate; Catalogue number (11360-070), nonessential amino acids; Catalogue number (11140050), L-glutamine; Catalogue number (25030-081) and MEM vitamins; Catalogue number (11140076). Caspase GLO 3/7 assay kit was purchased

from Promega; Catalogue number (G8091). XTT cell proliferation assay kit; Catalogue number (TOX2), human insulin; Catalogue number (I9278), Epidermal Growth Factor (EGF); Catalogue number (E9644) and Tamoxifen (TMX); Catalogue number (T5648-1G) were purchased from Sigma-Aldrich, Germany.

MCF-7 and MDA-MB-231 cell lines were propagated in DMEM cell culture medium supplemented with 10% FBS and 1% v/v PS solution. T47D cell line was propagated in RPMI 1640 cell culture medium supplemented with 10% FBS, 1% PS and insulin at 5 $\mu\text{g mL}^{-1}$. MCF-10A cell line was propagated in MEM cell culture medium supplemented with 10% FBS, 1% PS, 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% MEM vitamins, 5 $\mu\text{g mL}^{-1}$ insulin and EGF at 100 ng mL^{-1} . Cell culture work was carried out in sterile conditions in class II biosafety cabinet (ESCO, USA).

Preliminary Cytotoxicity Studies

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 the method developed by Francoeur and Assalian (1996) with minor modifications. The cells were seeded at $(1.5-2)\times 10^4$ cell well^{-1} in 180 μL of fresh culture medium and were allowed to attach for overnight at 37°C in 5% CO_2 . Next day the cells were treated with the extracts at 100 $\mu\text{g mL}^{-1}$ for 48 h. After the treatment XTT solution; prepared at 0.5 mg mL^{-1} in sterile PBS; was added to each well at 10% v/v and was incubated for 5 h at 37°C in 5% CO_2 . 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. DMSO at less than 0.5% v/v was used as a negative control, TMX was used as a positive control and cell-free medium was as a blank. The assay was performed in quadruplicates and the results were presented as a mean percent inhibition to the negative control (DMSO) \pm Standard Deviation (SD).

The following formula was used to calculate the percent of inhibition:

$$\text{Percent of Inhibition} = (1 - (\text{OD}_s/\text{OD}_c)) \times 100$$

Where:

OD_s = Optical Density of the samples

OD_c = Optical Density of the negative control

Dose Response Cytotoxic Properties of n-Hexane Extract

The dose-response relationship of n-hexane extract was also studied by XTT assay. 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 and 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, MCF-7 cells were seeded in white 96-well plate at 4×10^4 well^{-1} in 200 μL of DMEM and were allowed to attach for overnight. Next day the medium was changed and AE-17, TMX and DMSO were added at 100, 25 $\mu\text{g mL}^{-1}$ and 0.5%, respectively. Cell-free

medium was used as a blank. After treating cells for 2 h at 37°C in 5% CO₂, the medium was replaced with 200 µL of 1:1 v/v of DMEM: Glo 3/7 reagent and was incubated for 30 min at 37°C in 5% CO₂. Luminescence was measured by HIDEEX Plate CHAMELEON microplate reader (Multitechnology Plate Reader type, 425-100 Finland). The assay was conducted in triplicates and caspase 3/7 activity was presented as a mean of Relative Light Units (RLU)±SD. The following formula was used to calculate caspase 3/7 activity in RLU:

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

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. The differences between the means are considered significant at $\alpha = 0.05$. IC₅₀ values were calculated from the regression equations.

All experiments were carried out during the period of July 2008 to March 2009 at pharmacology labs at School of Pharmaceutical Sciences, University Sains Malaysia (USM), Malaysia.

RESULTS

Extraction

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

The Preliminary Cytotoxicity Studies

A preliminary cytotoxicity study at 100 µg 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. The n-hexane extract showed significant cytotoxic effects, the percent of inhibition on MCF-7, MDA-MB-231, T467 and MCF-10A; 96±1, 90±2, 83±2 and 95±3%, respectively. The methanolic extract showed a minor growth inhibition of the tested cells as following; 27±6, 30±3, 36±4 and 40±3%, respectively.

Dose Response Cytotoxic Properties of n-Hexane Extract

The cells were treated with n-hexane extract at different concentrations, the dose response curves of n-hexane extract of *S. koetjape* were obtained by blotting the average percent inhibition versus concentrations. Figure 1 and 2 show cytotoxicity results of AE-17 and TMX, respectively. Both n-hexane extract and TMX showed a dose dependent growth inhibition. IC₅₀ values for n-hexane extract were calculated from the linear regression equations shown in Fig. 1a-d. IC₅₀ values on MCF-7, MDA-MB-321 and MCF-10A were 44 µg mL⁻¹, whereas IC₅₀ value on T47D was 48 µg mL⁻¹. One-way ANOVA analysis showed no significant difference between IC₅₀ values of the extract on the tested cell lines, (p = 0.788). TMX was used as a positive control, it also showed a dose dependent growth inhibition (Fig. 2a-d), IC₅₀ values were calculated from for the non linear regression equations shown in Fig. 2. IC₅₀ values on MCF-7, MDA-MB-231, T47D and MCF-10A were 4.8, 6.3, 4.3 and 4.1 µg mL⁻¹, respectively. One way ANOVA analysis of TMX cytotoxicity results showed significant difference between IC₅₀ values on tested cell lines, (p<0.05). These results indicated that TMX was more active on normal breast cell line MCF-10A and less active on the hormone resistant breast cancer cell line MDA-MB-231.

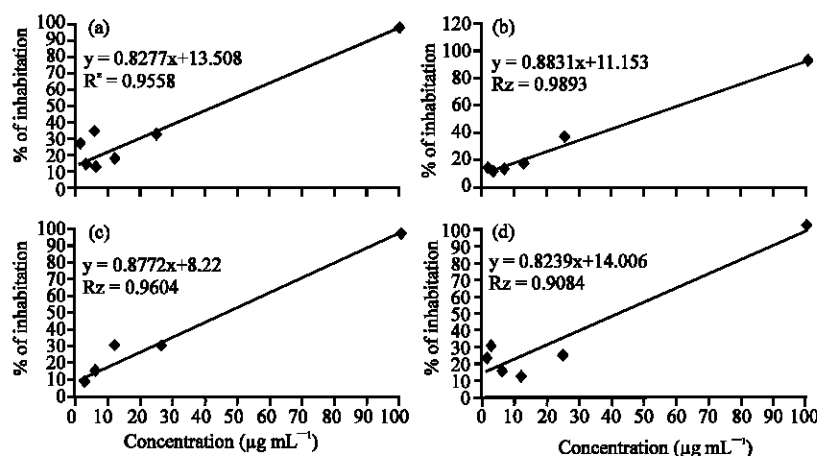


Fig. 1: Graphs show the dose response curves of AE-17 on (a) MCF.7, (b) T47D, (c) MDA-MB-231 and (d) MCF-10A cell lines

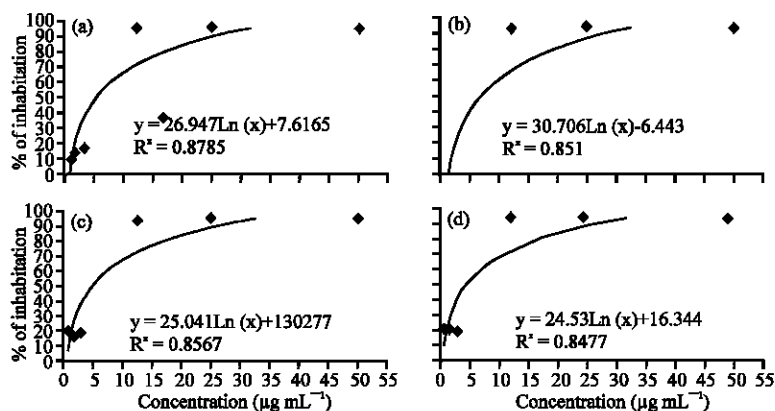


Fig. 2: Graphs show the dose response curves of TMX on (a) MCF.7, (b) T47D, (c) MDA-MB-231 and (d) MCF-10A cell lines

Caspase GLO 3/7 Apoptosis Assay

Caspase 3/7 assay results were presented as a mean $\text{RLU} \pm \text{SD}$. Caspase 3 and 7 activity induced by AE-17 at $100 \mu\text{g mL}^{-1}$, TMX at $25 \mu\text{g mL}^{-1}$ and DMSO were 430000 ± 1000 (RLU), 480000 ± 2300 RLU and 250000 ± 23000 (RLU), respectively. A student-t test showed significant difference of caspase 3/7 activity between AE-17 and the vehicle (DMSO), ($p < 0.05$).

DISCUSSION

XTT Cell proliferation assay is a simple method based on the activity of mitochondrial enzymes of living cells; the assay provides a rapid method for determination of the number of living cells to assess the rate of cell proliferation and to screen cytotoxic agents (Berridge *et al.*, 2005). The n-hexane extract of *S. koetjape* showed a significant growth inhibition with dose dependent cytotoxicity profiles against all tested cell lines, while methanolic extract showed minor growth inhibition, so the dose response and apoptosis

assays were performed on n-hexane extract only. Statistical analysis of IC_{50} values of n-hexane extract indicates nonselective cytotoxicity. 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). MCF.7 cell line was used as a model cell for apoptosis assay based on Simstein *et al.* (2003). The n-hexane extract at $100 \mu\text{g mL}^{-1}$ significantly induced apoptotic cell death of the hormone sensitive breast cancer cell line MCF-7 by inducing caspases 3 and 7 activity. Caspase 3/7 assay results suggest that the cytotoxic activity of n-hexane extract is due to activation of apoptotic cell death pathways.

Cytotoxicity results of n-hexane extract of *S. koetjape* provided additional support for the previous reports of Kaneda *et al.* (1992) and Ismail *et al.* (2003a) where cytotoxic and anti-carcinogenic triterpenes isolated from stem of *S. koetjape* were reported. However the results shown in this research article differ in terms of the cytotoxicity assays and the cell lines used in this study, also the results differ in terms of cytotoxic activity where the median inhibitory concentration of n-hexane extract is higher than that reported in the previous report of Kaneda *et al.* (1992), this difference can be explained by the use of pure compounds in the previous reports compared to the nature of crude extracts used in this study. Also these results provide additional support for a recent report of Aisha *et al.* (2009), in which *in vitro* cytotoxic and anti-angiogenic properties of n-hexane extract were reported. This cytotoxic activity might be explained by the presence of cytotoxic triterpenes such as katic acid, koetjapic acid and 3-oxo-olean-12-en-29-oic acid.

Anti-inflammatory drugs particularly the highly selective COX-2 inhibitors, show promise as anticancer drugs due to their ability to stimulate apoptosis and inhibit angiogenesis, two mechanisms that help to suppress malignant transformation and tumor growth (Thun *et al.*, 2002). *In vivo* anti-inflammatory activity of *S. koetjape* reported by Rasadah *et al.* (2004) and anti-angiogenic activity reported by Aisha *et al.* (2009), in addition to apoptotic activity reported in this article make *S. koetjape* a good candidate for further studies, as this combined activity is preferred in the treatment of cancer and it was reported to be effective in the treatment of metastatic colorectal carcinoma in case of combination of Avastin and 5-fluorouracil (Hurwitz *et al.*, 2004).

In summary, this study provides additional evidence for the presence of effective anti-cancer agents in the stem bark of *S. koetjape*. However, further studies are required to improve the percent yield of the extract, standardization of the extracts and both the mechanism of action and to study activity against *in vivo* tumor models.

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 (USM), 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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