Embelin (2,5-dihydroxy-3-undecyl-p-benzoquinone): A bioactive molecule isolated from Embelia ribes as an effective photodynamic therapeutic candidate against tumor in vivo

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\section*{A B S T R A C T}

The present study was carried out to assess the photosensitizing potential of embelin, the biologically active natural product isolated from Embelia ribes in photodynamic therapy (PDT) experiments in vivo. In vitro PDT clearly indicated that embelin recorded significant cytotoxicity in Ehrlich’s Ascites Carcinoma (EAC) cells, which is superior to 5-aminolevulinic acid, a known photodynamic compound. For in vivo experiments solid tumor was induced using EAC cells in the male Swiss albino mice of groups I, II, III and IV. Group I served as the control (without solid tumor), group II served as tumor bearing mice without treatment and groups III and IV served as treatments. At the completion of 4 weeks of induction, the tumor bearing mice from group III and IV were given an intraperitoneal injection with embelin (12.5 mg/kg body weight). After 24 h, tumor area in the Group III and IV animals was exposed to visible light from a 1000 W halogen lamp. The mice from groups I to III were sacrificed 2 weeks after the PDT treatment and the marker enzymes (myeloperoxidase [MPO], β-glucuronidase, and rhodanese) were assayed and expression of Bcl-2 and Bax were analyzed in normal and tumor tissues. Animals from group IV were sacrificed after 90 days of PDT treatment and the above mentioned parameters were recorded. Reduction in tumor volume and reversal of biochemical markers to near normal levels were observed in the treated groups. This is the first report on PDT using a natural compound for solid tumor control in vivo. The uniqueness of the mode of treatment lies in the selective uptake of the nontoxic natural compound, embelin from the medicinal plant E. ribes used in Indian system of medicine, by the solid tumor cells and their selective destruction using PDT without affecting the neighboring normal cells, which is much advantageous over radiation therapy now frequently used.

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\section*{Introduction}

PDT is fast developing as a treatment that has great potential in the areas of cancer, age related macular degeneration, psoriasis, etc. It is recently used experimentally and clinically for peritoneal carcinomatosis, non-small cell lung cancer, premalignant conditions of the upper aerodigestive tract, and prostate cancer (Bauer et al. 2001; Sanfilippo et al. 2001; Nathan et al. 2002; Friedberg et al. 2003; Little et al. 2003). The principle of PDT is that an administered photosensitizer is selectively retained by cancer cells and when the tumor is irradiated with light of the appropriate wavelength, the photosensitizer is activated and causes tumor cell death by the production of reactive oxygen species (ROS) and free radicals (Garg et al. 2012). The presence of the photosensitizer linked to cell structures is essential for an effective photodynamic reaction. PS distribution inside the cell is also related to the internalization process and to the damage caused by the oxygen pathways. The conventional approaches to combat cancer, namely surgery, chemotherapy and radiation are not successful in curing cancer completely. They lack the specificity needed to kill cancer cells, without simultaneously damaging normal cells, as evidenced by the serious side effects that accompany these treatments. The patients cannot usually be subjected to high enough doses of chemicals or radiation to eradicate all the cancer cells as they cause significant damage to the normal cells and other side effects. So, there is an

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urgent need for an alternative therapeutic approach for the selective eradication of cancer cells by the use of disease site directed strategies. The post genomic era has now opened new avenues for better targeted therapies, which is contemplated to be more effective and specific for tumor cells (Gayathri et al. 2008).

Embelin (2,5-dihydroxy-3-undecyl-p-benzoquinone) (Fig. 1), is a natural compound isolated from E. ribes, the medicinal plant used in the Indian system of medicine. Chemical structure of embelin is having quite resemblance with that of natural coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanisms (Kobaisy et al. 2008). More than 4 decades ago, the active component from this plant was isolated and named embelin (Siveen and Kuttan 2001) and later chemically synthesized (Du and Wie 1963). Embelin, 2,5-dihydroxy-3-undecyl-1,4-benzoquinone, has two intra-molecular hydrogen bonds between the quinone and hydroquinone groups present on the same ring. Therefore, it is expected to produce semiquinone radical in the oxidation as well as in the reduction reactions. Reduction reaction of embelin has been found to produce delocalized semiquinone anion radical. On the other hand, oxidation of embelin is expected to produce several transient species. However, the calculations suggested that oxidation of hydroquinone group is the first step, which is followed by transformation into carbon-centered radical.

Even though the compound is showing a wide spectrum of biological activities, there is no study regarding the PDT of embelin in in vitro or in vivo conditions. Embelin is a very stable compound and in methanol shows a peak absorbance ($\lambda_{max}$) at 450 nm (Beena et al. 2010). In vivo cellular uptake, distribution and toxicity assessment of embelin were undertaken before designing the systematic studies. As no previous reports are available on PDT of in vivo experimental cancer models using embelin, we designed this study so as to evaluate the therapeutic effect of embelin as a photosensitizer in solid tumor-induced Swiss albino mice. The uniqueness of the mode of treatment lies in the selective uptake and destruction of cancer cells safeguarding the neighboring normal cells. This area is much more advantageous over the radiation therapy now commonly used in cancer therapy.

Materials and methods

Chemicals

All reagents used were of analytical grade. All chemicals used in the present study were obtained from M/s. Sigma, St. Louis, MO, USA. A 1000 W halogen lamp (Philips PF 811) was used as the light source for the PDT studies.

Isolation of embelin and its purification

About 100 g of the powdered berries of E. ribes were extracted with n-hexane in the solvent ratio 1:15 (w/v). After the complete extraction, the solvent was removed by concentration. The yield of n-hexane extract was found to be 9% in dry material. This extract was subjected to column chromatography over silica gel (100–200 mesh). Elution of the column with benzene yielded an orange color powder. The purification of embelin has been done by the crystallization using diethyl ether which yielded orange plates of pure embelin. The spectral characterization of isolated compound has been done, and was satisfied with the structure of embelin. The purity of embelin has been confirmed by using analytical high pressure liquid chromatography (HPLC) (Beena et al. 2010). About 750 mg of pure embelin was isolated and was used in the present study.

Animal models

The animal models used for the in vivo study (Swiss albino mice-Male, weighing 20–25 g) were from the Departmental Animal House, University of Kerala, Kariavattom Campus, Thiruvananthapuram, Kerala, India. The animals were housed in polypolypropylene cages in rooms maintained at 25 ± 1°C. Mice were fed with standard laboratory diet supplied by Lipton India Ltd. For maintaining the experimental animals, the Institutional Ethical guidelines were absolutely followed as per CPCSEA rules [Sanction No.: IAEC- KU-08/2011-12-BC-AA (20)]. The animals were weighed weekly and the food and water consumption were recorded. The animals were observed regularly for the detection of any lesion and tumor development.

Ehrlich’s ascites carcinoma (EAC)

EAC cells were procured from Amala Cancer Research Centre, Thrissur, Kerala, India were used for the tumor development in mice models. EAC cells were maintained in the peritoneal cavity of mice. Approximately 3 weeks were taken for the development of tumor in the peritoneal cavity and matured cells were aspirated from the peritoneum and used for the in vivo and in vitro experiments.

In vitro photo dynamic therapy on EAC cells

The EAC cells were aspirated from peritoneal cavity of tumor bearing mice, washed three times with ice-cold PBS. The in vitro PDT of embelin on EAC cells was checked for the viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, the cells (1 x 10^6 cells/well) were planted in the 96-well culture plates overnight at 37°C. After that the cells were treated with different concentrations of embelin (2–12 mM). Another set of cells, after treatment with different concentrations of embelin (2–12 mM) were subjected to PDT for 8 h (600 nm, 338 kJ/m², 1000 W). A control set was also maintained which received PDT alone without embelin treatment and EAC cell alone without PDT and embelin treatment. 5-Aminolevulinic acid (5-ALA)
(Sigma Chemical Co., 1 mmol/l) was used as positive control. After 8 h PDT treatment, cell proliferation was recorded by using MTT. MTT reagent was added into each well, incubating for 4 h, then MTT reagent was removed and DMSO was added per well. After shaking for 10 min, optical densities (OD) were determined on a microtiter plate reader at 570 nm. Three independent experiments were done. Proliferation was calculated using the following equation: Proliferation (%)= 1 – (OD control group – OD treatment group)/OD control group × 100%.

Solid tumor induction using EAC cells

The mice were divided into four groups consisting of 6 mice in each group as

**Group I:** Control;
**Group II:** Solid tumor bearing mice;
**Group III:** Solid tumor bearing mice subjected to embelin PDT (animals sacrificed after 2 weeks of light treatment);
**Group IV:** Solid tumor bearing mice subjected to embelin PDT (animals sacrificed after 90 days of light treatment).

EAC cells were used for inducing solid tumor development in mice models. EAC cells were maintained in the peritoneal cavity of mice. Approximately 2 weeks were taken for the development of tumor in the peritoneal cavity of mice and matured cells were aspirated from the peritoneum and used to inoculate for solid tumor development. The solid tumor model was established by subcutaneous injection of EAC cells (1 × 10⁶ cells/animal) into the back right hind limb of mice (Dallacker and Lohnert 1972). Initial and final diameters of the hind limb were noted using Vernier calipers. The mice were subjected to PDT treatment when the tumor volume reached 4.5 ± 1.9 mm³.

The body weight of each animals and papillomas appearing on the shaved area of the skin were recorded at weekly intervals. Tumor promoting activity was evaluated by determining both the proportion of tumor bearing mice and the number of tumors per mouse (Budunova et al. 1999; Moore et al. 1999). Tumor volume and burden were calculated as described by Subapriya and Nagini (2003) using the following formulæ:

Mean Tumor volume = 4/3πr³  (r = mean tumor radius in mm)
Mean Tumor burden = mean tumor volume × mean number of tumors

Photodynamic treatment of tumors

For photodynamic treatment of tumors, we have followed our earlier report (Gayathri et al. 2008). Here, after the completion of the study period (4 weeks), the tumor bearing mice of Groups III and IV, were given an intraperitoneal injection with embelin [dissolved in phosphate buffered saline, pH 7.4] at a concentration of 12.5 mg/kg body weight (the concentration of the dye was fixed according to the results of previous dose dependent study). The mice were kept in dark for 24 h after injection to eliminate any possible tissue damage due to the exposure to sunlight. In Group III, the treatment with light was done after 24 h.

Prior to PDT treatment (of mice kept for 24 h in dark after the intraperitoneal administration of the embelin at a concentration of 12.5 mg/kg body weight, dissolved in PBS), skin except the target tumor was covered with aluminum foil and the mouse was kept in a cooled container. The mouse was restrained in the container using tape, whenever it was necessary. The tumor area was then exposed to visible light from 1000 W halogen lamp, keeping at a distance of 33 cm (the distance was fixed according to our previous reports [data not shown] and in this distance mice showed minimal signs of irritation) for different time intervals, corresponding to the different total light doses required in each case. A stream of air cooled the area under PDT treatment continuously. The total light dose required for the treatment was not delivered at a single stretch. Instead the methods of fractionated light delivery of 2 min each with short-term intervals (dark periods) of 8 min was used (Gayathri et al. 2008). This allows re-oxygenation of tumor tissue during the treatment and thus enhances efficacy of PDT by preventing thermal injury.

Two weeks after the light treatment, mice from Groups I to III were euthanized by sodium pentothal injection. Liver and kidney were collected, homogenized in appropriate buffers and used for various biochemical estimations. Tumor tissues from the back right hind limb of mice were excised. Tumorous tissues for various biochemical studies were immediately frozen at −80 °C. The mice of Group IV that received the treatment were monitored for a period of 90 days and assayed for various enzyme parameters.

**Biochemical parameters**

Various biochemical parameters assayed during this study include myeloperoxidase (MPO) (Desser et al. 1972), β-glucuronidase (Kawai and Anno 1971) and rhodanese (Sorbo 1955). PCR analysis of Bax and Bcl-2 were done to check the therapeutic efficacy of embelin PDT (Saiki et al. 1985).

**Statistical analysis**

The data were statistically analyzed using analysis of variance (ANOVA) and significant difference of means was determined using Duncan’s multiple range test at the level of p < 0.05 (Steel et al. 1997).

**Results**

**In vitro photo dynamic therapy on EAC cells**

No cytotoxicity was observed when the EAC cells were incubated with different concentrations of embelin, without PDT (Fig. 2). PDT alone without embelin also recorded no cytotoxicity. But when the cells were subjected to PDT with the different concentrations of embelin, significant cytotoxicity was observed in a concentration-dependent manner (Fig. 2). PDT with embelin was more superior to 5-ALA, a known photodynamic compound.

Fig. 2. Photodynamic effect of embelin against EAC cells in in vitro condition.
Table 1
Change in body weight of experimental animals.

<table>
<thead>
<tr>
<th>Body weight</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>24.24 ± 0.29</td>
<td>24.55 ± 0.30</td>
<td>23.52 ± 0.32</td>
<td>23.67 ± 0.25</td>
</tr>
<tr>
<td>Final</td>
<td>28.16 ± 0.40</td>
<td>18.242 ± 0.23</td>
<td>21.18 ± 0.34</td>
<td>24.19 ± 0.34</td>
</tr>
</tbody>
</table>

Values are mean of the body weights of six animals per group ± SD.

Tumor induction and PDT treatment

During the whole experimental period, the mice were observed at weekly intervals for changes in body weight and diet and water consumption. The average body weight changes of all the animal groups before and after the treatment are represented in Table 1. A significant decrease was observed in the final body weight of the animals in Groups II at the time of sacrifice. There was a gradual increase in the body weight of the animals after the light treatment than that of tumor induced animals. The body weight of Group IV animals reached almost normal value after 90 days of PDT. No significant changes were observed in the diet and water consumption by the mice in all the groups as compared to the control animals.

Tumor statistics

The effect of embelin PDT on tumor statistics is shown in Table 2. Tumor incidence was found to be 82% and 77% in groups that bear solid tumor. The mean tumor volume in the mice of Group II and III was found to be 522.02 mm³ and 448.90 mm³ respectively at the time of sacrifice. Ninety days after the light treatment, the mean tumor volume was found to be decreased significantly to 101.81 mm³. The mean tumor burden in the animals at 90 days after PDT was also found to be reduced significantly by 80.67%. From these results we can point out the efficacy of embelin PDT as a therapeutic agent against tumor. The tumor profile of animals is given in Table 2. Morphological changes of mice Groups I to III after 2 weeks of PDT and Group IV after 90 days of PDT were shown in Fig. 3.

Biochemical parameters

The therapeutic properties of embelin (2,5-dihydroxy-3-undecyl-p-benzoquinone) are attributed to the efficacy of the treatment in altering the biochemical parameters. Under tumor conditions the level of myeloperoxidase (MPO) (Fig. 4) and β-d-glucuronidase (Fig. 5) were found to be elevated while the level of rhodanese (Fig. 6) was reduced. All these markers show near normal values after the PDT treatment. The expression of Bcl-2 is significantly upregulated in tumor induced mice when compared to normal group (Fig. 7A). The PDT treatment using embelin significantly down regulated the Bcl-2 expression. In the case of Bax, the expression is significantly upregulated in PDT treated mice when compared to tumor-induced mice (Fig. 7B).

Table 2
Effect of PDT on tumor statistics.

<table>
<thead>
<tr>
<th>Tumor mice</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of tumors per mouse</td>
<td>Nil</td>
<td>5.6</td>
<td>5.3</td>
<td>2</td>
</tr>
<tr>
<td>Percentage tumor incidence</td>
<td>Nil</td>
<td>82</td>
<td>77</td>
<td>22</td>
</tr>
<tr>
<td>Mean tumor volume</td>
<td>Nil</td>
<td>522.02</td>
<td>448.90</td>
<td>101.81</td>
</tr>
<tr>
<td>Percentage reduction in mean tumor burden</td>
<td>Nil</td>
<td>Nil</td>
<td>16.2</td>
<td>80.67</td>
</tr>
</tbody>
</table>

Values are mean of the body weights of six animals per group ± SD.

Fig. 3. Photographs of PDT with embelin in mice. Groups I to III taken after 2 weeks of PDT, and Group IV taken after 90 days of PDT.

Fig. 4. Activity of myeloperoxidase. Liver and kidney were excised from mice of different groups, homogenized in 0.1 M citrate phosphate buffer pH 5 and used for the determination of MPO activity. Diaminobenzidine reagent was used for the estimation and optical density was read at 400 nm. The activity was expressed in OD per mg protein. Values expressed as average of six samples ± SD in each group. Different alphabets indicate significant difference at p < 0.05.
parameters were found to be normal. Apoptosis is a mechanism by which organisms initiate cellular death via an orchestrated sequence of cellular events. During cancer progression the apoptotic process is being arrested. Malignant cell types often exhibit an impaired ability to undergo apoptosis, an effect associated with the ability to survive chemotherapy (Vaux and Strasser 1996; Hildeman et al. 1999). In the present study also, apoptosis is found to be inhibited in the tumor induced group as indicated by the enhanced expression of Bcl-2 and lower expression of Bax. When the markers of apoptosis were analyzed after 2 weeks of PDT treatment in mice, it was observed that apoptosis is significantly induced in the treated area. Thus it can be inferred that in the tumor site, apoptosis was induced by embelin PDT mediated oxidative stress, and thus the apoptotic level was brought back to normal when the oxidative stress subsided.

The pattern of tumor markers like MPO, β-d-glucuronidase and rhodanese in our study clearly show that photodynamic therapy using embelin as a photosensitizer certainly possess clinical applications. Our study demonstrated for the first time that embelin, a plant based compound possess strong photodynamic therapeutic effects against solid tumor in 

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References


Du, Y.C., Wie, J.S., 1963. Study of vermifuge. I. Isolation of embelin from the fruit of 


Fig. 5. Activity of β-d-glucuronidase. Tissue homogenates were prepared in aqueous 0.1% Brij 35 solution. The substrate used for the assay was p-nitrophenyl-β-d-glucuronide. Activity was expressed as mg of p-nitrophenyl liberated/min/g protein. Values expressed as average of six samples ± SD in each group. Different alphabets indicate significant difference at p<0.05.

Fig. 6. Activity of rhodanese. The excised tissues were homogenized in a mixture of thioulate and albumin solutions. The supernatants were used as the enzyme source for the assay which involves the reaction with KCN. The unit in which the activity was expressed is μM thiocyanate liberated/min/g protein. Values expressed as average of six samples ± SD in each group. Different alphabets indicate significant difference at p<0.05.

Fig. 7. Expression of Bcl-2 and Bax. Bcl-2[A]: The expression of Bcl-2 is significantly upregulated in tumor induced mice (Group II) when compared to normal group. The PDT treatment (Group III and IV) using embelin significantly downregulated the Bcl-2 expression (Group III and IV). Bax [B]: The expression of Bax is significantly upregulated in PDT treated mice (Group III and IV) when compared to tumor induced mice (Group II) μM.

Discussion

PDT for the treatment of malignant tumors has been proved to be a feasible, safe and reliable method and has many advantages. In the tumor bearing mice, there was a sharp drop in the body weight. Similarly weight loss and tissue wasting are observed in cancer patients (Khan and Tisdale 1999). The increase in the body weight compared to the cancer induced group at 1 week after PDT treatment indicates the counteractive property of the treatment (Khan and Tisdale 1999; Gayathri et al. 2008). This effect of embelin PDT on tumor statistics also points toward the potency of embelin as a therapeutic agent against tumor, by acting as photosensitizer.

Myeloperoxidase, β-d-glucuronidase and rhodanese are commonly studied tumor markers (Gayathri et al. 2008). In the present study, after PDT, the level of the above mentioned marker