Biocatalysis and Agricultural Biotechnology 19 (2019) 101106

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

In vitro and *in vivo* antitumor activity of deoxyelephantopin from a potential medicinal plant *Elephantopus scaber* against Ehrlich ascites carcinoma

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ARTICLE INFO

Keywords:
Deoxyelephantopin
Ehrlich ascites carcinoma (EAC)
Apoptosis
Elephantopus scaber

ABSTRACT

Elephantopus scaber Linn. is a perennial medicinal herb widely used in Asian traditional medicine for the treatment of different kinds of neoplasm. The aim of the present study was to appraise the *in vitro* and *in vivo* antitumor activity of deoxyelephantopin (DOE) - the active principle isolated from *E. scaber* against murine Ehrlich ascites carcinoma (EAC). Intraperitoneal administration of DOE (25 mg/kg) reduced the ascites tumor volume to a better extend than the treatments with same concentrations of 5-Flourouracil (5-FU) in EAC ascites tumor model and increased the life span of treated mice with respect to control mice ($p < 0.001$). Moreover, treatment with 25 mg/kg DOE reduced the tumor volume and tumor burden in EAC solid tumor model and showed the best overall survival responses (Log-Rank p value < 0.001). DOE exhibited impressive cytotoxicity against EAC cells *in vitro*. Morphological analysis of DOE-treated EAC cells showed membrane blebbing, chromatin condensation and nuclear fragmentation-signs of apoptotic cell death. Assessment for DNA laddering in DOE-treated EAC cells confirmed that DOE caused EAC cell death by apoptosis. The present study showed that even lower doses of DOE has better *in vivo* antitumor activity, than the widely used chemotherapeutic agent- 5-FU. Thus, it is obvious that DOE shall be developed as a useful chemotherapeutic drug for cancer treatment in near future.

1. Introduction

Plant-derived compounds account for as much as 60% of the drugs currently used for cancer therapy (Gordaliza, 2007). Plant based drug discovery mainly relies on the existing traditional medicine system such as Ayurveda and Traditional Chinese Medicine. Due to the high levels of toxicity to normal cells, the usage of currently available chemotherapeutic drugs is highly restricted. Plant-derived compounds could serve as antitumor drugs with lesser side effects. Interestingly, phytochemicals exhibit their anticancer properties *via* a variety of distinct mechanisms. Inducing apoptosis of cancer cells has been considered as a major mechanism by which phytochemicals caused cancer cell death.

Elephantopus scaber Linn., a perennial medicinal herb that grows widely in many Asian countries, is effective in the treatment of cancer (Farha and Remani, 2014). Both the aqueous and chloroform extracts of *E. scaber* significantly reduced tumor growth and increased the life span of DLA ascitic tumor bearing Swiss albino mice (Rajkapoor et al., 2002; Geetha et al., 2003). Extract of *E. scaber* reduced skin papillomas induced by DMBA/croton oil as well as 20-methyl cholanthrene induced soft tissue sarcoma (Geetha et al., 2010). Previously, Geetha et al. (2017) reported the *in vivo* Ehrlich's ascites carcinoma (EAC) tumor cell suppression effect of active fraction of *E. scaber*. The studies revealed that intraperitoneal administration of the active fraction of *E. scaber* (100 mg/kg) showed remarkable antitumor activity against EAC cells and prolonged the life span of EAC tumor bearing mice. However, their study did not explore the active antitumor agent present in the active-fraction of *E. scaber*.

Sesquiterpene lactones isolated from medicinal plants can act as potent antitumor agents. Deoxyelephantopin (DOE), a sesquiterpne lactone isolated from *E. scaber* exerts many beneficial effects such as hepatoprotective (Huang et al., 2013), antitumor (Xu et al., 2006), antiprotozoal (Zahari et al., 2014) and wound healing (Singh et al., 2005). Previous studies reported that DOE could inhibit cancer cell growth by apoptosis induction (Huang et al., 2010; Su et al., 2011; Geetha et al., 2012; Kabeer et al., 2017). In this context, the present study was aimed to evaluate the *in vitro* and *in vivo* antitumor activity of DOE, an active

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https://doi.org/10.1016/j.bcab.2019.101106
Received 27 September 2018; Received in revised form 3 March 2019; Accepted 19 March 2019
Available online 22 March 2019
1878-8181/

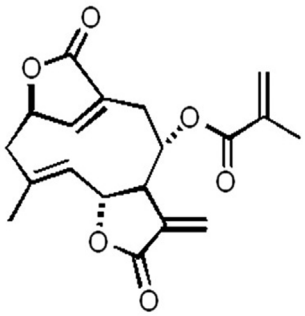

Fig. 1. Structure of DOE

principle isolated from *E. scaber* against murine cancer EAC cells.

2. Materials and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) (MTT), Fetal bovine serum (FBS), Acridine Orange (AO), Ethidium Bromide (EB), Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, USA); Dimethyl sulfoxide (DMSO) (Merck, India); Hoechst 33342 (Invitrogen) were used for the present study. DOE (Fig. 1) from *E. scaber* was dissolved in DMSO (20 mg/mL) and was diluted to the desired concentrations just before use.

2.2. MTT assay

EAC cells were maintained in Swiss albino mice by intraperitoneal (i.p.) inoculation of 2×10^6 cells per mice. For cytotoxicity studies, EAC cells, freshly harvested from Swiss albino mice were plated in 96-well plates (5×10^3 cells/well). The cells were treated with various concentrations of DOE (1.56–25 μg/mL) and incubated for 24, 48 and 72 h. At the end of incubation period, 20 μL MTT (5 mg/mL) was added in to each well and incubated further for 2 h. DMSO (100 μL) was added and the absorbance at 570 nm was measured. The cytotoxic effect of DOE on EAC cells was expressed as % of cytotoxicity, using the following formula:

% Cytotoxicity = 100-(A_{570} of treated cells / A_{570} of control cells) × 100).

2.3. Animals

Male Swiss albino mice obtained from Regional Cancer Centre, Trivandrum, Kerala, India were maintained under standard laboratory conditions. The mice were fed with standard rodent pellet feed and water ad libitum. Adult mice weighing 25 g were used for the experiments. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC/RCC.No.1/06).

2.4. Study design

Study I was carried out with EAC cells induced ascites tumor model while EAC cells induced solid tumor model was used for study II. Three doses of DOE *viz.* 5, 10 and 25 mg/kg was chosen based on the results of toxicity studies done previously. The mice were divided into five different groups (n = 6) as follows:

Group I: Tumor control (0.5% DMSO in phosphate buffered saline (PBS) *i. p*)
Group II: 5-Flourouracil (5-FU) 20 mg/kg
Group III: DOE 5 mg/kg
Group IV: DOE 10 mg/kg
Group V: DOE 25 mg/kg

For tumor induction in study I, each mouse was injected with 2×10^6 EAC cells in 0.2 mL of PBS intraperitoneally on day '0'. After 24 h of transplantation, 5 mg/kg, 10 mg/kg, and 25 mg/kg body weight dose of DOE were administered intraperitoneally on every day to the EAC bearing mice (**Group III to V**) up to 14th day. The control group (**Group I**) and positive control group (**Group II**) was treated with 0.5% DMSO in PBS and 5-FU (20 mg/kg/day) respectively. The effects of DOE on tumor growth were examined by measuring ascites tumor volume, mean survival time (MST) and percentage increase in life span (% ILS).

V3 = (V1 + V2) − V2, where V3 is the total ascites fluid volume, V1 is the volume of the ascetic fluid obtained from the peritoneum, and V2 is the volume of the added saline

Mean survival time (MST) (days) = ΣSurvival time (days) of each mouse in a group/ Total number of mice

ILS (%) = (MST of treated group/ MST of control group) × 100

In study II, the solid tumor was established by injecting EAC cells (2×10^6/0.2 mL PBS) on right hind limb of each mouse intramuscularly. After 24 h of EAC cells inoculation, the drug administration (i.p.) was continued for next 10 days according to their respective groups. The mice were followed till death or up to 35 days. The antitumor activity was assessed by the reduction of solid tumor volume. The solid tumor volume was measured on the seventh day after tumor inoculation and then it was repeated every fifth day until the end of the study.

Tumor volume = 4/3 π r_1^2 × r_2 (where, r_1 is the minor radius and r_2 is the major radius)

The percentage of inhibition of tumor volume in mice

$$= \left(\frac{\text{Tumor volume of control group on } 35^{\text{th}} \text{ day} - \text{Tumor volume of treated groups on } 35^{\text{th}} \text{ day}}{\text{Tumor volume of control on } 35^{\text{th}} \text{ Day}} \right) \times 100$$

2.5. Apoptotic detection assays

To elucidate the mechanism(s) associated with the antitumor activity of DOE, EAC cells treated with DOE for 48 h *in vitro* and EAC cells collected from the peritoneal cavity of ascites tumor model after *in vivo* treatment with 25 mg/kg DOE were subjected to following assays.

2.5.1. AO/EB staining and Hoechst staining

EAC cells were stained with 1 μL of AO and EB mix (100 μg/mL AO; 100 μg/mL EB). Cells were washed twice with PBS and visualized under fluorescence microscope (Olympus, Japan) (magnification x20). For Hoechst staining assay, EAC cells were stained by adding 10 μL of Hoechst 33342 and visualized under fluorescence microscope (Olympus, Japan).

2.5.2. DNA fragmentation assay

EAC cells were mixed with 500 μL DNA extraction buffer and 10 μL proteinase K and incubated in water bath at 55 °C. The DNA pellet was precipitated by the addition of isopropanol and washed with 70% ethanol. DNA was subjected to 1.5% agarose electrophoresis, and bands were visualized by staining with EB.

2.6. Statistical analysis

Data were expressed as mean ± S.D. The statistical significance

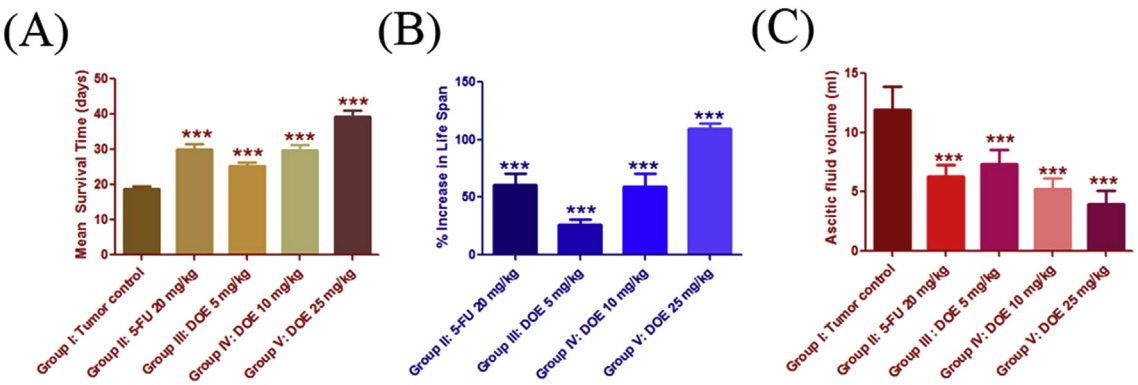

Fig. 2. Effect of DOE on EAC ascites tumor model. EAC cells were injected intraperitoneally into mice and were then administered with vehicle (0.5% DMSO), DOE (5 mg/kg, 10 mg/kg or 25 mg/kg), and 5-Flourouracil (5-FU) (20 mg/kg) intraperitoneally and the following parameters were assessed. (A) Mean survival time (B) % increase in life span (C) tumor volume. Data are mean + SD; (n = 6 in each group); *p < 0.05; **p < 0.01; ***p < 0.001 vs. the control group.

was evaluated using one way analysis of variance (ANOVA) followed by Dunnett's *t*-test. Comparison of survival time between control and treated mice was done by Kaplan-Meier analysis. P < 0.05 was considered to be statistically significant when compared to control.

3. Results

3.1. In vivo antitumor activity of DOE

In vivo antitumor effect of DOE was evaluated using EAC ascites tumor model and EAC solid tumor model established in Swiss albino mice. An increase in mean survival time (Fig. 2A) and % life span (Fig. 2B) was observed in the ascites tumor model when treated with 10 and 25 mg/kg of DOE and 5- FU. In addition, a decrease in ascites fluid volume was noted in all the treatment groups except the control mice group. (Fig. 2C). It has been noticed that treatment with 10 mg/kg DOE is as potent as 20 mg/kg 5FU in terms of decrease in ascites fluid volume and tumor reduction ($p < 0.05$). Treatment with 25 mg/kg DOE has shown more pronounced effect in tumor bearing mice with significant reduction of tumor volume ($p < 0.001$) than the 20 mg/kg 5FU treated mice.

In solid tumor model, tumor reduction was highly significant in 25 mg/kg DOE treated mice in group V ($p < 0.001$). Both 20 mg/kg 5-FU and 10 mg/kg DOE showed similar effects and showed significant tumor reduction ($p < 0.01$) (Fig. 3A). Compared to positive control 5-FU group II, 25 mg/kg DOE showed the best antitumor effects in both ascites tumor model and solid tumor model. Both 10 mg/kg DOE treated mice and 20 mg/kg 5FU treated mice have shown similar overall survival pattern. Treatment with 25 mg/kg DOE prolonged the overall survival of EAC solid tumor mice model (Fig. 3B).

3.2. In vitro cytotoxicity studies

The cytotoxic effect of DOE against EAC cells was evaluated by MTT assay. DOE caused dose and time dependent reduction in the viability of EAC cells (Fig. 4A). The IC_{50} values were 5.8 µg/mL, 2.7 µg/mL and 2.1 µg/mL on EAC cells after 24, 48 and 72 h of DOE treatment respectively.

3.3. DOE induced apoptosis of EAC cells both in vitro and in vivo conditions

To elucidate the mechanism associated with the antitumor activity of DOE, we studied the morphological changes in DOE-treated EAC cells. The EAC cells from the ascites fluid of control and DOE treatment mice (25 mg/kg) groups were stained with AO/EB dual stain and Hoechst 33342 stain. The DOE-treated cells showed marked morphological changes as compared to the tumor control cells. Membrane blebbing, chromatin condensation and nuclear fragmentation were

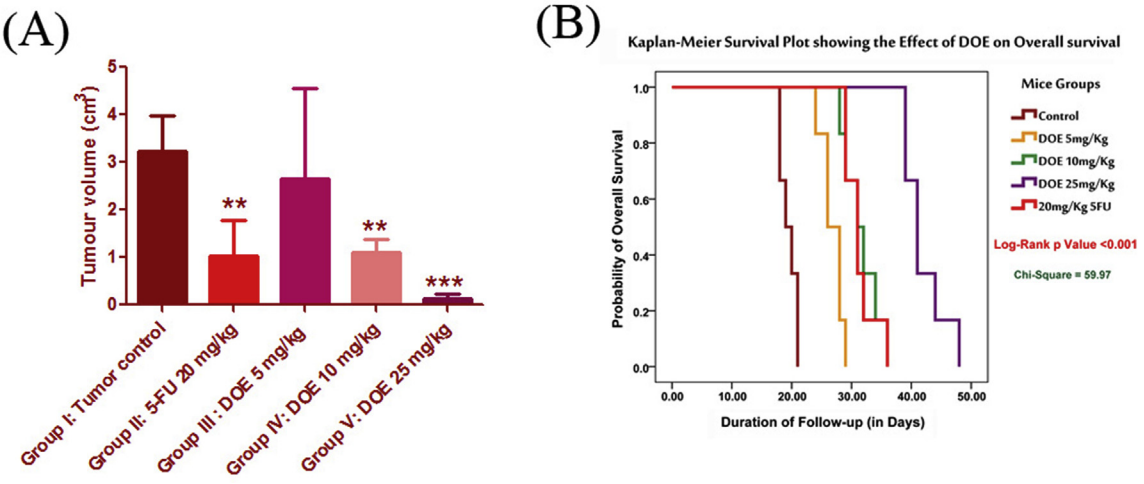

Fig. 3. Effect of DOE on EAC solid tumor model. (A) EAC cells were intramuscularly injected into right hind limb of the mice and were then administered with vehicle (0.5% DMSO) or DOE (5 mg/kg, 10 mg/kg or 25 mg/kg), and 5-Flourouracil (5-FU) (20 mg/kg) intraperitoneally and tumor volume was measured. Data are mean + SD; (n = 6 in each group); *p < 0.05; **p < 0.01; ***p < 0.001 vs. the control group. (B) Kaplan–Meier overall survival curve of EAC solid tumor model (n = 6).

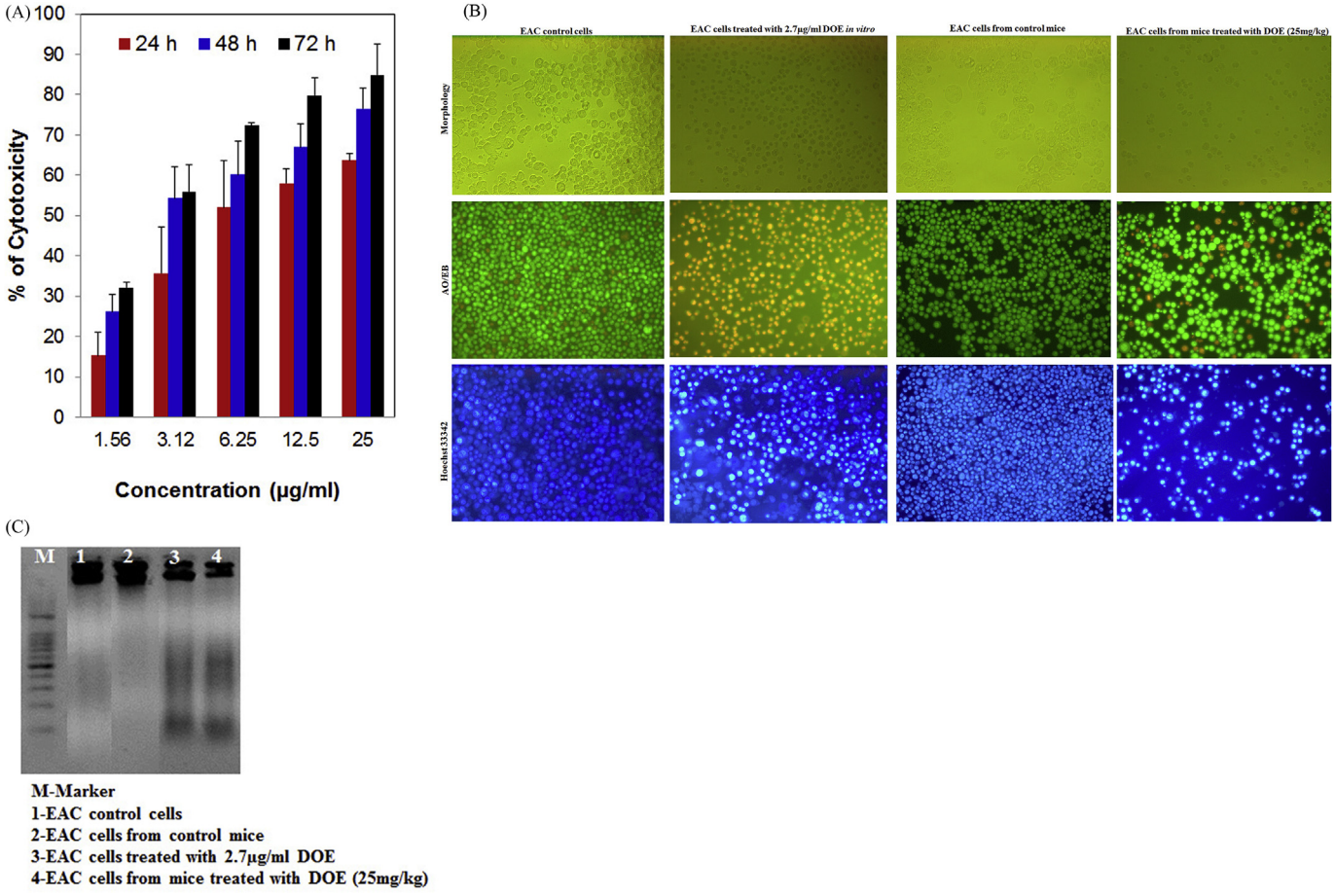

Fig. 4. DOE induced murine EAC cell death *via* apoptosis induction. (A) *In vitro* cytotoxic effect of DOE on EAC cells by MTT assay. (B) Light microscopy (first row), Acridine orange/Ethidium bromide (AO/EB) (second row) and Hoechst 33342 staining (third row) (magnification: × 20); (C) Agarose gel showing DNA fragmentation.

evident in DOE-treated EAC cells, suggesting the induction of apoptosis by DOE. AO/EB dual staining and Hoechst 33342 staining of EAC cells aspirated from DOE treated mice showed a significant increase of apoptotic cells than the control mice (Fig. 4B). DOE (25 mg/kg) could induce apoptosis within 48 h in the ascites tumor bearing mice, as evident from the characteristic ladder-like fragmentation of DNA, a sign of apoptosis induction (Fig. 4C).

In the present study, DOE was able to inhibit the *in vitro* growth of EAC cells. The toxicity of DOE was correlated with apoptotic induction, as evidenced by the morphological analysis of DOE (2.7 µg/mL) treated cells showing membrane blebbing, chromatin condensation, and DNA fragmentation, the characteristic features of apoptosis (Fig. 4B and C). Our results suggest that DOE can exhibit antitumor effects both *in vitro* and *in vivo*.

4. Discussion

Plants have been a source of medicine to cure various ailments since ancient time. Recently, traditional medicines, such as Traditional Chinese Medicine and Ayurveda are widely accepted as complementary and alternative therapies for cancer. During the last few years, attempts were made in search of novel anticancer agents from medicinal plants due to the presence of high content of bioactive compounds (Cragg and Newman, 2013).

In the present study, we evaluated the antitumor effects of DOE, a plant derived sesquiterpene lactone, against murine cancer EAC cells both *in vitro* and *in vivo*. EAC cells are transplantable, poorly differentiated and rapidly growing murine mammary adenocarcinoma with a very aggressive behavior. EAC cells can develop both solid and ascites forms of tumor in almost all strains of mice (Ozaslan et al., 2011). To investigate *in vivo* antitumor activity of DOE, EAC ascites tumor model and EAC solid tumor model were established in Swiss albino mice.

Prolongation of life span of animal represents the criterion for determining the efficacy of antitumor drugs (Hoagland, 1982). In ascites tumor model study, DOE treatment significantly increased the mean survival time and % of life span of mice compared to mice in control group. There is a correlation between ascites fluid accumulation and tumor growth since ascites fluid provides essential nutrients for the growth of cancer cells (Feng et al., 2001). Our data showed that administration of DOE could inhibit the accumulation of ascites fluid in the peritoneal cavity of the EAC tumor mice, subsequently reduced the tumor burden. Likewise, DOE treatment decreased the volume of solid tumor in EAC solid tumor model. Compared to 5-FU, DOE was more effective in reducing the progressive development of EAC solid tumor. The antitumor effect of 25 mg/kg DOE was highly significant in both tumor models and showed better activity than 5-FU. Moreover, 25 mg/kg of DOE showed the best overall survival response compared to all other groups.

The inhibition of ascites/solid tumor growth might be related to the cytotoxic effect of DOE. *In vitro* cytotoxicity studies showed that DOE significantly inhibited the growth of EAC cells in a dose- and time-dependent manner. Previous studies reported the cancer cell growth inhibition effect of DOE (Kabeer et al., 2017). Growth inhibitory potential of DOE might be due to the presence of α, β-unsaturated γ-lactone moiety, which confers cytotoxicity (Rodriguez et al., 1976). The anticancer effect of a compound is associated with its ability to induce

apoptosis in cancer cells. Because defective apoptosis is considered as one of the hallmarks of cancer development, apoptosis inducing agents can offer a new therapeutic approach. The main features of cell apoptosis are membrane blebbing, size reduction, chromatin condensation and DNA fragmentation (Kerr et al., 1972). Numerous studies demonstrated the apoptosis inducing capability of sesquiterpene lactones in cancer cells (Woynarowski et al., 1997). DOE inhibited the proliferation of a wide range of cancer cells through apoptosis (Huang et al., 2010; Su et al., 2011; Kabeer et al., 2017). Typical apoptotic features such as membrane blebbing, condensation and fragmentation of chromatin were observed in EAC cells after DOE treatment, indicating the apoptosis induction ability of DOE. DNA fragmentation, the hallmark of apoptosis (Bortner et al., 1995) was also evident after the treatment of DOE.

The results from the present study suggest that DOE has potential antitumor activity both *in vitro* and *in vivo*, which can be compared to the effect of 5-FU treatment. Since DOE shows lesser side effects than conventional chemotherapeutic agents, this compound shall be developed as a therapeutic agent for the treatment of human cancers. The mechanistic approaches in the present study revealed that DOE inflicts its growth inhibitory effects on EAC cells through the induction of apoptosis. Moreover, this study has revealed that DOE has considerably enhanced the overall survival of tumor bearing mice as compared to control group. The information from this study adds to the current knowledge of chemotherapeutic potential of DOE and will be helpful in developing DOE as an effective anticancer drug in future.

5. Conclusion

Our study reported significant *in vitro* and *in vivo* antitumor effects of DOE on EAC cells. Even lower doses of DOE gave better results than the standard chemotherapeutic drug (5FU). Notably, DOE mediated its cytotoxicity towards EAC cells by inducing apoptosis-both *in vitro* and *in vivo*. The new information obtained from present study could be helpful in developing DOE as a potential anticancer agent in near future.

Conflicts of interest

None declared.

Acknowledgements

The first author is thankful to University Grant Commission (UGC), New Delhi (India), for financial support. The authors would like to thank Indian Council for Medical Research, New Delhi, India (Grant no. 59/47/2011/BMS/TRM) and Kerala State Council for Science Technology and Environment (KSCSTE) (035/SRSLS/2007/CSTE), Kerala, India, for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101106.

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