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Research Article

Dentatin from *Clausena excavata* induces apoptosis and reduces the tumors size of La-7 induced mammary carcinogenesis in Sprague Dawley rats

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Keywords

Dentatin;
Clausena excavata;
Apoptosis;
Mammary tumors;
Tumor regression.

Abstract

Clausena excavata Burm.f., has been used as folk medicines in the eastern of Thailand for the treatment of cancer. Dentatin (DTN) was isolated from this plant. DTN-induced cytotoxicity was observed with the MTT assay. An *in vivo* study was conducted to determine the effect of DTN on LA-7 cell-induced rat mammary tumor as a confirmation of occurrence of apoptosis. After the first tumors appearance, the thirty rats were divided into five groups (n=6).Group I served as normal control animals. Group II served as non-treated LA-7 cell-induced mammary tumors rats. The other three groups comprised mammary gland tumor-bearing animals treated with 30mg/kg DTN dissolved in Tween 20 oil (Group II), 60mg/kg DTN dissolved in Tween 20 oil (Group III) and 10mg/kg tamoxifen (TAM) dissolved in Tween 20 oil (Group IV). After treatment, significant reduction in tumor volume was observed in the treated groups of dentatin compared to control groups. From the TUNEL assay, the number of apoptotic cells were significantly ($P<0.05$) higher in rats treated with DTN than those untreated. Soft X-ray imaging showed that, tumors in the mammary tumors control group grew rapidly, while groups treated with DTN-Low Dose, DTN-High Dose and TAM exerted a significant ($P < 0.05$) reduction in tumors volume. The *in vivo* study suggests that DTN inhibits proliferation, induces mitochondria-regulated apoptosis, and therefore, minimizes LA-7-induced carcinogenesis in rat mammary glands. It can be suggested that DTN may have therapeutic value in breast cancer treatment worthy of further development.

1. Introduction

Plants are considered as the oldest source of pharmacologically active bio-compounds that contribute most significantly in disease treatments throughout mankind history (Rates, 2001). *Clausena excavata* Burm. f. is a wild shrub, belonging to the Rutaceae family (Arbab*et al*., 2013; Taufiq-Yap *et al*., 2007). Dentatin is one of promising bio-compounds originally isolated from the roots of *Clausena excavata*. It is a secondary metabolite that belongs to the coumarin class (Arbab *et al*., 2011; Mowat and Murray, 1973). Cancer is a disease having complex problems that continues to intrigue researchers in the fields of plant chemistry, medicine and ethnopharmacology (Arnold, 2002). It is a malady characterized by abnormal uncontrollable cell growth (Kim 2001; Sierra *et al.*, 1995). Breast cancer,

specifically invasive ductal carcinoma (IDC), is the ultimate prevalent type of malignancies widespread in the world with remarkable annual incidence (Bachman *et al*., 2004).

Breast cancer is considered as the most frequent cancer after lung cancers, amongst 17 most common cancers reported in Malaysia (18 %). It ranked 13th by type among top 50 causes of death in Malaysia and 10th by age (Arbab *et al*., 2013; Jemal *et al*., 2011). Deaths due to breast cancer in Malaysia attained 1,716 or 1.68% of total deaths. The age adjusted Death Rate is 15.83 per 100,000 of population ranks Malaysia 100 in the world (Arbab *et al*., 2013; Jemal *et al*., 2011).The foretelling for breast cancer is mostly relying on the stage of the ailment at diagnosis. Five-year survival rates range from 84% for early disease to just 18% for advanced cancers (Sainsbury *et al*., 2000). Therefore, the goal of treatment also depends on the cancer stage at diagnosis (Parkin *et al*., 2001).

Apoptosis is a term used to describe programmed cell death believed that apoptosis is the major form of patho (Kerr *et al*., 1972) or cell suicide. This process is a normal development in multicellular organism. Apoptosis is also illustrated as "a genetically encoded cell death program with defined morphological and biochemical features" (Elmore 2007). Plants are considered as the oldest source of pharmacologically active bio-compounds that contribute most significantly in disease treatments throughout mankind history (Rates 2001). The plant *Clausena excavata* however, is easily available in Malaysia and the coumarin class compound isolated is worth investigating of its biological importance as anti-cancer agent. Therefore, this study provided detailed investigations to the characterization and potential use of dentatin, isolated from *Clausena excavata* for treatment of human breast cancer *in vivo* in rats.

2. Materials and Methods

2.1 Plant raw materials

The plant used in this study *Clausena excavata* Burm.f. was collected from Pendang, Kedah, Malaysia in February 2011. The taxonomic identification of this plant was carried out by Dr. Khamis S., (2011), (A resident Botanist) at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen of this plant was deposited in the herbarium of the institute (TI-013201-CE). Stem barks and roots Fig. 1. (c) & (d), were cut into thin slices and dried at room temperature. The dry plant materials were ground with a mill grinder into coarse powder so as to be used for extraction of biologically active substances via bioactivity guided approach.

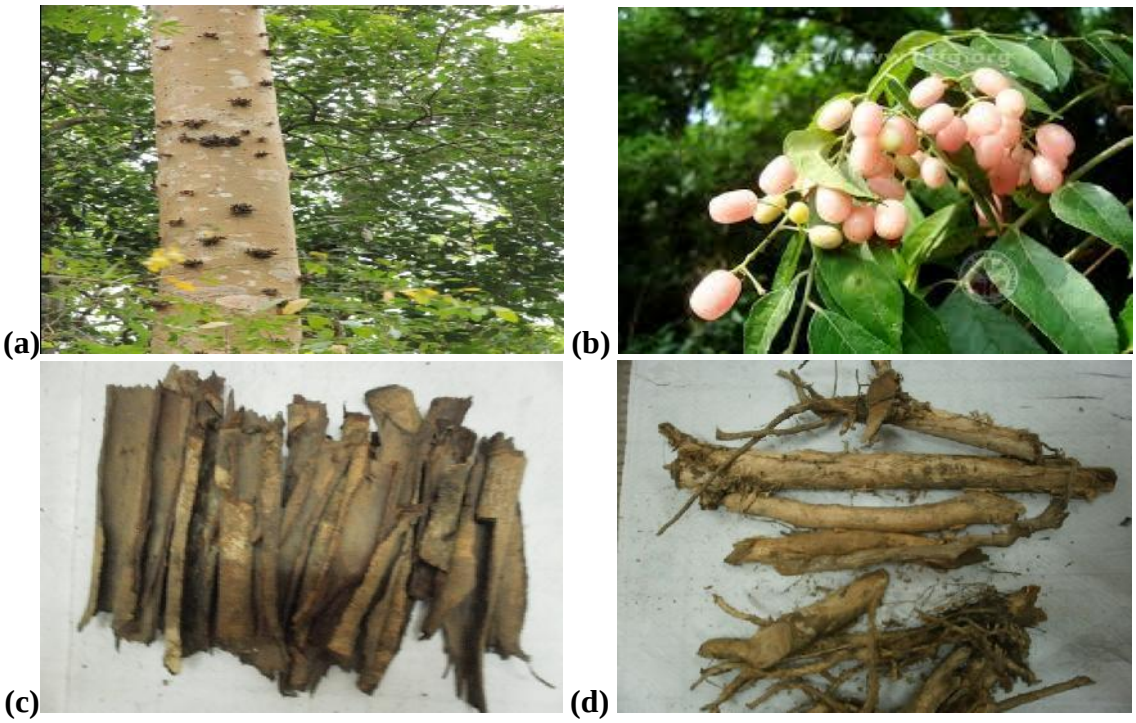

Fig.1. *Clausena excavata* Burm. F. (Rutaceae); (A) the appearance of the overall tree; (B) the fruits and leaves; (C) The dried stem barks; (D) The dried roots. Sources: (A), (B), (C) & (D) Photos before and after collection and drying of the plant parts used in isolation of DTN.

2.2 Isolation and characterization of DTN

Fresh stem barks (2.00 kg) and roots (2.5 kg) of *Clausena excavata* were extracted at room temperature (ca. 25-27 ˚C) successively with analytical grade solvents of hexane (5L), chloroform (4.5L), ethyl acetate (4L) and methanol (4L). Eight extracts were obtained. The extracts then fractionated using mini-column. DTN has been isolated from one of the root chloroform fractions. The isolated DTN was subjected to Direct Infusion Mass Spectroscopy and HPLC techniques for its characterization. The DTN crystals were kept for further chemical and pharmacological analyses.

2.3 Cell culture and cell preparation for injection

LA-7 rat mammary gland tumor cells were purchase from ATCC (VA, USA). The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) at 37°C in a humidified atmosphere of 5% CO_2 supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 IU/ml penicillin. After one week acclimation period, twenty four rats were anesthetized using an intraperitoneal injection with a mixture of ketamine-HCl(150 mg/kg body weight) and xylazine(10 mg/Kg body weight). The LA-7 cells (300 µL containing 6 x 10^6 cells) were inoculated subcutaneously into the mammary fat pad (right flank or left flank) of each rat using a tuberculin syringe and 26-Gauge needle.

2.4 Animals used in this protocol

Thirty virgin adult female Sprague-Dawley rats aged 6 to 8 weeks, weighing 180-200 g were purchased from Sapphire Enterprise, Malaysia. The animals were housed at two rats per plastic cages and allowed to acclimate in standard conditions (under a 12h light/dark cycle) for one week. The rats were given free access to distilled water and commercialized food throughout the experiment. The animals were randomly divided into five groups of six animals. The summary of the groups of animals under investigation was shown in Table1.

Table 1: Experimental protocol & treatment duration of the animals under investigation

Grouping	Experimental design (n=6)	No. of cages and No. of animals/cage	Treatment duration
Group I	Normal control (NC)	2 cages (n = 3)	–
Group II	Mammary tumor control(MTC)	3 cages (n =2)	–
Group III	DTN30mg/kg (DTN-LD)	6 cages (n= 1)	4 weeks
Group IV	DTN 60 mg/kg(DTN-HD)	6 cages (n = 1)	4 weeks
Group V	TAM10mg/kg (TAM)	6 cages (n = 1)	4 weeks

The treatments were given orally for 4 consecutive weeks to the animals using gastric intubations.

All study protocols and the experimental design were approved by Institutional Animal Care and Use Committee, University of Malaya (FAR/30/03/03/2012/IAA(R)), and research was conducted according to the guidelines for the care and use of laboratory animals.

2.5 Tumor regression study

The animals were weighed twice weekly and the tumors measured using a digital caliper. The radius of individual palpable tumor was horizontally and vertically measured and averaged. The two dimensional tumor areas were calculated a sanellipse The volume of tumor (V) was calculated by the formula given by (Carlsson *et al*., 1983):$V=ab^2/2$.Where,'a'is the longest diameter and 'b' is the shortest diameter of the tumor.

2.6 TUNEL Assay

Terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. A landmark of cellular self-destruction by apoptosis is the activation of nucleases that eventually degrade the nuclear DNA into fragments of approximately 200 base pairs in length. Detection of these DNA fragments is relatively straightforward, making this assay among the most reliable methods for identifying apoptotic cells. Flourometric TUNEL assay measures fragmented DNA of apoptotic cells by incorporating flourescein-12-dUTP (a) at 3'-OH DNA ends using the terminal recombinant deoxynucleotidyltransferase enzyme (rTdT). This assay was performed on FFPE mammary gland tissues using DeadEnd^{TM}Flourometric TUNEL system (PromegaInc, Madison, USA), according to the manufacturer's instructions.

The samples were analyzed under a confocal microscope (ZIESS, LSM 70) using standard flourescein filters. DNAase was used as positive control. Percentages of cells undergo apoptosis in the TUNEL assay were calculated based upon the corresponding negative control.

2.7 X-ray imaging

The tumor mass in rat breast area was confirmed by radiographs using a soft X-ray instrument (Shimadzu, Japan). Prior to imaging, animals were shaved and anesthetized as described earlier. The rats were exposed to the X-ray at44 kV, for2.5 milliseconds.

3. Results and Discussion

In this study, rat mammary tumor cell, LA-7 was used to induce mammary tumors in right or left flank of rats to produce malignant tumors. Apoptosis is 'silent death' and happens without inflammation, because dying cells are phagocytosed by intraepithelial macrophages and alveolar epithelial cells (Tatarczuch *et al*., 2002). Based on the bioactivity guided fractionation of the extracts of roots and stem barks of *Clausena excavata*, dentatin was isolated as colorless needles-shaped crystal Fig.2a from chloroform extract (fraction 5) of the roots of *Clausena excavata*. The DIMS of DTN, Fig.2c indicated that the presence of molecular ion peak at *m/z* 263 which corresponds to the molecular formula of $C_{20}H_{22}O_4$. The compound was obtained with 95.76% purity. HPLC was run on DTN as a validation for its purity, which gave one peak, Fig.2d.

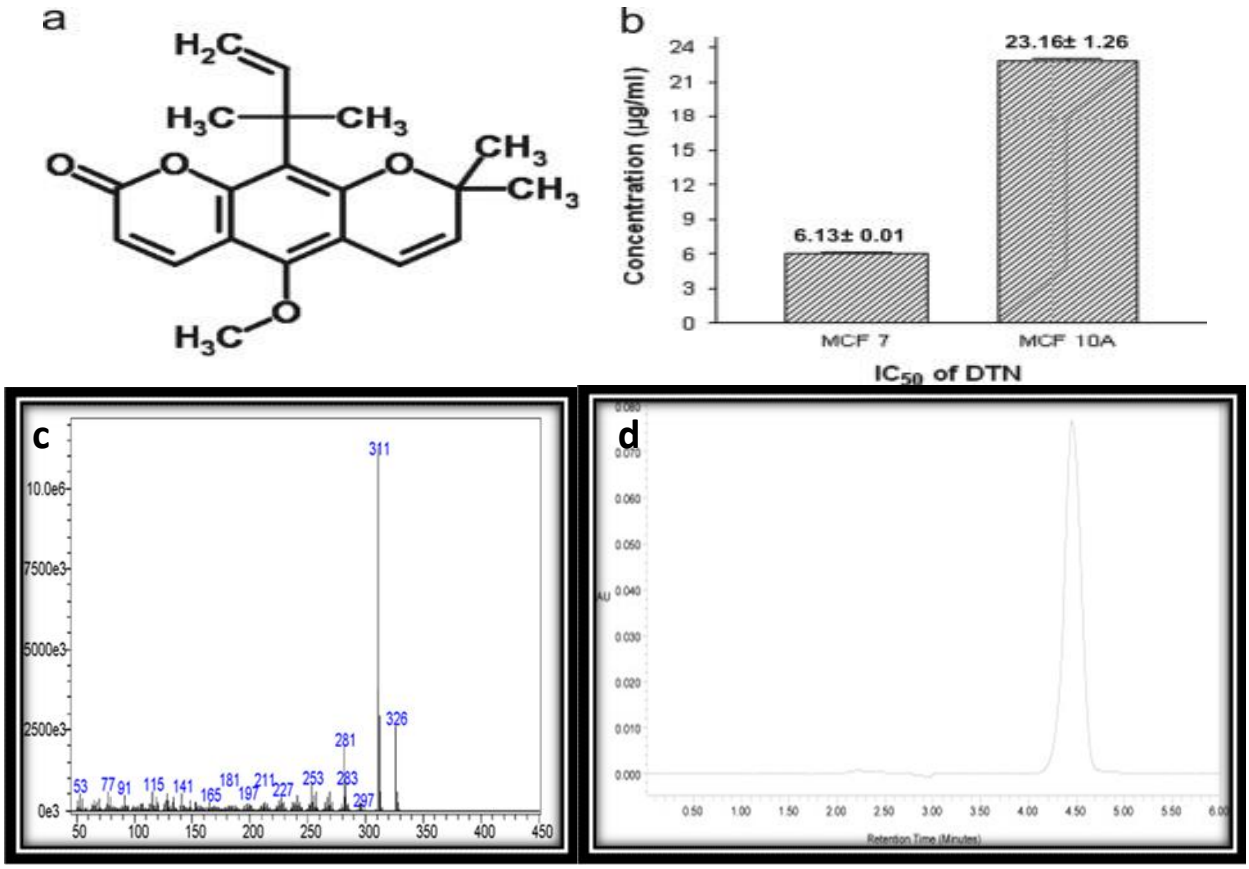

Fig. 2. Identification, characterization and MTT of DTN: Chemical structure of Dentatin (molecular weight: 326.15) (a), IC_{50} of DTN on MCF-7 and MCF-10A cells (b), DI-MS showing presence of molecular ion peak at m/z 326 which corresponds to the molecular formula of $C_{20}H_{22}O_4$ (c) and HPLC Profile of DTN (95.76% Purity) (d).

Dentatin Inhibits Cell Growth of MCF-7 breast Cancer Cells. To study anticancer potential of dentatin on breast cancer cells, we treated MCF-7 cells with various concentrations of dentatin. Cell viability at each time-point was determined by MTT colorimetric assays. As shown in Figure 2b, the half-maximal inhibition concentration (IC_{50}) readings of dentatin-treated MCF-7 cells at 6.13 µM. On the other hand, dentatin showed less cytotoxic effect on MCF-10 normal breast epithelial cell line with IC50 > 20 µM (23.16).

In our study, the rate of tumorigenicity of LA-7cells ($6X10^6$cells/300µL) in these rats was 80%. As shown in Table 2, Tumors in the mammary tumor control group grew rapidly, while groups treated with DTN-LD, DTN-HD and TAM showed a significant ($P<0.05$) reduction in tumor volume. This finding is similar to those reported earlier, when TAM alone and TAM plus riboflavin, niacin and Co Q_{10} were used (Perumal *et al*., 2005).

Table 2: Effect of treatment with TAM, DTN-LD and DTN-HD on animal tumor volume (mm^3) compared to the control groups.

Group	Drug treated days			
	0	**7**	**14**	**28**
MTC	657±209	1090±177	1445^a±549	1564^a±540
TAM	441±159	874±210	490^b±217	99^b±49
DTN-LD	384±217	914±316	350^b±207	48^b±25
DTN-HD	467±340	807±351	448^b±218	60^b±29

Both cell proliferation and apoptosis have been used clinically for assessment of tumor prognosis because there is a relationship between proliferation index and malignancy in many tumors (including breast cancer)and for the analysis of the response of cancer cells to clinical interventions (Railo *et al*., 2007). Mammary tumor control group (LA7 induced none treated) animals Fig.3d shows that the cells which were negative to TUNEL staining. It is clear that the propidium iodide (PI) stain was dominant in these samples. In these cells, the mammary ducts were lined with cells of well-defined DNA containing nucleus with the PI red staining. Mammary gland sections of rats with LA7-induced adenocarcinoma after treatment with 30 and 60 mg/kg of DTN (Fig.3 a & b) respectively, showed significantly (*P* <0.05*)* higher numbers of apoptotic cells than the LA7 induced none treated sections. Apoptotic cells were evident in the mammary gland sections of all DTN-treated rats. The highest percentages of apoptotic cells were observed in animals treated with dentatin, both high and low dosages (Table 3).

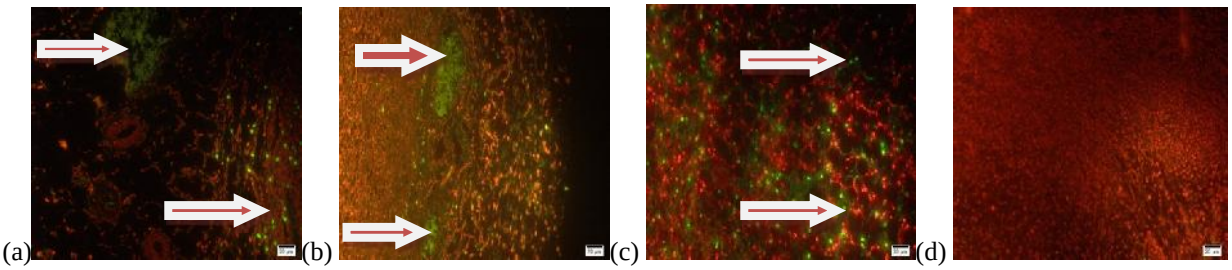

Fig. 3. TUNEL labeling of cancer with: (a) treatment of 30 mg/kg of DTN group. Note small dots of green fluorescent stained cells (arrows) overlapping the PI red fluorescent staining for DNA nucleus; (b) treatment of 60 mg/kg of DTN group. Note the sum of the cells was stained with dense green fluorescent (arrows); (c) treatment of 10 mg/kg of TAM group. Note the nuclei were stained with dense red (around the blue circles) and green fluorescent and d) mammary tumor control group (LA7 induced none treated) animals. No green fluorescent stain was noted on these sections. (Magnification 40x).

Table 3: Percentages of cells undergo apoptosis in TUNEL assay.

LA7-induced non-treated	TAM (10mg/kg)	DTN (30mg/kg)	DTN (60mg/kg)
2 %	51%	63%	69%

Palpation of mammary gland was performed by rolling up the skin and pinching the mass between the fingers (Perumal *et al*., 2005) in 10-14 early days after injection of LA7 cells. The tumors that developed in 80% of the rats were soft, rubbery and as they grew they become irregular, lobulated and more adhesive to the skin than to the body wall. Mammary tumors were observed as early as 7 to10 days after LA7cell injections, which indicated that the cells have strong tumorigenicity properties. The results of soft X-ray images of tumor bearing rats are presented in Fig.4 (a) and (b). The results showed that treatment with DTN-High Dose (60mg/kg) did not negatively affect the body weights of these animals and simultaneously reduced the tumor size Fig.4a compared to the tumor size of LA7-induced none treated animals.

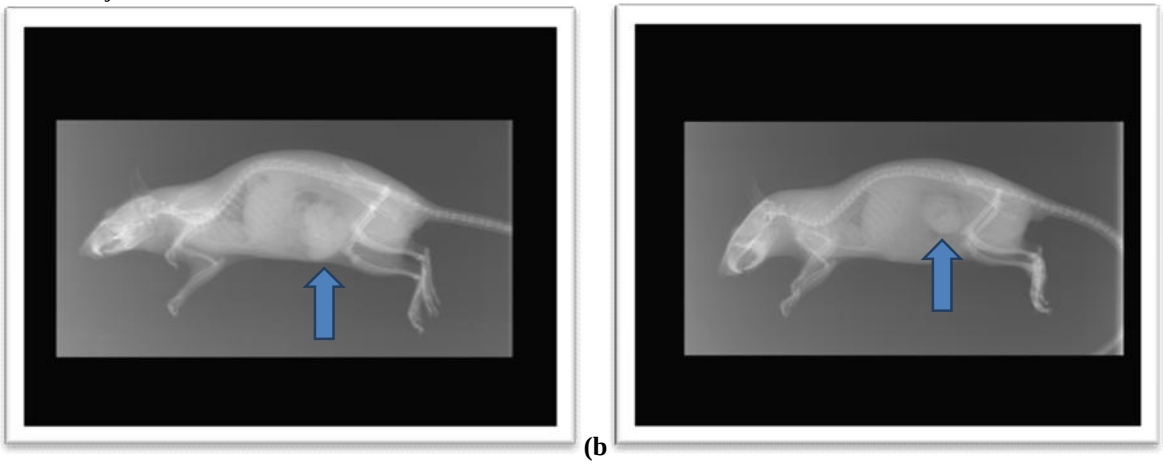

Fig.4.Soft X-ray images from two rats bearing mammary gland tumor obtained after two weeks injection of cancer cells without treatment (a) and after two weeks injection of cancer cells treated with 60mg/kg DTN (two weeks) (b). Blue arrows indicate the area and the size of tumors Total6×10^6 LA7 cells/rat were injected on the right or left flank of 25 female *Sprague Dawley* rats.

4. Conclusion

The result suggests that DTN has better effect on the tumor compared to TAM, which promoted apoptosis in the rat mammary gland tumor. However, the DTN-High Dose treatment showed a more prolonged effect suggesting that DTN could be a vital future drug in the chemotherapy of breast cancers, while decreasing the hepatotoxic effects. Thus, further studies are warranted to investigate and develop a drug delivery system for DTN in the treatment of cancers.

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