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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.09.008

Anti-breast cancer properties and toxicity of *Dillenia suffruticosa* root aqueous extract in BALB/c mice

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ARTICLE INFO

Article history:
Received 29 Jul 2015
Received in revised form 12 Aug, 2nd revised form 19 Aug 2015
Accepted 30 Aug 2015
Available online 20 Oct 2015

Keywords:
Dillenia suffruticosa
4T1 Tumor-bearing mice
Breast cancer
Toxicity

ABSTRACT

Objective: To determine the anti-breast cancer activities and the safety oral consumption of *Dillenia suffruticosa* root aqueous extract (DRAE) in BALB/c mice.

Methods: In the anti-breast cancer study, female BALB/c mice were divided into five groups (n = 12), which were (1) positive control (with breast cancer, untreated), (2) negative control (without breast cancer, untreated) and other three groups of mice with breast cancer treated with 1 000, 500 and 250 mg/kg of DRAE, respectively, by oral gavage for 28 days. All mice except from the negative control group were injected into the mammary fat pad with 4T1 cells (1×10^5 4T1 cells/0.1 mL of phosphate buffer solution). DRAE was administered orally on Day 11 after the tumor has developed.

Results: The tumor volume of the 1 000 mg/kg of DRAE group reduced significantly compared to the positive control while treatment with 500 mg/kg of DRAE had significantly inhibited metastasis to the heart. In the acute toxicity study, treatment with up to 5 000 mg/kg of DRAE was not toxic to the animals, indicating its safety when a large amount of this plant extract was ingested. Based on the sub-acute toxicity study, treatment of the highest dose of DRAE (1 000 mg/kg) had mild liver toxicity indicated by mild focal hemorrhage.

Conclusions: DRAE possesses anti-breast cancer properties but at the same time it shows mild toxicity to the liver. The non observable adverse effect dose for DRAE is 500 mg/kg.

1. Introduction

Breast cancer is the most common cancer in women worldwide. It is also the principle cause of death from cancer among women globally. The incidence of breast cancer has been on the rise in the developing countries due to the increasing life expectancy, urbanization and adoption of western lifestyles [1]. Statistics show that nearly 1.7 million people were diagnosed with breast cancer and 522 000 of reported breast cancer-related deaths in 2012 worldwide [2]. Majority of the breast cancer-related death is the results of uncontrollable metastasis. Although it affects only 10%–15% of the cases, breast cancer can spread to other parts of the body within 3 years of its initial diagnosis, and metastasis tends to recur later, up to 10 years or more after the detection of the primary tumor [3].

Surgery, hormone therapy, chemotherapy, radiation therapy and selective combination of aforementioned therapies have been the standard treatments of breast cancer. Nevertheless, they are not completely effective in the treatment of metastatic breast cancer [4]. Moreover, chemotherapy comes with unpleasant adverse effects such as hair loss, nausea, vomiting, anemia, joint pain, leukemia and heart failure [5]. Patients may develop

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Peer review under responsibility of Hainan Medical University.
Foundation Project: Supported by the Universiti Putra Malaysia with Grant No. 9366600.

resistance to the chemotherapeutic drugs. The emergence of tumor cells resistant to anticancer drug during the course of treatment frequently has resulted in failure to subsequent therapy, relapse of rapid tumor growth and patient mortality [6].

Therefore, scientists have begun to search for new more effective alternative anticancer drugs that derive from natural products including plants. Indeed, plants have become an important source of currently clinically available anti-cancer drugs such as vinca alkaloids (vinblastine and vincristine) extracted from the *Catharanthus roseus* [7], taxane diterpenoids (paclitaxel and irinotecan) from *Taxus brevifolia* [8], and camptothecins quinoline alkaloid derivatives (topotecan and irinotecan) from *Camptotheca acuminate* [9]. It has been reported that more than 50% of all modern drugs in clinical use are of natural products, many of which have been recognized to have the ability to induce apoptosis [9].

Dillenia suffruticosa Griffith ex Hook. F. and Thomson (Martelli Dilleniaceae) (*D. suffruticosa*) is locally known as "Simpoh Air" by the Malays and "Shrubby Simpoh" by the people of Hawaii [10]. It is an evergreen tree that has red colored fruits and attractive yellow colored flowers. In East Malaysia, the Rungus ethnic had been using *D. suffruticosa* to treat cancerous growth [11]. This plant has been traditionally used in the state of Perak, Peninsular Malaysia, as traditional remedies to treat microbial and fungal infections [12,13]. It is reported that *D. suffruticosa* has been long used by the indigenous and the local people of Sabah to treat headache and has wound healing properties [14,15].

Our previous studies showed that the root extract of *D. suffruticosa* exhibited stronger cytotoxic activities against human cancer cell lines including HeLa, MCF-7, MDA-MB-231, A549 and HT-29, as compared to other parts of the plant [16]. The ethyl acetate extract of *D. suffruticosa* root exhibited cytotoxic effect by induction of oxidative stress in MCF-7 cell line [17]. In addition, the root dichloromethane extract of *D. suffruticosa* induced apoptosis and cell cycle arrest via up-regulation of NF-κB and JNK1, and down-regulation of AKT1 and ERK1 [18].

Although *D. suffruticosa* is traditionally used for treatment of cancerous growth, no information of its anti-breast properties and safety is available. This study determined the anti-breast cancer activities and the safety for oral consumption of *D. suffruticosa* root aqueous extract (DRAE) in BALB/c mice.

2. Materials and methods

2.1. Plant material

The fine powder of root of *D. suffruticosa* was supplied by Primer Herber Sdn. Bhd., Malaysia. The plant was identified and authenticated at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (Voucher specimen number SK 1937/11).

2.2. Preparation of DRAE

Briefly, 500 g of *D. suffruticosa* dry root powder was soaked in distilled water (1:4, w/v) and boiled for 5 min. After cooling down, the mixture was filtered through Whatman No. 1 filter paper. The residue was re-extracted twice. The filtrates were pooled and lyophilized by freeze-drying (VirTisbenchtop K, Bieleveld, Germany). The yield of extraction was approximately 5% (w/w). The lyophilized powder was stored at −20 °C for further use. The percentage of yield was calculated by using the formula as follows [19]:

$$\text{Yield } (\%) = \frac{\text{Weight of crude extract}}{\text{Weight of dried plant materials}} \times 100$$

2.3. Cell line

The 4T1 mouse mammary carcinoma cells (4T1 ATCC® CatalogNo. CRL-2539™) were purchased from the American Type and Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, and maintained in a humidified incubator at 37 °C in atmosphere of 5% CO_2.

2.4. Experimental animal

The protocol of the study was approved by the Animal Care and Use Committee, Faculty of Medicine and Health Sciences, UPM (Approval No. UPM/FPSK/PADS/BR-UUH/00505). Female BALB/c mice with 20–30 g in weight at the age of 6–8 weeks were used in this study. They were housed individually in cages under standard laboratory conditions with a period of 12/12-h light/dark cycle, at 20–24 °C with 40%–50% relative humidity. The animals were acclimatized for one week before the actual experiment. The mice were fed with a standard chow pellet (Specialty Food, Australia) and allowed to drink water *ad libitum*.

2.5. Experimental design

2.5.1. In vivo anti-breast tumor study

Sixty female BALB/c mice were divided into 5 groups (n = 12), which were (1) positive control (with breast cancer, untreated), (2) negative control (without breast cancer, untreated) and other three groups of mice with breast cancer treated with 1 000 mg/kg, 500 mg/kg and 250 mg/kg of DRAE. All mice except from the negative control group were injected into the mammary fat pad with 4T1 cells (1×10^5 4T1 cells/0.1 mL of PBS). On Day 11, DRAE was administered orally to the animals via gavage for 28 days, following injection of 4T1 cells. Mice were weighed three times a week. The tumor volume was measured twice weekly by using a vernier caliper. The following formula was used to determine the volume of the tumors [20]:

$$\text{Volume of the tumors} = \text{Length} \times \text{width}^2 \times 0.52$$

Mice that were moribund were sacrificed and the date of sacrifice was recorded for calculating the survival time. The major organs (kidneys, liver, heart, lungs and spleen) were harvested, weighed and observed grossly. The presence of metastasis was recorded [20]. For each mouse, the organ weight to body weight percentage ratio was calculated.

2.5.2. Acute toxicity study

Twenty mice were randomly assigned into five groups (n = 4), which were the control and four treatment groups of

single escalating dose (625, 1 250, 2 500 and 5 000 mg/kg of DRAE). Prior to the acute toxicity study, the dose of DRAE was determined through a preliminary test. Control group received distilled water only. For the treatment groups, the lyophilized DRAE was dissolved in distilled water and orally administered by gavage only on the first day. The animals were observed in the first 4 h after dosing and then daily for 14 days for general appearance, behaviour, toxicity symptoms and mortality. The body weight was measured daily by using a table top electronic balance (A&D SK-5001WP, Japan).

2.5.3. Sub-acute toxicity study

The mice were randomly assigned into four groups (n = 8), which were the control and three treatment groups of escalating dose (250, 500 and 1 000 mg/kg of DRAE). Control group received distilled water only. For the treatment groups, the lyophilized DRAE was dissolved in distilled water and orally administered by gavage daily for 28 days. The animals were observed twice daily (before and after dosing) for general physical conditions such as general appearance, behaviour, toxicity symptoms and mortality in 28 days. The body weight was measured every 3 days (Days 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28).

2.5.4. Biochemical analysis

Blood collection was carried out on Day 29 in the sub-acute toxicity study. Prior to blood collection, the mice were anesthetized with diethyl ether. The blood samples (1 mL) were collected by cardiac puncture by using a 26 G × ½ needle" (Terumo®, Belgium, Europe) and centrifuged at 14 000 r/min for 10 min to separate the serum. Blood analysis was carried out by using a chemistry analyzer (Selectra XL, Dieren, Netherlands). For hepatic function, level of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltranspeptidase (GGT) and alkaline phosphatise (ALP) were evaluated. For renal function, level of blood urea nitrogen and serum creatinine was determined.

2.5.5. Histological examination

Following blood sampling, the mice were sacrificed by suffocating with diethyl ether in a glass desiccator. Tumors and organs including heart, lungs, kidneys, spleen and liver were harvested and fixed in 10% formalin. After fixation for at least 24 h, the tissues were processed for 16 h by an automated tissue processer (Leica ASP6025, Leica Microsystems, Wetzlar, Germany). Processed tissues were then embedded in paraffin. Four micrometres thick sections were cut from the paraffin block and stained with hematoxylin and eosin (H&E). Each slide was examined under a light microscope with assistance of a pathologist. At least 10 fields from each slide of each group were examined to evaluate the histological changes.

For anti-breast cancer study, the scoring was done based on the presence of metastatic cells in the organs. Slides were screened for any metastatic cell and any organ that had one metastatic cell and then recorded as "with metastasis". For toxicity studies, the scoring system for histological changes of liver and kidney was according to Jihen *et al.* on degree of chromatin condensation, nucleus fragmentation, necrosis of hepatocytes, light cytoplasm and sinusoidal widening in liver while degree of dilation in the glomeruli and tubular necrosis in kidney [21].

2.6. Statistical analysis

Data were analyzed with One-way ANOVA, Dunnett's, Mann–Whitney, and Duncan's multiple range test by using SPSS version 20.0. All the data were expressed as mean ± SD. Probability of $P < 0.05$ was considered significant.

3. Results

3.1. Anti-breast cancer study

3.1.1. Body weight

Figure 1 illustrates that the weight between all DRAE-treated groups and controls were not significantly different ($P > 0.05$).

3.1.2. Organ to body weight ratio

The organ to body weight ratios of DRAE-treated groups and controls are illustrated in Figure 2. The treatment groups (250, 500 and 1 000 mg/kg of DRAE) and the positive control showed significant increase of lung, spleen and liver to body weight ratio ($P < 0.05$) compared to the negative control. The spleen to body weight ratio of the 1 000 mg/kg DRAE-treated group decreased significantly ($P < 0.05$) compared to the positive control.

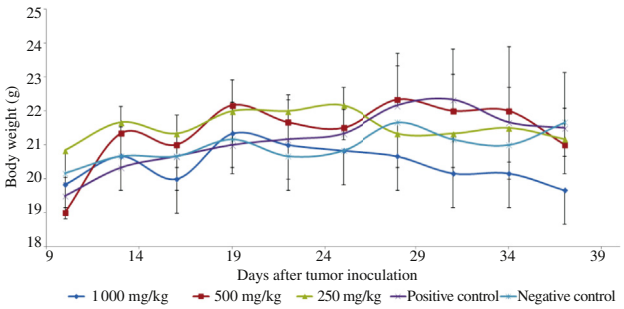

Figure 1. Change in the body weight of 4T1 tumor-bearing mice treated with different dose of DRAE.

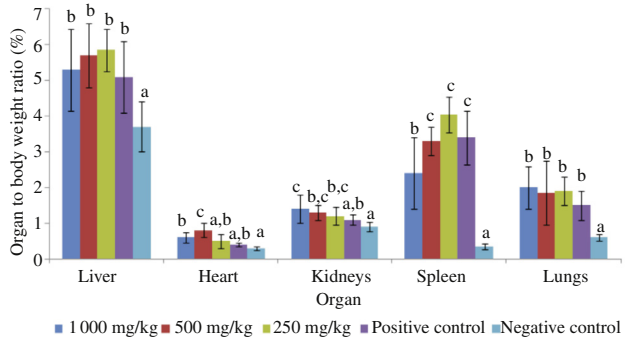

Figure 2. Organ to body weight ratio of 4T1 tumor-bearing mice treated with different dose of DRAE.
a–c: Mean with different superscripts differs significantly ($P < 0.05$).

3.1.3. Tumor volume

Figure 3 shows the volume of tumors from DRAE-treated groups and positive control. There was no significant difference in the tumor volume between the treatment groups and the positive control. On Day 30, the tumor volume of the 1 000 mg/kg DRAE group reduced significantly compared to the positive control ($P < 0.05$).

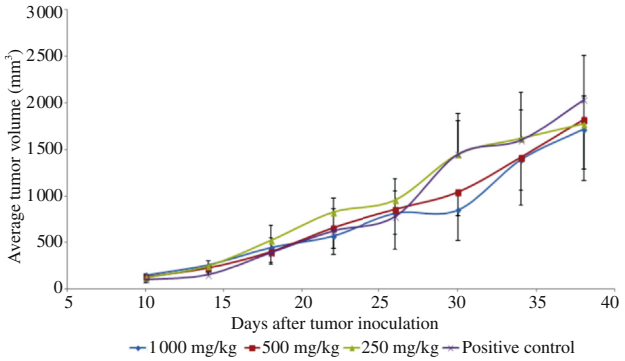

Figure 3. Volume of tumors of 4T1 tumor-bearing mice treated with different dose of DRAE.

3.1.4. Inhibition of metastasis

The percentage of organs that is metastasis-free is shown in Table 1. Treatment with 500 mg/kg of DRAE has significantly inhibited metastasis to the heart ($P < 0.05$).

Table 1

Percentage of organs free of metastasis following treatment with DRAE.

Group	Metastasis-free organ (%)			
	Lung	Liver	Heart	Spleen
1000 mg/kg	0	60	60	100
500 mg/kg	0	60	80*	100
250 mg/kg	0	80	40	100
Positive control	0	100	0	100

*: Significantly ($P < 0.05$) differs from the positive control group according to Mann–Whitney test.

3.1.5. Histopathological changes

Figure 4 shows the representative of H&E staining sections of lungs of mice treated with different doses of DRAE and the control groups as observed under a light microscope. All DRAE-treated and positive control mice exhibited infiltration of neoplastic cells in the lungs and structural destruction of the pulmonary alveoli. There was a reduction of alveolar sacs, which were spaces at the termination of the alveolar ducts that were surrounded by alveoli, due to infiltration of the neoplastic cells. The neoplastic cells were poorly differentiated (bear minimal resemblance to the cell from which they arose) and characterized by the presence of large hyperchromatic nuclei and small amount of cytoplasm. The metastatic cells in lungs appeared in clusters or clumps and were marked by arrows.

Figure 5 shows the representative of H&E staining sections of hearts of mice treated with different doses of DRAE and the control groups as observed under a light microscope. The DRAE-treated and positive control mice exhibited infiltration of neoplastic cells in the heart. The neoplastic cells were poorly differentiated (bear minimal resemblance to the cell from which they arose) and characterized by the presence of large hyperchromatic nuclei and small amount of cytoplasm. The metastatic cells in lungs appeared in clusters or clumps and were marked by arrows.

Figure 6 shows a representative of H&E staining section of a primary tumor from the positive control. The neoplastic cells were poorly differentiated as they bore minimal resemblance to

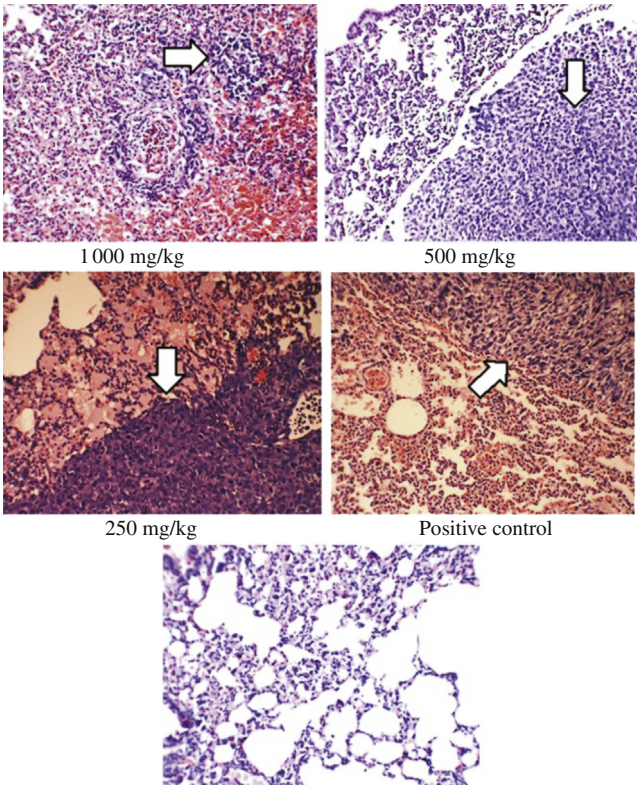

Figure 4. Representative sections of lungs sections from mice untreated and treated with different dose of DRAE following H&E staining as observed under a light microscope (200×).
Metastatic cells were marked by arrows.

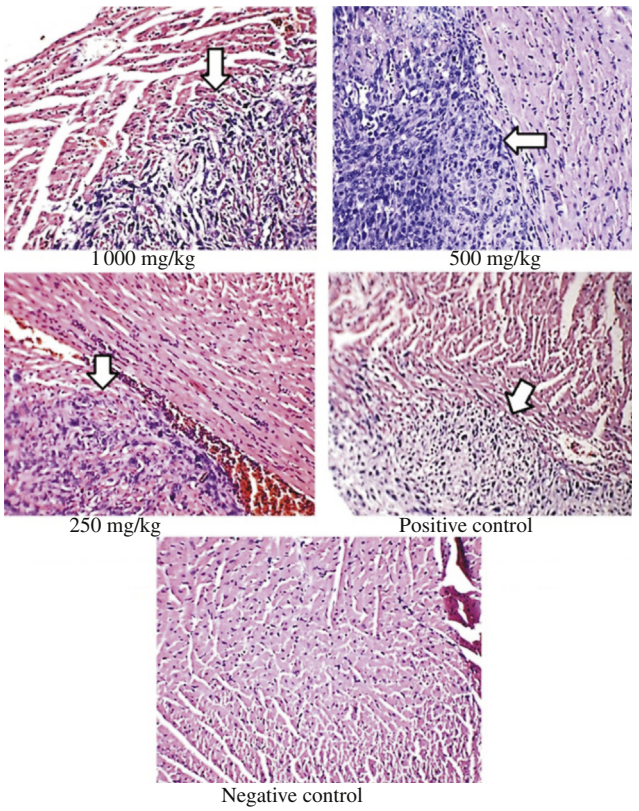

Figure 5. Representative sections of heart from mice untreated and treated with different dose of DRAE following H&E staining as observed under a light microscope (200×).
Metastatic cells were marked by arrows.

the cells and arose from in terms of shape and size of the nuclei. Large hyperchromatic nuclei and relatively small amount of cytoplasm were noted. The primary tumor showed the presence of necrosis infiltration by leucocytes. The areas of necrosis were marked by arrows.

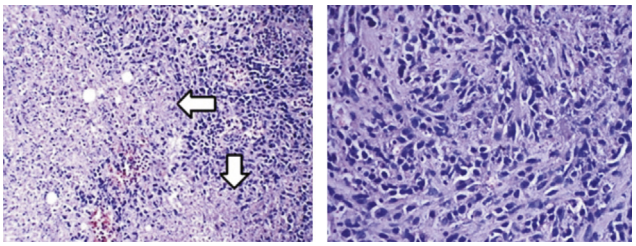

Figure 6. A section from a primary tumor illustrating area of necrosis (arrows) following H&E staining as observed under a light microscope. The neoplastic cells were poorly differentiated and characterized by small amount of cytoplasm.

The spleens harvested from the positive control showed signs of splenomegaly, which were changes in splenic architecture and increased number of immature splenic granulocytes (Figure 7a). An expansion of the granulocyte rich red pulp (marked by R) with reduction in white pulp area (marked by W) in the spleen of the positive control as compared to the negative control is shown in Figure 7b and Figure 7c. White and red pulps were named based on the color of fresh section. The white pulp consisted of lymphatic tissue, mostly lymphocytes and appeared basophilic because of the dense heterochromatin in the nuclei of the numerous lymphocytes. The red pulp had a red appearance in fresh state as well as in histologic sections because it contained large number of red blood cells. Spleens of positive control showed prominent megakaryoblast (arrows) in the splenic red pulp (Figure 7d).

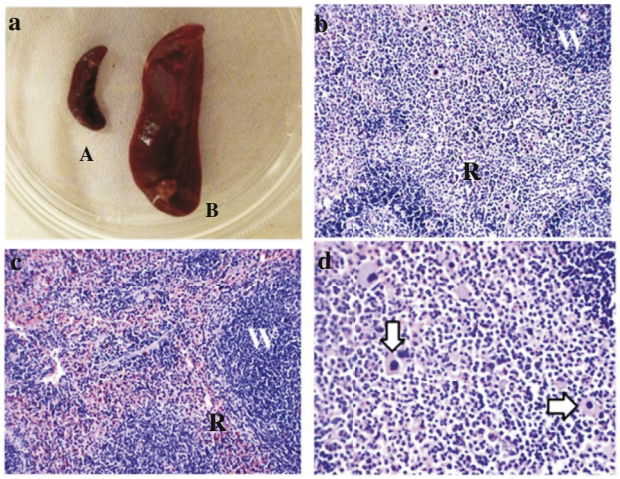

Figure 7. Representative sections of spleen from mice untreated and treated with different dose of DRAE following H&E staining as observed under a light microscope (200×).
a: Difference in spleen size between negative control (A) and tumor bearing mouse (B); b: H&E staining section of a spleen of a tumor bearing mouse (200×); c: H&E staining section of a mouse from the negative control group (200×); d: H&E staining section of a spleen of a tumor bearing mouse (400×).

Figure 8 shows the representative of H&E staining sections of livers of mice treated with different doses of DRAE and the control groups as observed under a light microscope. The DRAE-treated groups and positive control showed infiltration of neoplastic cells in the liver. The neoplastic cells were poorly differentiated (bear minimal resemblance to the cell from which they arose) and characterized by the presence of large hyperchromatic nuclei and small amount of cytoplasm. The metastatic cells in lungs appeared in clusters or clumps and were marked by arrows.

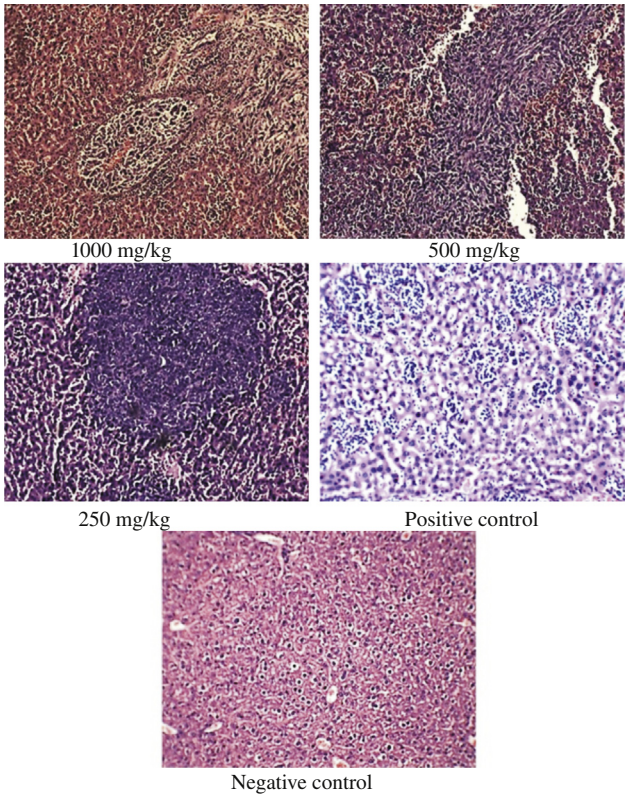

Figure 8. Representative sections of liver from mice untreated and treated with different dose of DRAE following H&E staining as observed under a light microscope (200×).
Metastatic cells are marked by arrows.

3.2. Acute toxicity study

There was no treatment-related mortality observed in mice treated at any dose level of DRAE (625, 1 250, 2 500 and 5 000 mg/kg body weight). All the mice appeared healthy with normal eyes, fur and skin condition during 14 days of observation period. No abnormalities such as hemorrhage, lesions, enlargement or atrophy in organs were observed following macroscopic post-mortem examinations of the animals. Percentage of body weight change in mice treated with DRAE for 14 days is illustrated in Figure 9. None of the mice experienced significant weight loss throughout the experimental period. However, there was a significant increase ($P < 0.05$) in body weight of mice treated with 5 000 mg/kg of DRAE after Day 10 compared to the control. Figure 10 illustrates the percentage of organ to body weight ratio of various organs (kidneys, liver, spleen and lungs) in BALB/c mice treated with DRAE. The

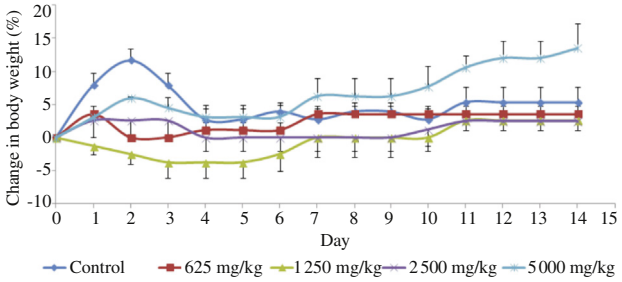

Figure 9. Change in body weight of mice treated with different dose of DRAE in the acute toxicity study.

mice treated with 5 000 mg/kg of DRAE showed a significant increase ($P < 0.05$) in the kidney weight compared to the control. There was an absence of any histological changes such as chromatin condensation, nucleus fragmentation, light cytoplasm and sinusoidal widening in the liver. The kidneys of mice in all treatment groups did not show any changes in glomeruli dilation and tubular necrosis (data not shown).

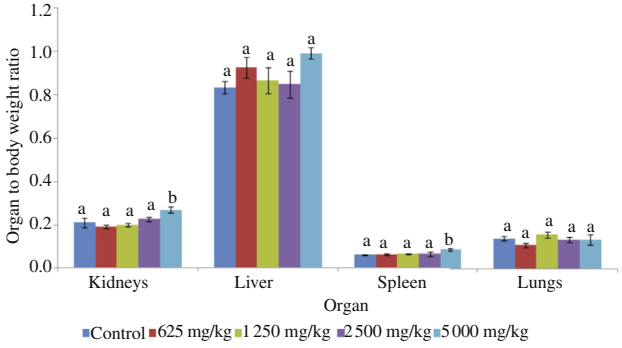

Figure 10. Organ to body weight ratio of mice treated with different dose of DRAE in the acute toxicity study.
a–b: Mean with different superscripts differs significantly ($P < 0.05$).

3.3. Sub-acute toxicity study

3.3.1. Clinical observations and mortality

There was no treatment-related mortality of mice at any dose level of DRAE tested (250, 500 and 1 000 mg/kg body weight). During treatment for 28 days, DRAE did not induce any toxicity symptom in the mice even at the highest dose (1 000 mg/kg). All the mice appeared healthy with normal eyes, fur and skin condition. No abnormalities such as hemorrhage, lesions, enlargement or atrophy in organs were observed following macroscopic post-mortem examinations of the animals.

3.3.2. Body weight change and organ to body weight ratio

Percentage of body weight change in BALB/c mice treated with DRAE for 28 days is illustrated in Figure 11. At the beginning of the treatment, there was a slight body weight drop in mice of all the treated groups (250, 500 and 1 000 mg/kg). Figure 12 illustrates the percentage of organ to body weight ratio of various organs (kidneys, liver, spleen, lungs, heart and stomach) of mice treated with DRAE. There was no significant difference ($P > 0.05$) in the percentage of organ to body weight ratio between all the treatment groups and the control.

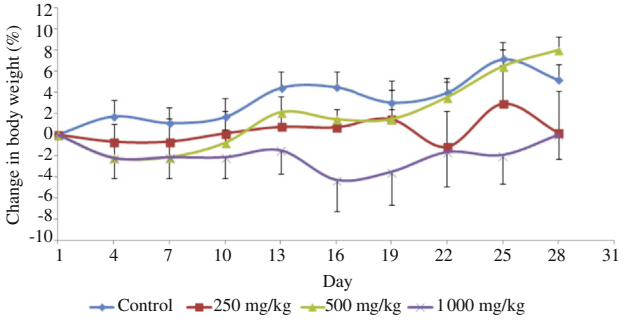

Figure 11. Change in body weight of mice treated with different dose of DRAE in the sub-acute toxicity study.

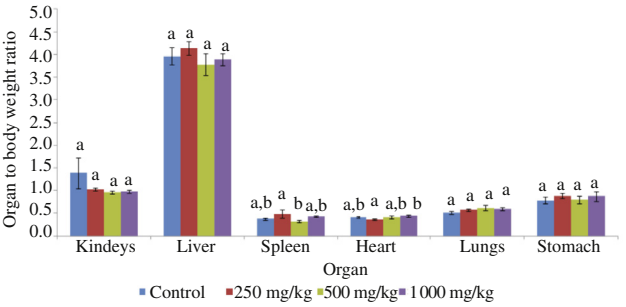

Figure 12. Organ to body weight ratio of mice treated with different dose of DRAE in the sub-acute toxicity study.
a–b: Mean with different superscripts differs significantly ($P < 0.05$).

3.3.3. Level of liver enzymes and kidney functions

Figure 13 illustrates the plasma level of AST, ALT, GGT, ALP, urea and creatinine in BALB/c mice treated with DRAE in the sub-acute toxicity study. There was no significant difference ($P > 0.05$) in all the biochemical parameters between all the treatment groups and the control.

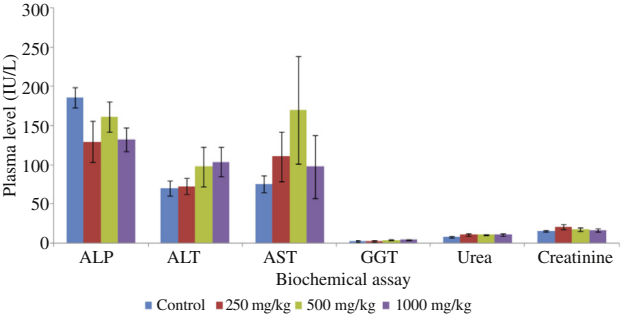

Figure 13. Plasma level of ALP, ALT, AST, GGT, urea and creatinine in mice treated with different dose of DRAE in the sub-acute toxicity study.

3.3.4. Histological changes

There was an absence of any histological changes such as chromatin condensation, nucleus fragmentation, light cytoplasm and sinusoidal widening in the liver. However, there was a focal mononuclear cell infiltrations in the mice treated with 1 000 mg/kg DRAE (Figure 14). The kidneys of mice in all treatment groups did not show any changes in glomeruli dilation and tubular necrosis (data not shown).

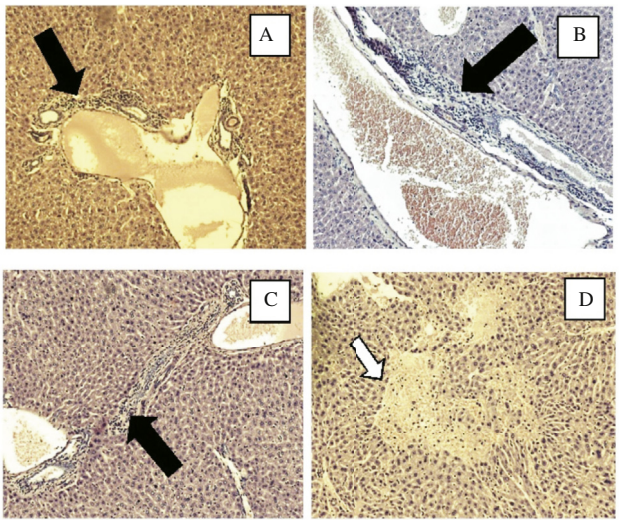

Figure 14. The liver tissue of control and mice treated with different dose of DRAE following H&E staining as observed under a light microscope (400× magnification).
A: Control; B: Mice fed with 250 mg/kg DRAE; C: Mice fed with 500 mg/kg DRAE; D: Mice fed with 1000 mg/kg DRAE showing mild focal hemorrhage (white arrow).

4. Discussion

The acute toxicity study of DRAE was conducted in accordance with Organization for Economic Co-operation and Development (OECD) 420 guideline for testing of chemicals with modification in selection of dose level [22], while the sub-acute toxicity study was conducted under OECD 407 guideline for the testing of chemicals [23]. Any moribund mouse will be euthanized. The endpoints of the experiment are the time when the animal experiences body weight loss of 20% in one week, inability to eat or drink, immobility and lack of responsiveness to manual stimulation.

The anti-breast cancer properties of DRAE were evaluated in this study. The weight of the DRAE-treated groups and positive control increased for the first 5 weeks after breast cancer induction and started to reduce at the end of the experiment. The increase is probably due to the growth of the tumors. The tumors increased in volume from Day 10 till Day 38 after breast cancer induction. The tumor-bearing mice showed the characteristics of cancer anorexia. Anorexia is an involuntary weight loss, tissue wasting and poor performance, which will ultimately result in death [24]. According to the UK Home Office Regulation, weight loss is expected in mice induced with breast cancer, and the mice can lose up to 25% of their body weight after 4 weeks of breast cancer induction [25]. From this study, even though the weight loss of all the treated groups and controls was not significant, weight loss was still one of the symptoms of cancer anorexia-cachexia syndrome. Cancer cachexia is defined as a wasting syndrome involving loss of muscle mass and fat directly caused by tumor factors or indirectly caused by an aberrant host response to tumor presence [26].

Liver and the spleen of the DRAE-treated and positive control mice showed significant increase in weight compared to the normal mice due to the extramedullary hematopoiesis (Figure 8). The presence of tumor increased the circulating neutrophils and other leukocytes, which caused the enlargement of the spleen and the liver [27]. Splenomegaly has been reported in breast tumor-induced leukemoid reaction [28–30]. Neoplasia associated with leukemoid reaction, although uncommon in both human and animals, has been reported in several animals and human cancers. Breast cancer has rarely been reported to induce granulocytosis in humans but there are several granulocytosis-inducing tumors described in animals [27,31]. The average weight of spleen for 1000 mg/kg group showed a significant reduction compared to the positive control. This suggests that at 1000 mg/kg of DRAE, breast cancer-induced extra-medullary hematopoiesis in spleen can be reduced.

The volume of tumors (Figure 9) between the DRAE-treated groups and the control group was insignificantly different throughout the study, except for treatment at 1000 mg/kg DRAE on Day 30, where the tumor volume did not increase compared to Day 26. Nevertheless, it was a transient growth inhibition whereby the tumor volume continued to increase after Day 30. The mechanism of the transient growth inhibition remains unclear. DRAE alone is probably not that effective in treating metastatic breast cancer. In the adjuvant setting, combination chemotherapy is used routinely due to widespread evidence that polychemotherapy offers a survival advantage compared with single-agent therapy [32]. Studies showed that combination and sequential therapy were effective in treating metastatic breast cancer. Drug combinations, such as paclitaxel/trastuzumab or capecitabine/docetaxel, showed survival advantages over single-agent therapy [33]. Despite not showing any significant reduction in tumor volume as compared to the control group, the treatment groups however showed a reducing trend in tumor volume. The size of tumors of the DRAE-treated group was lower as compared to the positive control.

Mice treated with DRAE showed no metastatic inhibition in the lungs. Lungs are by far the most likely organ to be affected by metastasis [34]. The 4T1 cells formed distinct metastatic foci, mostly in the lung. The present study also showed that metastasis was not found in the spleen. The enlargement of the spleens of the DRAE-treated and positive control mice was due to extramedullary hematopoiesis. There was a significant reduction of metastasis in the heart of tumor-bearing mice treated with 500 mg/kg DRAE as compared to the positive control. Eighty percent of heart of mice treated with 500 mg/kg DRAE showed no metastasis. It is speculated that DRAE at certain dose (effective dose) has the ability to prevent the formation of heart metastasis [20].

4.1. Acute toxicity study

In both acute and sub-acute toxicity studies of DRAE, female mice were used as recommended by OECD 420 as females were generally slightly more sensitive [22].

There was no dose-related toxicity effect observed since the mice appeared healthy with normal eyes, mucous membranes, fur and skin condition. Mortality was absent in both treatment and control groups. All the treated mice did not show any significant weight loss throughout the experiment, suggesting that the animals were free from wasting syndrome. According to Peterson *et al.*, loss of body weight or wasting syndrome is a characteristic sign observed in most animals in toxicity studies [35]. The weight loss usually manifests within a few days after exposure and results in a substantial reduction of the adipose and muscle tissue observed at autopsy [36]. However, the mice treated with the highest dose of DRAE (5000 mg/kg) experienced a significant weight gain compared to the control. It may be one of the indicators of general systemic toxicity

according to Keller and Banks where disturbance of metabolic, hormonal and homeostatic mechanisms could have occurred that leads to weight gain [37]. The body weight gain can be explained by an increase in percentage of kidney to body weight ratio in the mice treated with 5 000 mg/kg. Nevertheless, the enlargement of kidney is not considered attributable to the treatment because the histological examination illustrates normal architecture of kidney. Enlargement of kidney might be the result of a physiological response to exposure to a very high dose of DRAE, which is not part of the normal diet [38]. The enlargement is most likely adaptive and not toxicologically relevant. In addition, according to OECD, histopathological examination is considered to be a more sensitive marker of organ toxicity than organ weight [23].

According to the OECD 420 guideline, acute toxicity study provides information on the hazardous properties and allows the substance to be classified according to the Globally Harmonized System of classification and labeling of chemicals. Since the LD50 cut-off value of DRAE can be considered to exceed 5 000 mg/kg, no hazard classification or labeling is required. It is suggested that DRAE is considered safe or practically non-toxic.

4.2. Sub-acute toxicity study

Oral toxicity evaluation by using a 28-day toxicity test is an accustomed practice in a sub-acute study. There was no dose-related toxicity effect observed in the all treatment groups (250, 500 and 1 000 mg/kg of DRAE) since the mice appeared healthy with normal eyes, mucous membranes, fur and skin condition. Mortality was not detected in both control and treatment groups.

There was no weight loss of more than 20% in all the DRAE-treated mice, rejecting the hypothesis that the mice might be experiencing wasting syndrome, a sign of toxicity as mentioned by Peterson *et al.* [35]. Moreover, all the treatment groups were having normal percentage of organ to body weight ratio of kidneys, liver, spleen, heart, lungs and stomach, indicating that treatment of DRAE didn't affect the development of these organs [39].

There was no significant difference in both urea and creatinine serum level indicating absence of toxicity to the kidney caused by DRAE. Reinforcing these data, the histopathological findings of kidney revealed no abnormality of the organ. Based on the both biochemical analysis and histopathological examination, DRAE did not affect the renal function. Treatment with all doses of DRAE also did not change the plasma level of liver enzymes. Nevertheless, the liver of mice treated with 1 000 mg/kg of DRAE exhibited area of mild hemorrhage. It shows that high dose of DRAE may cause some toxic effects to the liver but not to the extent of altering the functions of the organ. It is suggested that the no observable adverse effect level of DRAE in this study is 500 mg/kg. From the no observable adverse effect level, human equivalent dose is calculated by normalized to body surface area. The safety dose of DRAE to be consumed by human daily is 2.4 g.

DRAE possesses anti-breast cancer properties that are dose-dependent. Based on the acute toxicity study, treatment with 625, 1 250, 2 500 and 5 000 mg/kg of DRAE was not toxic to the animals, indicating its safety when a large amount of this plant extract is ingested. Based on the sub-acute toxicity study, treatment of the highest dose of DRAE (1 000 mg/kg) has mild liver toxicity as indicated by mild focal hemorrhage.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors express their gratitude to the staff of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The work was funded by Universiti Putra Malaysia, Malaysia with Grant No. 9366600.

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