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ORIGINAL RESEARCH

Bioactive component, cantharidin from *Mylabris cichorii* and its antitumor activity against Ehrlich ascites carcinoma

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Abstract The anticancer activity of the extract of blister beetle, *Mylabris cichorii* has been documented earlier by us. In the present study, the active principle of *M. cichorii* was isolated and its anticancer efficacy was evaluated against murine Ehrlich ascites carcinoma (EAC). The isolated bioactive compound was characterized to be cantharidin which showed potent antitumor activity and inhibited the proliferation of Ehrlich ascites carcinoma, both in vivo and in vitro. Cantharidin-treated EAC-bearing mice showed about 82% increase in lifespan at the dose of 0.5 mg/kg/day. In vitro cytotoxicity assay with the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test revealed about 50% cell death at the concentration of 25.8 μg/ml. The fluorescence and transmission electron microscopy revealed that EAC cells treated with cantharidin depicted typical apoptotic morphology with chromatin condensation, nuclear fragmentation into discrete masses, and plasma membrane blebbing which deduce towards the death of these cells. Histological examination of the kidney of cantharidin-treated mice showed glomerular and tubular congestion with abnormal Bowman's capsule, thus, indicating a renal toxicity in the host. Cantharidin-induced renal damage in the host was also manifested by the decreased lactate dehydrogenase isozymes and its possible release from the cells.

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Keywords Apoptosis · Anticancer activity · Cantharidin · Ehrlich ascites carcinoma · *Mylabris cichorii* · Toxicity

Abbreviations

CC	Column chromatography
EAC	Ehrlich ascites carcinoma
ILS	Increase in lifespan
IR	Infrared
MTT	{3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide}
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
TEM	Transmission electron microscope
TLC	Thin layer chromatography

Introduction

The wide ranges of plants and animals have attracted the human kind for their use as traditional, folk medicine throughout the world (Roja and Heble 1994; Gupta et al. 2004). Ingredients sourced from plants and animals are not only used in traditional medicines, but are also increasingly valued as raw materials in the

preparation of modern medicines and herbal preparations (Shoeb 2006; Alves and Alves 2011). The treatment of human ailments with remedies made from animals and their products is called zootherapy (Wang 1989; Alves and Rosa 2005). The animal-derived remedies as an integral part of folk medicine may constitute an important alternative among many other known therapies practiced worldwide. Various animals and its parts have been used for treating different diseases such as asthma, rheumatism, wounds, thrombosis, bronchitis, epilepsy, cancer, renal failure, etc. (Alves and Rosa 2007). In India, nearly 15–20% of the Ayurvedic medicine is based on animal-derived substances (Mahawar and Jaroli 2008).

There are many reports on the use of animals or animal-derived products in the treatment against cancer-suspected disease. Anticancer peptide of molecular mass 6,280 Da was isolated from *Buthus martensii* Karsch that prevented proliferation of the mouse S-180 fibrosarcoma cells and murine Ehrlich ascites carcinoma (EAC) cells (Kapoor 2010). It has been reported that charybdotoxin, 37 amino acid neurotoxin from the venom of the scorpion *Leiurus quinquestriatus* hebraeus, induces depolarization in human breast cancer cells, arrests the cells in the early G1, late G1, and S phase and accumulated cells in the S phase (Cavallucci et al. 2010). The skin extract of common Indian toad *Bufo melanostictus*, schneider exhibits significant antineoplastic activity on EAC cells and human leukemic cell lines U937 and k562 (Upadhyay and Ahmad 2010).

Beetles are one of the insects of medical importance, mainly due to the presence of broad spectrum of chemical substances within their hemolymph. The blister beetle, *Mylabris cichorii* has been used by the traditional healers of some parts of Assam, India for the treatment of cancer suspected diseases. The antitumor potential of this beetle extract against murine ascites Dalton's lymphoma has been reported earlier by us (Prasad et al. 2010b).

Most of the reported work on the importance of beetles deals with Chinese blister beetles *Mylabris phalerata* (Pall.) and Spanish fly, *Lytta vesicatoria*. Chinese blister beetle, *M. phalerata* is 15–30 mm long and 5–10 mm wide. Each elytron has a large orange–yellow spot at its base where it joins the thorax and also two wide transverse orange–yellow bands. Both bands and the black background have stiff black hairs (Singh 2001). The Spanish fly, *L. vesicatoria* is actually not a fly, but a member of the blister beetle family and it is an emerald green beetle, 15–22 mm long and 5–8 mm wide (Moed et al. 2001).

M. cichorii is found in parts of India as well as China. *M. cichorii* is about 12–20 mm long and 3–6 mm wide. The bands of the elytra are pale oechre yellow and the basal one often joins the middle band along the inner margin of each elytron. Yellow hairs occur upon the yellow bands and black hairs on the black background (Singh 2001). These beetles belong to the Family Meloidae, Order Coleoptera in class Insecta. Various reports have shown the presence of cantharidin in the blister beetles (Moed et al. 2001; Bonness et al. 2006; Rauh et al. 2007). In contemporary studies, cantharidin has been shown to be active in cervical, tongue, gingival, mucoepidermoid carcinoma, adenocystic carcinoma, neuroblastoma, bone, ovarian, and colon cancer cell lines among others (Wu et al. 1992; McCluskey et al. 2000; Sakoff et al. 2002)

The present study was carried out on the blister beetle, *M. cichorii* which is very commonly found in our studies areas and very less work has been done on the beetles of this region. Moreover, the details of its active compound and its effect on EAC cells have not been explored. Therefore, in an attempt to recognize the anticancer active principle of *M. cichorii*, the present study was undertaken to isolate the bioactive compound from these beetles and evaluate its effect on the EAC cells in vivo and in vitro. The findings from the present studies demonstrate that the bioactive compound in the *M. cichorii* is cantharidin, which exhibits potent anticancer activity and induces apoptosis in EAC cells.

Materials and methods

Animals and tumor model

Inbred Swiss albino mice were maintained under conventional laboratory conditions (20±2°C) with free access to food (Amrut Laboratory, New Delhi) and water ad libitum. EAC is being maintained in vivo in 10–12-week-old mice by serial intraperitoneal (i.p.) transplantations of 1×10^6 viable EAC cells (Gothoskar and Ranadive 1971) per animal (0.25 ml in phosphate-buffered saline (PBS), pH 7.4). Tumor-transplanted mice usually survived for 18–20 days. The use of

animals in the present study was as per the ethical norms and has been cleared by the institutional ethical committee of North-Eastern Hill University, Shillong, India.

Collection and identification of *M. cichorii*

Blister beetle, *M. cichorii* was collected from different locations of Karbi Anglong and North Cachar Hills districts of Assam, India. Species identification was done in Zoological Survey of India, Kolkata, bearing identification report no 9/2007 and a voucher specimen (no. SBP 101) was deposited in the department of Zoology, North-Eastern Hill University, Shillong, India.

Extraction and purification of the active component

The powdered beetle (1 kg) was extracted three times with 2 l of absolute methanol each time. The accumulated extract was concentrated under reduced pressure using rotary evaporator at 40°C. The dried free-flowing sample (80 g) was subjected to column chromatography, using a 45× 4 cm glass column filled with silica gel 60 (mesh size, 60–120) in *n*-hexane. Prepared methanol extract sample was added to the free volume at the head of the column. Total 75 fractions were collected using *n*-hexane: ethyl acetate (1:10 and finally with 100% ethyl acetate) as the eluting solvents and each fraction was tested for the activity against tumor model. Based on the similar thin layer chromatography (TLC) profile (R_f values) and high antitumor activity, fractions 16–25 were combined and single active compound (white crystals) was purified using second-column chromatography over silica gel eluted with *n*-hexane: ethyl acetate (1:15 and 1:20). The purity of compound was confirmed by TLC and proton nuclear magnetic resonance (^{1}H-NMR).

Spectral measurements of the isolated compound

Infrared spectrum was recorded in chloroform on a Perkin-Elmer system 2000 Fourier-transformed infrared (IR) Spectrophotometer calibrated against the polystyrene absorption at 1,601 cm^{-1}. Mass spectrum was recorded on a gas chromatography–mass spectrometry in a Bruker Daltonic Data Analysis 2.0 Spectrometer. ^{1}H-NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded using $CDCl_3$ as solvent in a Bruker Advance DPX-300 NMR machine considering TMS as an initial standard and chemical shift values were in delta parts per million (δ ppm) values. Spectra were referenced to tetramethylsilane (^{1}H) or solvent (^{13}C) signals.

Antitumor activity study

Cantharidin was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4 mg/ml and stored at 4°C. Its anticancer activity was determined following the method described by Ahluwalia et al. (1984). Tumor cells were transplanted intraperitoneally in 11–12-week-old male mice (30 g) and the day of transplantation was taken as day 0. The tumor-transplanted animals were randomly divided into eight groups with 10 mice in each group. On the sixth day of tumor transplantation, mice were treated with different doses of cantharidin (0.5, 1, 1.5, and 2 mg/kg body weight/day; i.p.) and the LD_{50} was determined based on these doses. Subsequently sublethal doses of cantharidin were selected and diluted with PBS to get the desired concentration, i.e., 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/kg body weight/day. The mice in various groups were treated with different concentration of cantharidin for five consecutive days starting from the sixth day of tumor transplantation. The control group of tumor-bearing mice received the same volume of cantharidin vehicle (the same volume of DMSO diluted with PBS) alone. The deaths of animals, if any in different treatment groups, were recorded daily. The anticancer efficacy was determined in percentage of average increase in life span (%ILS) using the formula: $(T/C \times 100) - 100$, where, T and C are the mean survival days of treated and control groups of mice, respectively. The dose of cantharidin, i.e., 0.5 mg/kg body weight showing highest anticancer activity was selected for further apoptotic study using transmission electron microscope (TEM) and fluorescence microscope.

To have a comparative analysis of the cantharidin's antitumor effect, in other set of experiment, a known anticancer drug, cisplatin (2 mg/kg body weight/day, i.p.) was given as the reference drug to the tumor-bearing mice on the sixth day of transplantation daily up to tenth day. The cisplatin has been used as a reference anticancer drug by other workers also (Ajith and Janardhanan 2003).

In vitro cytotoxicity assay (MTT)

Cell growth inhibition was determined by {3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (MTT) assay. MTT assay is a nonradioactive colorimetric assay (Campling et al. 1988) to measure cell cytotoxicity, proliferation, or viability. Briefly 1×10^6 cells in 1-ml culture medium were seeded on 24-well plates and the cells were treated with different concentration (10, 20, 30, 40, and 50 μg/ml) of cantharidin for 12 h. At the end of the incubation, culture medium was removed and MTT (5 mg/ml) was added and the cells were further incubated for 4 h. After removing the media, DMSO (100 μl) was added in each well to solubilize the formazan crystals. The absorbance was read at a wavelength 595 nm. Cell death was expressed as percentage over the control. The same treated cells were also processed for in vitro apoptosis assay using acridine orange and ethidium bromide (AO/EtBr) staining method as described below.

Apoptosis study using fluorescence microscopy

Fluorescence-based in vivo apoptosis was determined by using AO/EtBr staining method as described by Shylesh et al. (2005). After 24, 48, 72, and 96 h of the treatment of the EAC-bearing mice with cantharidin, tumor cells were collected, washed with PBS, and treated with AO/EtBr (100 μg/ml PBS of each dye). The cells were thoroughly studied under fluorescent microscope (Leica) using a blue filter and photographed. Viable cell's nucleus stain green due to permeability of only acridine orange whereas, apoptotic cells appear yellow–red due to costaining of both stains.

Transmission electron microscopy

The EAC cells from mice in different groups were collected and processed for transmission electron microscopy as described by Prasad et al. (2010a). Briefly, each cell suspension was mixed rapidly with an equal volume of 2% glutaraldehyde solution in 0.1 M cacodylate buffer and fixed for 2 h. The cells pellet obtained after centrifugation (1,000×*g* for 5 min) was resuspended twice in an excess of 0.1 M cacodylate buffer with a 15-min interval. Cells were resuspended in 1% osmium tetroxide in 0.1 M cacodylate buffer and fixed for 30 min and then centrifuged at 1,000×*g* for 5 min. Then, 0.1 M cacodylate buffer was added and this step was repeated twice. The samples were stored in 2% glutaraldehyde solution at 4°C until further processing for embedding, cutting ultrathin sections, and viewing under transmission electron microscope *JEOL 100CX II*.

Kidney histopathology

For the analysis of kidney toxicity, normal mice (25–30 g) were divided into three groups with 10 mice in each group. Mice in group I, serving as normal control, received (i.p.) vehicle alone from days 1–. Mice in group II, serving as toxic control or positive control, received single dose of cisplatin (8 mg/kg of body weight; i.p.) as described by Prasad et al. (2006). In group III, serving as treated group, mice were administered with cantharidin (i.p., 0.5 μg/kg of body weight/day) for 6 days. Mice in different groups were killed after 14 days of the treatment and kidneys were collected for histopathological studies as described by Yang et al. (2006). Slices of the left kidney (from five animals of each group) were fixed in 10% formalin for 48 h and were embedded in paraffin. Thin sections (4–5 μm thick) collected on glass slides were deparaffinized and stained with hematoxylin and eosin stain. The stained sections were examined under a light microscope (Leica DFC425 C) and the cellular features and any deformities were recorded.

Lactate dehydrogenase isozymes profile

To understand further on the cantharidin-induced damage/toxicity on kidney, lactate dehydrogenase (LDH) isozymes pattern and its intensity pattern was also determined for the kidney, which were collected and used for histology. Polyacrylamide slab gel (6%) was prepared and electrophoresis was performed following the method of Davis (1964). Tissue homogenate (20% in PBS, pH 7.4) was prepared and centrifuged at 8,000×*g* for 15 min at 4°C and the supernatant was collected. Equal amount of (40 μl, i.e., 25 mg protein) tissue homogenate supernatants were loaded on the gel. After electrophoretic separation, the gel was processed for LDH-specific staining. The gel was dipped in LDH specific reaction solution (10 mg NAD, 10 mg MTT, 1 mg PMS, and 2 ml of 60% L-lactate as substrate in 50 ml millipore water), and incubated at 37°C for 15 min and the reaction was stopped by adding tap water and the gel was fixed and stored in 7% acetic acid. The net band intensity analysis of all five LDH isoforms

(LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5) was carried out using Transilluminator Bioview UXT-20 M-8E Gel logic 100 imaging system.

Statistical analysis

The results were expressed as mean±SD. Statistical significance was determined by one-way analysis of variance. The difference among multiple groups was analyzed by a post hoc test, Bonferroni. P value ≤0.05 were considered as statistically significant.

Results

Isolation, characterization, and structural elucidation of active compound

TLC profile [ethyl acetate/chloroform (1:10)] under UV light showed the presence of total 15 spots; whereas in visible range, only three spots were visible in methanol crude extract. The anticancer activity was shown by the single purified compound having R_f value 0.78 [ethyl acetate/chloroform (1:10)]. The instrumental analysis data of isolated active compound is shown in Table 1.On the basis of spectroscopic data mentioned above and comparing with the literatures (Walter and Cole 1967; Wang et al. 2000), this isolated compound sample code Cry 01 was identified as cantharidin and showed the purity over 98% (TLC and ^{1}H-NMR). The spectrogram for IR, TLC, and NMR profiles has been shown in Fig. 1.

Antitumor activity study

The determination of LD_{50} value from the different doses of the isolated cantharidin was found to be 1 mg/kg body weight in Swiss albino mice (SB Prasad, personal communication). Out of the different sublethal doses of isolated cantharidin used, the dose of 0.5 mg/kg was found to be the most effective against EAC. The effect of cantharidin at this dose on the survival of tumor-bearing mice is shown in Table 2. Mean survival time for the control group was about 20 days, which increased to about 36 and 37 days for the groups treated with cantharidin (0.5 mg/kg/day) and cisplatin (2 mg/kg/day), respectively. The increase in the lifespan of tumor-bearing mice treated with cantharidin and cisplatin was found to be about 82% and 87%, respectively, as compared to the control (Table 2).

In vitro cytotoxicity assay (MTT)

The effect of cantharidin on viability of tumor cells was checked using the MTT assay. The cantharidin treatment decreased the viability of the EAC cells in a dose-dependent manner as shown in Fig. 2. Cantharidin at about 25.8 μg/ml decreased the viability of EAC cells to 50% of the initial level and this was chosen as the $IC_{50.}$ However, in case of cisplatin, it was 32 μg/ml. Longer exposures resulted in additional cytotoxicity to

Table 1 Physical and spectral data for the purified compound (cantharidin) from *M. cichorii*

Sl no.	Parameter data	
1	Sample code	Cry 01
2	Yield	7% *w*/*w*
3	Nature	Crystalline
4	Color	White
5	Solubility	Chloroform, alcohol, ethyl acetate and dimethyl sulfoxide
6	R_f value (TLC)	0.78 [Ethyl acetate/chloroform (1:10)]
7	Molecular formula	$C_{10}H_{12}O_4$
8	Molecular weight	196.1
9	EI–MS (m/e,% 70ev)	96 (100%), 128 (83%), 70 (28%), 109 (11%), 95.1 (19%)
10	IR (Chloroform)	cm^{-1}, 3000 (C–H), 1780−1850 (C=O), 1240 (C–O)
11	^{13}C-NMR ($CDCL_3$, 75 MHz) δ	12.69 (CH3), 23.42 (C-5, C-6), 55.26 (C-3a, C-7a), 84.74 (C-4, C-7), 175.99 (C-1, C-3)
12	^{1}H-NMR ($CDCL_3$, 300 MHz) δ	1.248 (6H, s, CH3), 1.827 (2H, m, H-5, H-6), 4.734 (2H, t, J=4.9 Hz, H-4, H-7)

TLC thin layer chromatography, *IR* infrared, *NMR* nuclear magnetic resonance

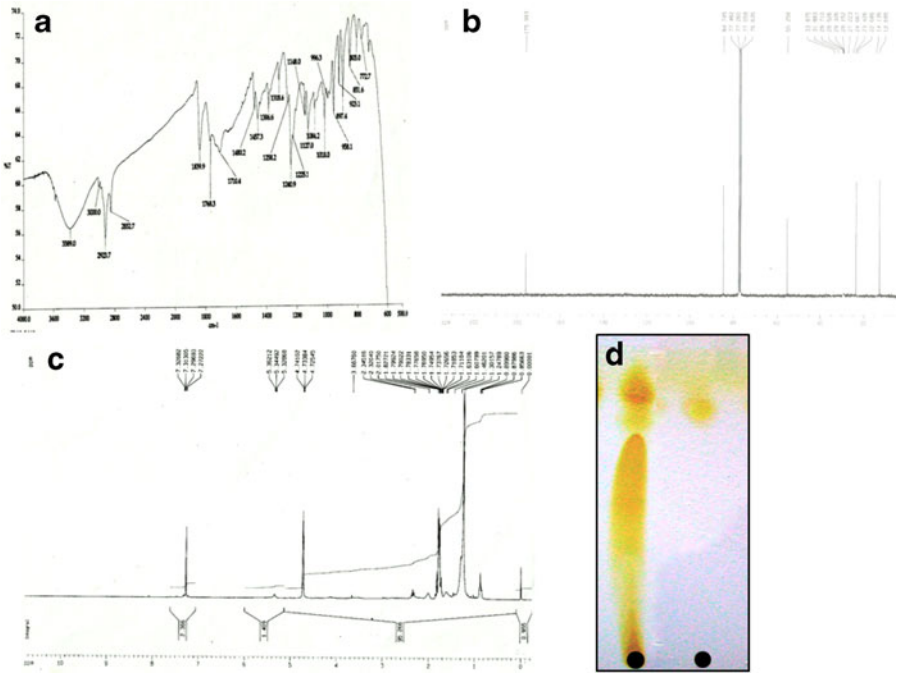

Fig. 1 Spectrogram for infrared (IR), thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) profile of the pure isolated compound, cantharidin. **a** Infrared (IR) spectrophotometry profile. **b** ^{13}C- NMR showing the number of carbon atoms. **c** ^{1}H-NMR showing the number of proton. **d** TLC profile of crude extract showing many spots (*lane 1*) and a single spot for isolated pure compound (*lane 2*)

the cells. Comparison of the doses of cantharidin and cisplatin and the determination of cytotoxicity by in vitro MTT assay suggest that cantharidin seems to be more effective/cytotoxic to EAC cells as compared to the reference drug cisplatin after 12 h of exposure (Fig. 2).

Apoptosis study using fluorescence microscopy

Acridine orange is a vital dye that stains both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity (Shylesh et al. 2005). Cells stained green represents viable cells, whereas yellowish–red staining represents apoptotic cells. The control EAC cells were rounded in shape with deep green fluorescence in blue filter (Fig. 3a). After 24-h treatment, nuclei constriction and early apoptotic features were very much prominent (Fig. 3b) while at 48 h of treatment, reduction in cell volume, cell shrinkage, and loss of cell membrane integrity and appearance of membrane blebbing were observed (Fig. 3c). At 72 h of incubation period, severe nucleus fragmentation was observed in more than 70% of cells with many late apoptotic cells and few early apoptotic cells. At 96 h of treatment, changes in cellular

Table 2 Effect of cantharidin treatment on mean survival time and percentage ILS of Ehrlich ascites carcinoma-bearing mice

Groups	Treatments (mg/kg)	Mean survival time (days)	% Increase in life span (ILS)
Control	Vehicle	20±1.3	–
Cisplatin	2	37.5±2.5*	87.50
Cantharidin	0.5	36.45±1.2*	82.25

Values are mean±SD, n=6. Significance of difference between control and treated groups was tested by one-way ANOVA

*P≤0.001, significant with respect to control

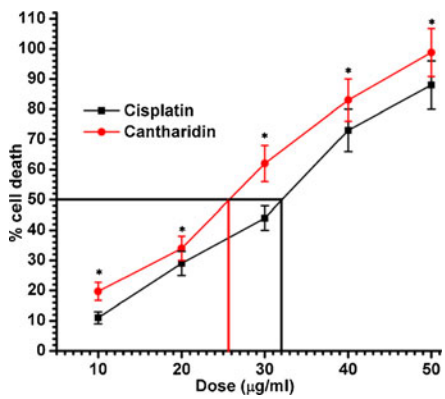

Fig. 2 Cytotoxicity of cantharidin against Ehrlich ascites carcinoma cells determined by MTT assay after 12 h of incubation at different doses. Control group is treated with vehicle alone whereas, cisplatin is used as a positive reference drug. Results are expressed as mean±SD. ANOVA, n=5, *P≤0.05 as compared to cisplatin treatment

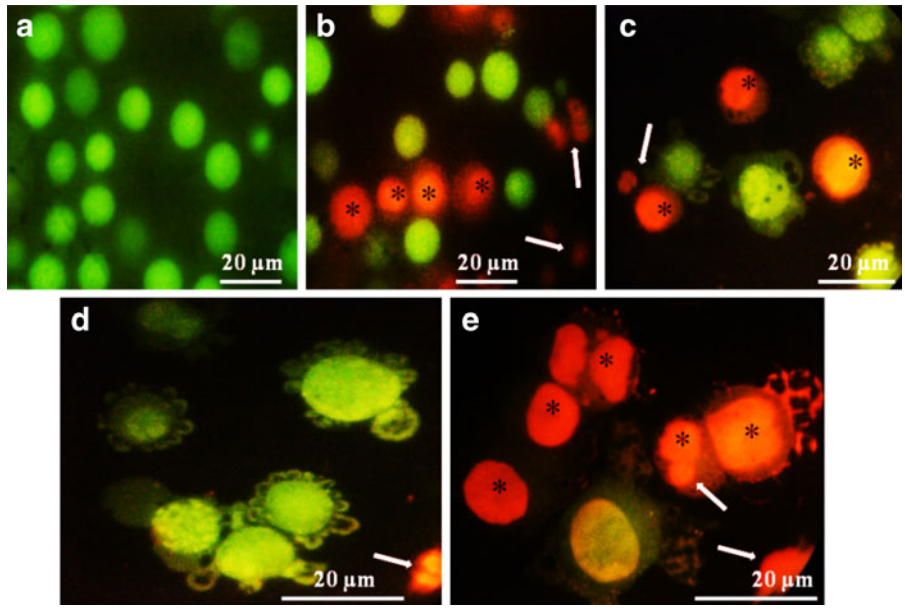

Fig. 3 AO/EtBr staining of EAC cells. **a** Control, EAC cells from the mice treated with vehicle alone is *rounded* in shape, with green fluorescence. After the treatment of mice with cantharidin (0.5 mg/kg body weight) for 24 h, **b** EAC cells depict appearance of membrane blebbing and formation of some fragmented nuclei. At 48 h of the treatment, **c** cells show chromatin condensation and cell membrane abnormality. At 72 h of the treatment, **d** cells are seen with severe membrane blebbing with some fragmented nuclei while at 96 h of treatment **e** formation of fragile membrane, membrane vacuoles, and presence of apoptotic bodies can be noticed. Each experiment was performed in triplicate and generated similar morphological features. *Arrow* indicates fragmented nuclei whereas *asterisk* showed apoptotic cells

morphology, including chromatin condensation, membrane blebbing, fragmented nuclei, large size cytoplasmic, and membrane vacuoles were seen with complete loss of membrane integrity (Fig. 3e). Thus, the morphological features of cantharidin-treated EAC cells showed the involvement of apoptosis.

The percentage of apoptotic cells in vitro at different doses of cantharidin and cisplatin for 12 h exposure is shown in Fig. 4. Cisplatin-treated cells also showed the apoptotic morphology but the apoptotic cells were comparatively lower at different doses as summarized in Fig. 4. Here, cell deaths were observed but nucleus fragmentation and membrane blebbing was not visible.

Transmission electron microscopy

Here, TEM study was carried out to corroborate the observations made by AO/EtBr staining. Ultrastructural examination of the cantharidin-treated EAC cells showed typical morphological features of apoptosis (Fig. 5). The morphological changes observed were reduction in cell volume, cell shrinkage, reduction in chromatin condensation, and nucleus fragmentation. Control

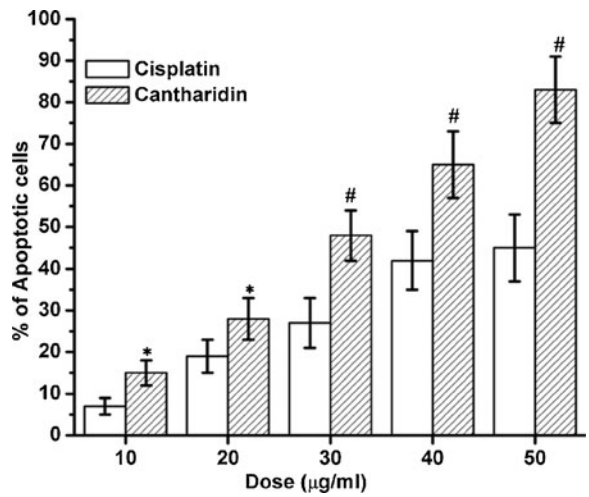

Fig. 4 Graph showing the percentage of apoptotic cells in vitro treated with different doses of cantharidin and cisplatin for 12 h. The result is based on the AO/EtBr staining method, apoptotic cells appears *red* in color whereas viable cells were *green*. Thousand cells were analyzed and percentages of apoptotic cells were counted. Results are expressed as mean±SD. ANOVA, $n=6$, as compared to respective cisplatin treatment. *$P \leq 0.05$ and #$P \leq 0.001$

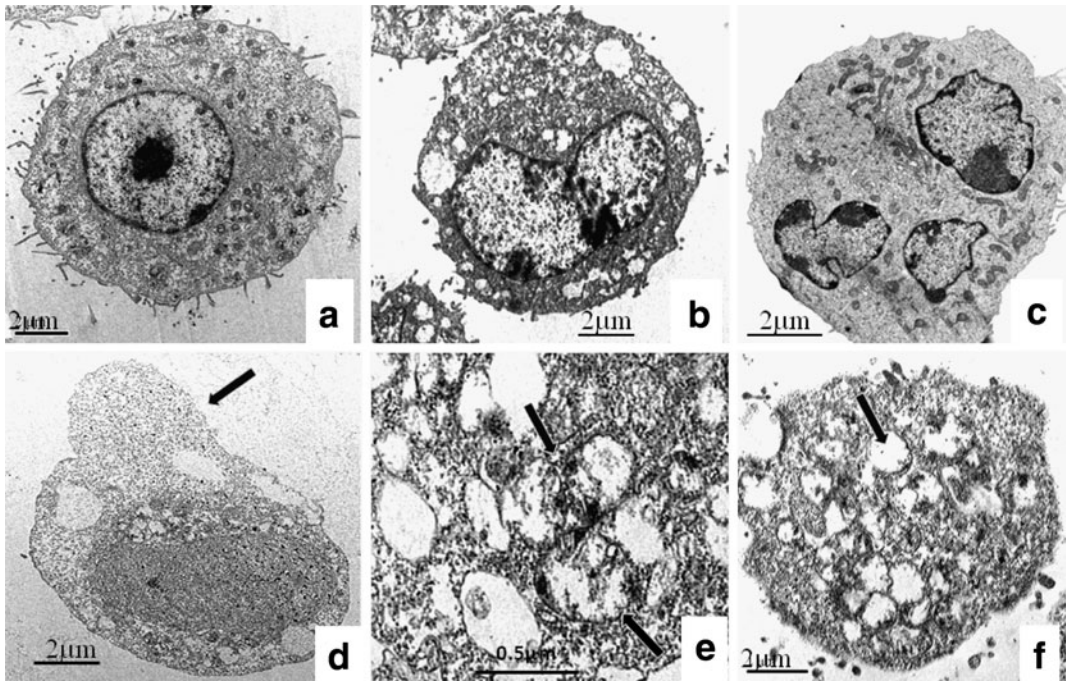

Fig. 5 Ultrastructural features of Ehrlich ascites carcinoma cells. Tumor-bearing control (**a**), showing a more or less rounded shape, normal nucleus with microvilli like processes over the cells surface. Cantharidin treatment (0.5 mg/kg body weight) of mice for 24 h (**b**) shows the appearance of nucleus abnormality with condensation of chromatin, appearance of cytoplasmic vacuoles. At 48 h of treatment (**c**), severe fragmented nuclei with disorganized cell membrane were noted. At 72 h of the treatment (**d**), formation of cytoplasmic vacuoles, disruption in the nuclear membrane and disintegration in the cell surface membrane is prominent; arrow indicates the apoptotic budding of the cells. Magnified view of the cells indicates (*arrow*) abnormal swelling of mitochondria with loss of cristae (**e**). At 96 h of treatment, major loss of cellular framework with both cytoplasmic (*arrow*) and membrane vacuoles as well as loss of nuclear membrane leading to lysis of cancer cells may be noted

tumor cells were rounded in shape without any apoptotic morphology with normal round nucleus (Fig. 5a). Cantharidin treatment (0.5 mg/kg/day) of mice for 24 h (Fig. 5b) showed the appearance of constricted nucleus with condensation of chromatin in EAC cells, appearance of cytoplasmic vacuoles were also observed. Membrane disorganization and severe fragmented nucleus was observed after 48 h of treatment (Fig. 5c); while at 72 h of treatment (Fig. 5d and e), there was a reduction in cell volume showing cell shrinkage, compaction of the nuclear chromatin, fragmentation of nuclei, condensation of the cytoplasm, and appearance of the apoptotic bodies. At the same time, large numbers of cytoplasmic vacuoles were also observed (Fig. 5d); magnified view of the cells indicates abnormal swelling of mitochondria with loss of cristae (Fig. 5e). At 96 h of treatment (Fig. 5f), the appearance of cytoplasmic as well as membrane vacuoles and complete loss of cellular framework with gradual disintegration of plasma membrane leading to lysis of the tumor cells was visible. Moreover, after 96 h of treatment, appearance of apoptotic bodies and fragmented nuclei were scattered outside the cells which indicated severe cells damage by apoptosis (Fig. 5f).

Kidney histopathology

Various histopathological features of kidney from different groups are presented in Fig. 6 and mentioned in Table 3. Kidney of normal mice showed the normal structures of the renal cortex, which comprised renal corpuscles, glomerulus, proximal, and distal convoluted tubules. Blood vessels congestion and tubular cast were absent (Fig. 6a and d). In the cisplatin-treated mice, which served as positive control, kidney showed immense histological damages as evidenced by the glomerular and tubular congestion with abnormal Bowman's capsule, blood vessel congestion, epithelial cell desquamation, and presence of tubular cast with few inflammatory cells (Fig. 6b). Magnified view of glomerulus depicts loss of capsular wall with abnormally

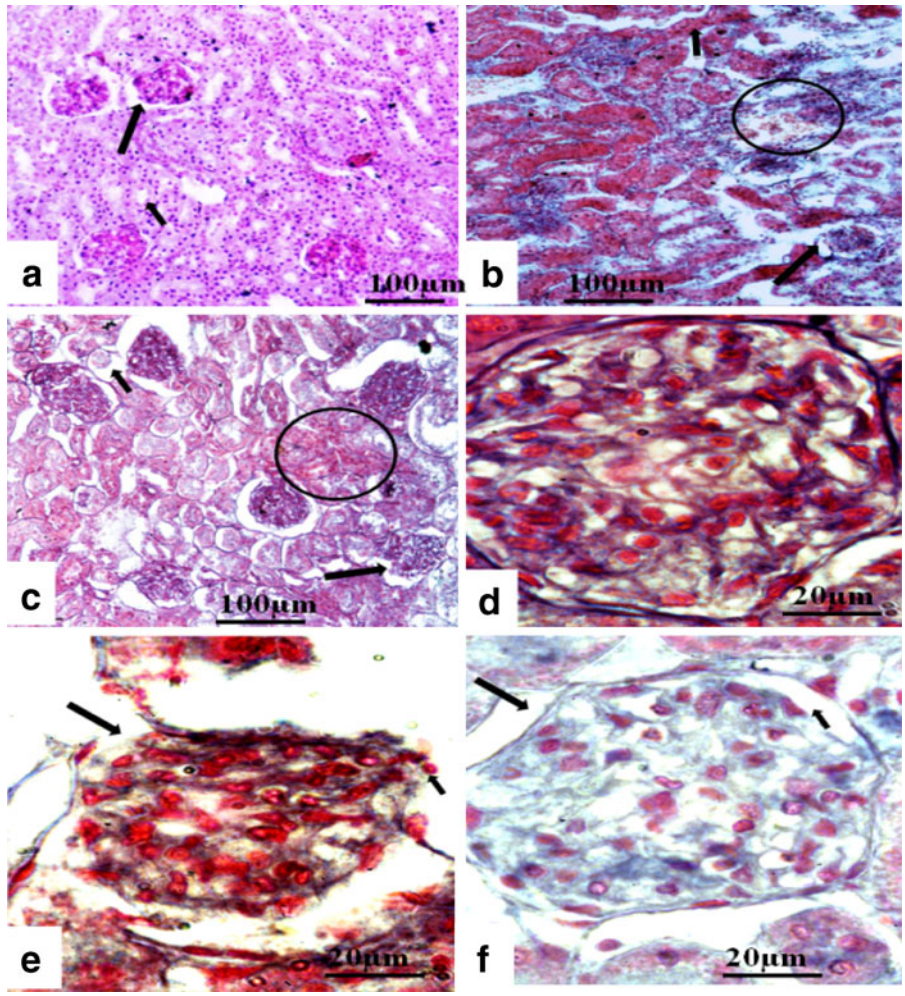

Fig. 6 Photomicrographs of LS of kidney of different groups. **a** The histological studies of normal group showed normal glomerular (*big arrow*) and tubular (*small arrow*) arrangements with normal Bowmen's capsule; **b** cisplatin-treated group showing congested vein, damaged tubule, degenerate glomeruli with leucocyte infiltration shown in *circle* and dilatation of subcapsular space; **c** cantharidin-treated group showing vacuolated cells with pyknotic nuclei, abnormal glomeruli with subcapsular space and leucocyte infiltration shown in *circle*; **d** magnified view of normal group glomerulus showing normal arrangement and compact capsular wall surrounded by renal tubules; **e** cisplatin-treated glomerulus showing loss of capsular wall (*big arrow*) with abnormal fragmented dispersed nucleus (*small arrow*); **f** cantharidin-treated glomerulus showing dilatation of subcapsular space (*big arrows*) and formation of large vacuoles inside the glomerulus (*small arrows*)

dispersed nucleus (Fig. 6e). In cantharidin-treated mice, the kidney tubular epithelia were exfoliated from their underlying basement membrane and their lining cells exhibited cytoplasmic vacuolation and pyknotic nuclei (Fig. 6c). Some glomeruli seemed to have lost their attachments and mesangial stromas were atrophied with dilatation in the subcapsular space (Fig. 6f). Thus, it is evident that cantharidin treatment caused some nephrotoxicity and cellular damage on the host but it was lower as compared to cisplatin.

LDH isozymes profile

The analysis of LDH isozymes patterns revealed the presence of all the five isozymes forms (i.e., LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5) in the kidney (Fig. 7a–c). After cantharidin treatment, the expression profile as shown by band intensity (Fig. 8) of all the isozymes decreased (Fig. 7b) significantly as compared to control. Cisplatin treatment caused more decrease in the isozymes (Fig. 8) intensity as compared to that of

Table 3 Histological features from longitudinal section of kidneys of normal and different treated groups of mice

Histological features	Normal (vehicle alone)	Cisplatin treated (8 mg/kg body weightt/day)	Cantharidin treated (0.5 mg/kg body weight/day)
Tubular congestion	−	+++	++++
Tubular cast	−	++	++
Epithelial disquamation	−	++	+++
Glomerular congestion	−	++++	++
Blood vessel congestion	−	++++	++++
Hyperaemia of medullary part	−	++	++
Inflammatory cells	−	++	++++
Necrosis	−	++++	+++

(++++) very high, (+++) high, (++) medium, (+) low, (−) negative

cantharidin treatment (Fig. 7c). The decrease in isozymes after treatments in both cisplatin and cantharidin groups may suggest the leakage of LDH from kidney and damage to tissue.

Discussion

Our field survey with the indigenous people of Karbi Anglong and North Cachar Hills districts of Assam, India revealed that the people of this region frequently use blister beetles, *M. cichorii* against cancer suspected cases. It has been reported earlier that the methanol extract of these beetles has antitumor potential (Prasad et al. 2010b). In an attempt to understand further on the mechanism(s) of the antitumor activity, the isolation and characterization of bioactive compound from these beetles was undertaken along with the evaluation of antitumor activity of isolated compound against EAC.

Other studies to isolate cantharidin from beetles have used different solvent system like 50% aqueous ethanol/methanol, chloroform, *n*-butanol, etc. In the present studies, we used absolute methanol as it is rapidly evaporated while drying the extract using rotary evaporator. Before extraction in methanol, the beetle powder was washed three times with petroleum ether to remove fats and pigment which existed in extract as it may cause hindrance in the column run.

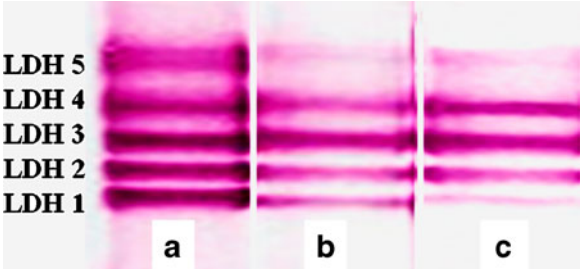

Fig. 7 Photograph showing lactate dyhydrogenase (LDH) isozymes patterns in kidney in various treatment conditions in a slab gel. Each lane is about 0.8 cm in width. **a** LDH isozymes pattern of normal control; **b** LDH isozymes of cantharidin treated; **c** represent cisplatin treatment for 14 days. In case of normal **a**, the band intensity of all isozymes were high while it decreased significantly in treatment groups (**b** and **c**) as indicated by band intensity shown in Fig. 8

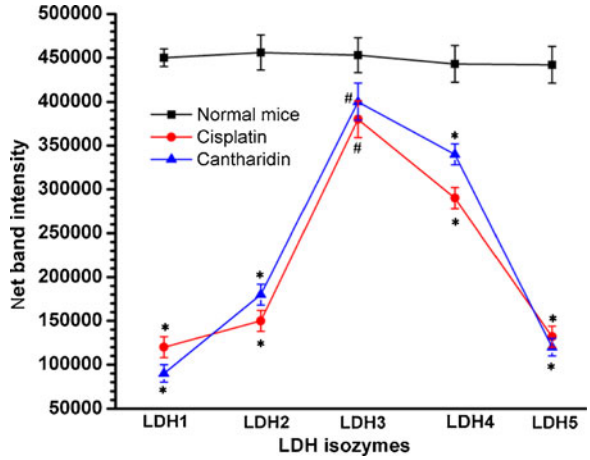

Fig. 8 Graph showing the net band intensity of different LDH isozymes of kidney in normal, cisplatin- and cantharidin-treated mice. The band intensity was calculated using transilluminator bioview UXT–20M–8E Gel logic 100 imaging system. The net band intensity is mean of three different gels. As compared to the normal, band intensity of all the five isozymes decreased significantly. Results are expressed as mean±SD. ANOVA, as compared to normal, $n=3$,$^{*}P\leq 0.001$; $^{\#}P\leq 0.05$

The findings after the simulation of different spectroscopic data (Fig. 1) of the isolated compound from the present studies showed that the major bioactive compound from *M. cichorii*, is cantharidin which exhibited potent antitumor activity against EAC. The details of the characteristic features of the identified compound, cantharidin is given in Table 1.

Cantharidin ($C_{10}H_{12}O_4$) with molecular weight of 196.1 is a monoterpene anhydride having the chemical name as 2-endo, 3-endo-dimethyl-7-oxabicyclo (2.2.1) heptanes-2-exo, 3-exo-dicarboxylic anhydride, most abundantly found in blister beetles (Eldridge and Casida 1995; Wang et al. 2000). Cantharidin is absorbed by the lipid layers of cell membranes (Moed et al. 2001). It was observed that cantharidin-treated EAC-bearing mice showed about 82% increase in life span (Table 2). This antitumor effect is in conformity with the earlier reports showing the presence of cantharidin as the major component of *M. cichorii* having anticancer potentials. Cantharidin, a vesicant produced by beetles in the order Coleoptera, has a long history in both folk and traditional medicine. Cantharidin has been reported to produce cytotoxic effects in a number of human cancer cell lines and primary cancer cells (Huan et al. 2006; Nikbakhtzadeh and Ebrahimi 2007; Rauh et al. 2007). The use of cantharidin and its analogs in cancer therapy has been widely suggested (Cirrito and Bergstein 2008). The first documented use of cantharidin to treat cancer is by the physician Yang Shi-Ying dating back to 1264 (Wang 1989). Cantharidin has been shown to be active in cervical, tongue, gingival, mucoepidermoid carcinoma, adenocystic carcinoma, neuroblastoma, bone, ovarian, and colon cancer cell lines among others (Wu et al. 1992; McCluskey et al. 2000; Sakoff et al. 2002). Effect of cantharidins against hepatocellular and colorectal tumors (Wang et al. 2000; To et al. 2005; Chen et al. 2005) and leukemic stem cells (Dorn et al. 2009) has been well documented.

Modifications of cantharidin's skeleton permitted the development of a new series of analogs (McClusky et al. 2003; Hill et al. 2007; Liu and Zhiwei 2009). Analogs possessing good protein phosphatases 1 (PP1) and 2A (PP2A) inhibition exhibited good anticancer activity (McClusky et al. 2003). PP1 and PP2A are serine/threonine protein phosphatases which are inhibited by cantharidin (Honkanen 1993; Efferth 2005; Li and Casida 1992). Protein serine/threonine phosphatases have recently been used as viable therapeutic targets in the development of drugs (McConnell and Wadzinski 2009). Other natural product extracts have proven to be a rich source of small molecules that potently inhibit the activity of family ser/thr protein phosphatases. Some of these inhibitors include, okadaic acid (produced by marine dionoflagelates, *Prorocentrum* sp. and *Dinophysis* sp.), calyculin A, dragmacidins (isolated from marine sponges), microcystins, nodularins (isolated from cyanobacteria, *Microcystis* sp. and *Nodularia* sp.), tautomycin, tautomycetin, cytostatins, phospholine, leustroducsins, phoslactomycins, and fostriecin (isolated from soil bacteria, *Streptomyces* sp.). Fostriecin and cantharidin possess antitumor activity, but okadaic acid and microcystin LR have been touted to act as tumor-promoting agents. Microcystin, a nonselective inhibitor primarily affects the liver, causing minor to widespread damage, depending on the amount of toxin absorbed (Swingle et al. 2007).

Cantharidin has also been reported to cause delays in cell cycle progression following DNA replication with no apparent effect on G(1)-S or S-G(2) phase progression (Bonness et al. 2006). However, cantharidin can rapidly arrest growth when added during G(2) or early M phase (Dongwu and Zhiwei 2009). Reference drug used in present studies was cisplatin which has been established to be one of the most effective cancer chemotherapeutic agents. It has been well documented that cellular DNA could be the primary target of cisplatin's anticancer activity. Cisplatin is water-soluble square planar coordination complex containing a central platinum atom surrounded by two-chloride atoms and two ammonia moieties. Cisplatin is an alkylating drug and its anticancer activity has been attributed mainly to its ability to bind with cellular DNA involving intrastrand and interstrand cross-links (Fuertes et al. 2003). Cisplatin used here as a reference drug showed ILS value of about 87% which was quite close to the ILS, 82% noted for cantharidin treatment (Table 2). It may be of importance to mention that almost similar anticancer activity observed with cantharidin is at much lower concentration as compared to reference drug, cisplatin. The cytotoxic effects of cantharidin on EAC cells in in vitro were evident at 1 h of continuous exposure. In vitro cytotoxicity assay with the MTT test revealed an IC_{50} at 25.8 μg/ml while for cisplatin under the same conditions, it was 32 μg/ml (Fig. 2). This may suggest that as compared to cisplatin, cantharidin is able to cause heightened injury to cells and this may be due to

a better diffusion of cantharidin through the cell membranes, due to its nonpolar nature and low molecular size.

Uncontrolled proliferation and a defect in apoptosis constitute crucial elements in the development and progression of malignant tumors (Bryan et al. 2011; Finkel et al. 2007). Among many other biological response modifiers known to influence these mechanisms, the efficacy of drugs in the treatment of various malignant entities is currently matter of discussion (Bao-ying et al. 2011; Zhang et al. 2010). Apoptosis is formally defined by morphological criteria and this remains an important means of characterizing an apoptotic cell (Kerr et al. 1972). The assay based on TEM and AO/EtBr fluorescence staining is a good reliable analysis for the authentication of apoptotic features (Zakeri et al. 1995; Mattes 2007) compared to other methods (Leite et al. 1999). Cantharidin treatment caused changes in cellular morphology, including chromatin condensation, membrane blebbing, fragmented nuclei, large size cytoplasmic, and membrane vacuoles with complete loss of membrane integrity (Fig. 3). The ultrastructure of cantharidin-treated EAC cells depicted typical apoptotic morphology with chromatin condensation, fragmented nucleus into discrete masses, cells shrinkage, etc. (Fig. 5) and membrane blebbing as supported by fluorescence study (Fig. 3). Finally, the whole cell buds, producing apoptotic bodies, vary in size and structure. The apoptic features were seen in more than 70% of cells. Thus, it may obviously be suggested that cantharidin treatment could induce apoptosis in EAC cells.

The effects of cantharidin and cantharidin derivates on tumor cells have been illustrated which also indicated that cantharidin induces apoptosis in many types of tumor cells (Liu and Zhiwei 2009). It has been reported that cantharidin induces caspase-3, -8, and -9 activities in myeloma cells (Sagawa et al. 2008) and it inhibits the activity of serine/threonine protein phosphatase 4 (PP4; Cohen et al. 2005). During apoptosis, many functional molecules may undergo post-translational modification, including phosphorylation, dephosphorylation, and caspase cleavage. Some apoptosis-regulating genes also undergo alternative splicing, generating splice variants that antagonize normal transcripts on apoptosis (Hoof and Goris 2003). It is also found that PP2A acts in the apoptotic signal transduction pathway not only upstream but also downstream of the effector caspases. PP2A activates pro-apoptotic and inhibits anti-apoptotic proteins of the Bcl-2 family; hence, PP2A is involved in the regulation as well as the cellular response of apoptosis. Probably, various PP2A holoenzymes with distinct regulatory subunits altering the PP2A substrate specificity are implicated at different levels of the apoptotic signal transduction pathway (Hoof and Goris 2003).

It has been found that the mitogen-activated protein kinase (MAPK) family, the MAPK ERK kinase and ERK become active after cantharidic acid stimulation, and result in a significant increase in caspase-3-mediated apoptosis of tumor cells (Schweyer et al. 2007). The cancer cells that are treated with cantharidin may also undergo death by autophagy. For example, breast carcinoma cells that are treated with the estrogen-receptor antagonist tamoxifen accumulate autophagic vacuoles shortly before dying. Extensive autophagic degradation of the Golgi apparatus, polyribosomes, and endoplasmic reticulum were seen in high magnification (Fig. 5e and f). Morphologically, the dead cells lack the features of cells that have undergone apoptosis. Unusually large size of putative autophagic vacuoles may also provide a clue to an apoptotic origin (Abedin et al. 2007).

The full use of cisplatin for the management of cancer is usually limited by the development of nephrotoxicity (Borch 1987). Histological changes in kidney after cantharidin treatment of mice revealed tubular necrosis, atrophy of glomerulus, and marked dilation of proximal convoluted tubules with slogging of almost entire epithelium due to desquamation of tubular epithelium which indicate injury to kidney. There was an increase/infiltration of inflammatory cells in the kidney after treatment (Table 3) which may also indirectly suggest the renal irregularity/toxicity. The simultaneous decrease in LDH isozymes from kidney and histological abnormality is a fair indication of altered membrane permeability of cells in kidney. A correlation between tissue cytotoxicity and LDH release has been demonstrated and used as a parameter of tissue damage by many workers (Takema et al. 1991; Hasan et al. 2005). Slight damage to the plasma membrane will easily lead to leakage of LDH from the cell to the extracellular environment (Akanji et al. 2008). However, the decrease in kidney LDH activity as shown by band intensity in present study (Fig. 8) may be due to labialized plasma membrane (Akanji et al. 1993). At the same time, the possibility

of decreased synthesis and/or increase leakage from the cells due to cell membrane injury may also exist. The release of LDH is higher after cisplatin treatment as compared to that of cantharidin suggesting more nephrotoxic effects induced by cisplatin. It has been reported that LDH-1 and LDH-2 isoenzymes can be released by cellular injury to cardiac muscle or kidney (Kopperschlager and Kirchberger 1996; Akanji and Yakubu 2000). In the present studies, it is also found that the band intensity of LDH-1, LDH-2, and LDH-5 decreases significantly after treatment as compared to control, supporting the damage to kidney.

In conclusion, the result of the present studies showed that cantharidin-mediated anticancer activity against EAC may involve apoptosis. Cantharidin treatment caused plasma membrane disintegration and the appearance of membrane vacuoles and blebbing on the tumor cells which may lead to cell death. It also exerts some kidney damage in the host. However, the detailed molecular mechanism(s) involved in the antitumor activity of cantharidin against EAC needs to be elucidated.

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