Anti-tumor-Promoting Activity of Diterpenes from *Excoecaria agallocha*¹)

Tenji Konishi,a Midori Takasakib, Harukuni Tokuk,b Shu Kyosawac, and Takao Konošimaa

Kyoto Pharmaceutical University,a; Misasagi, Yamashina-Ku, Kyoto 607–8414, Japan and Kyoto Prefectural University of Medicine,a; Kawaramachi-Hirokaji, Kamigyo-ku, Kyoto 602–0841, Japan.

Received March 25, 1998; accepted June 8, 1998

To search for possible anti-tumor-promoters, we carried out the primary screening of seventeen diterpenes (1—17) isolated from the resinus wood of *Excoecaria agallocha* (Euphorbiaceae) using an *in vitro* synergistic assay system. Of these diterpenes, ent-16-hydroxy-3-oxo-13-epi-manoyllose (5), (13R,14R)-ent-8β,13:14,15-diepoxy-13-epi-labda-3β-ol (8) ent-3β-hydroxy-15-beyeren-2-one (10) and ent-15-hydroxy-labda-8(17),13E-dien-3-one (14) exhibited significant inhibitory effects on Epstein-Barr virus (EBV) activation induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Furthermore, 10 exhibited remarkable anti-tumor-promoting activity *in vivo* on a two-stage carcinogenesis test of mouse tumor using 7,12-dimethylbenz(a)-anthracene (DMBA) as an initiator and TPA as a promoter.

**Key words** diterpene; *Excoecaria agallocha*; Euphorbiaceae; anti-tumor-promoter; two-stage carcinogenesis

*Excoecaria* species (Euphorbiaceae) are distributed in tropical Africa and East Asia.² The latex and leaves of *E. agallocha* L. have been used as a dart poison and fish poison in New Caledonia,³ India,⁴ and Malaysia,⁵ and are used in traditional medicine in Thailand.⁶ In Okinawa, Japan, the resinus wood, including the latex of the so-called “Okinawa-jinko” (in Japanese), has been used as a substitute for the incense of agarwood (Jinko).⁷ The piscicidal constituent of the twigs and bark of *E. agallocha* native to Okinawa has been characterized as the daphnane diterpene ester, excoecaritoxin.³ This diterpene ester and some related compounds were also obtained from the latex of *E. agallocha* from Thailand.²⁵ Daphnane diterpene esters are known as skin irritants and tumor promoter substances.²⁵ Recently, Erickson *et al.* have reported a new phorbol ester as an anti-HIV principle which was isolated from the leaves and stems of *E. agallocha* collected in Northwest Australia.⁷ Also, we have previously reported on the isolation and structural elucidation of some new labdane and beyerane-type diterpenes isolated from *E. agallocha* collected in Okinawa.⁸

On the other hand, to search for novel tumor-promoters and/or anti-tumor-promoters from natural resources, we have carried out a primary screening of many kinds of natural products (flavonoids,⁹ euglobals,¹⁰ triterpenoids,¹¹ crude drugs¹²) and kampo prescriptions¹³) by assessing their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). It has also been reported that many compounds which inhibit EBV-EA induced by tumor promoters have been shown to act as inhibitors of tumor promotion *in vivo*, and some natural products which exhibited anti-tumor-promoting activities on a two-stage carcinogenesis test of mouse skin tumor have also exhibited inhibitory effects on the two-stage carcinogenesis test of mouse pulmonary and hepatic tumors.¹²,¹³ In the course of our continuing search for these tumor-promoters or anti-tumor-promoters (cancer chemopreventive agents), the acetone extract of *E. agallocha* collected in Okinawa was examined by an EBV-EA activation test. From the reported data of the existence of daphnane and phorbol diterpenes, the activated effect of the extract on EBV-EA induction was presumed.²,³ However, in our experiments, this acetone extract exhibited no activation of EBV-EA and showed a weak inhibitory effect on EBV-EA induced by TPA.¹⁴ Therefore, in this paper, we report the results of a primary screening test for the inhibitory effects of seventeen diterpenes (1—17) isolated from *E. agallocha* on EBV-EA activation. The result of an *in vivo* two-stage carcinogenesis test on mouse skin tumor promotion with ent-3β-hydroxy-15-beyeren-2-one (10), which was potently active on the inhibition of EBV-EA activation, is also reported.

**MATERIALS AND METHODS**

Isolation of diterpenes  Compounds 1—17, as shown in Chart 1, were isolated from the resinus wood of *Excoecaria agallocha* (Euphorbiaceae) collected in Okinawa, Japan, using column chromatography on silica gel and reversed-phase silica gel (ODS). The details of the isolation and structural elucidation of these compounds have been reported previously.⁸

**Chemicals** The cell culture reagents, n-butryc acid and other reagents, were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA and DMBA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC), used for an immunofluorescence test, was a gift from Prof. H. Hattori, Department of Otorhinolaryngology, Kobe University.

**Cells** The EBV genome carrying lymphoblastoid cells (Raji cells derived from Burkitt’s lymphoma) were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) under the conditions described previously.⁸—¹² Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

**Animals** Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan), and housed in polycarbonate cages in a temperature-controlled room at 24±2°C, and given food and water *ad libitum*.

**In Vitro EBV-EA Activation Experiments** The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer), the EBV genome-carrying human lymphoblastoid cells which were cultivated in 10% FBS RPMI 1640 medium. The indicator cells (Raji, 1×10⁴/ml) were incubated at 37°C for 48 h in 1 ml of medium containing n-bu-
tyric acid (4 mmol, inducer), TPA [32 pmol; 2 μl of 20 ng/ml in dimethylsulfoxide (DMSO)], and various amounts of the test compounds dissolved in 5 μl of DMSO. Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique. The details of the in vitro assay on EBV-EA activation have been reported previously. In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was expressed by making a comparison with that of the positive control experiment (100%) which was carried out with n-butyric acid (4 mmol) plus TPA (32 pmol). In the experiments, the EBV-EA induction was ordinarily at around 35% and these values were taken as the positive control (100%). Four millimolar n-butyric acid alone induced 0.1% EA-positive cells.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas** Each group was composed of 15 mice housed five per cage and given water ad libitum. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) for the initiation treatment. One week after the initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) on the skin. Group I received this TPA treatment alone, and groups II and III received a topical application of each test compound (85 nmol) and glycyrrhetic acid (85 nmol) in acetone (0.1 ml) 1 h before each TPA treatment, respectively. The incidence and numbers of papillomas were observed and detected weekly for 20 weeks; only typical papillomas larger than about 1 mm in diameter were counted.

**RESULTS AND DISCUSSION**

The primary screening test of 1—17 was carried out utilizing a short-term in vitro synergistic assay on EBV-EA activation, and the results are shown in Table 1.

of these compounds, 1, 5, 7, 8, 9, 10 and 14 exhibited remarkable inhibitory effects on EBV-EA activation (100% inhibition of activation at both $1\times10^3$ and $5\times10^2$ mol ratio/TPA) and preserved a high viability of Raji cells, and 3 and 4 exhibited inhibitory effects (100% and more than 80% inhibition at $1\times10^2$ and $5\times10^2$ mol ratio/TPA, respectively). In our experiments, the inhibitory activities of these compounds are stronger than those of glycyrrhetic acid and retinoic acid, which are known to be strong anti-tumor-promoters. Especially, ent-3β-hydroxy-15-beyerene-2-one (10) exhibited the most significant inhibitory effects on EBV-EA activation (more than 85 and 37% inhibition of activation even at $1\times10^2$ and $1\times10$ mol ratio/TPA). Further, the EBV-EA activation was not shown by the single treatment with compound 10 at 32 nmol/ml. These results in vitro strongly suggested that compound 10 might be a valuable anti-tumor-promoter. The inhibitory effects of 10 on the two-stage carcinogenesis test of mouse skin papillomas induced by
DMBA as an initiator and TPA as a promoter were therefore investigated. The activities, evaluated by both the rate (%) of papilloma-bearing mice and the average number of papillomas per mouse were compared with both those of the positive control group and the group treated with glycyrhetic acid.

On the positive control, 80 and 100% of mice bore papillomas at 8 and 9 weeks of promotion, respectively. Further, more than 4 and 9 papillomas were formed per mouse at 10 and 20 weeks of promotion, respectively, as shown in Fig. 1. In the group treated with glycyrhetic acid, about 43 and 95% of mice bore papillomas at 10 and 20 weeks of promotion, respectively. Furthermore, about 5 and 6 papillomas were formed per mouse at 15 and 20 weeks of promotion, respectively.

When 10 was applied before each TPA treatment, the formation of papillomas in mouse skin was significantly delayed as follows. In the group treated with 10, only about 20, 60 and 70% of mice bore papillomas even at 10, 15 and 20 weeks of promotion, respectively. Also, 10 reduced the number of papillomas per mouse as follows. In the case of 10, less than about 1 and 3.5 papillomas were formed per mouse after 10 and 15 weeks of promotion, respectively, and only about 4.5 papillomas were formed per mouse even after 20 weeks of promotion. Therefore, diterpenes 10 exhibited more than 50% inhibition, even at 20 weeks of promotion, as shown in Fig 1B, and the inhibitory effect of 10 on the two-stage carcinogenesis of mouse skin tumor were apparently more potent than those of glycyrhetic acid.

The results of this study strongly suggest that ent-3β-hydroxy-15-beyer-en-2-one (10) isolated from E. agallocha could be a valuable anti-tumor-promoter (cancer chemopreventive agent) in chemical carcinogenesis. Further studies on the details of the mechanism of anti-tumor-promoting activity and the inhibitory effects of 10 on pulmonary and hepatic carcinogenesis by oral administration are now underway.

REFERENCES AND NOTES

1) A part of this work was presented at the 118th Annual Meeting of the Japan Pharmaceutical Association, Kyoto, March, 1998, Abstracts Papers, p. 122.
8) Konishi T., Azuma M., Itoh R., Kiyosawa S., Fujimura Y., Shimada

---

**Table 1. Percentages of EBV-EA Induction in the Presence of Diterpenes (1—17) with Respect to Positive Control (100%)**

<table>
<thead>
<tr>
<th>Samples</th>
<th>1×10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>5×10&lt;sup&gt;2&lt;/sup&gt;</th>
<th>1×10&lt;sup&gt;2&lt;/sup&gt;</th>
<th>1×10&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>0.00 (60)</td>
<td>0.00 (60)</td>
<td>63.8 (80)</td>
<td>92.5 (80)</td>
</tr>
<tr>
<td>Compound 2</td>
<td>13.5 (70)</td>
<td>31.8 (80)</td>
<td>57.5 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 3</td>
<td>0.00 (60)</td>
<td>14.7 (80)</td>
<td>57.0 (80)</td>
<td>87.2 (80)</td>
</tr>
<tr>
<td>Compound 4</td>
<td>0.00 (50)</td>
<td>17.7 (80)</td>
<td>33.6 (80)</td>
<td>67.0 (80)</td>
</tr>
<tr>
<td>Compound 5</td>
<td>0.00 (70)</td>
<td>0.00 (80)</td>
<td>31.7 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 6</td>
<td>7.3 (40)</td>
<td>51.3 (60)</td>
<td>82.9 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 7</td>
<td>0.00 (60)</td>
<td>0.00 (80)</td>
<td>54.2 (80)</td>
<td>86.9 (80)</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.00 (70)</td>
<td>0.00 (80)</td>
<td>39.9 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 9</td>
<td>0.00 (70)</td>
<td>0.00 (80)</td>
<td>83.6 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 10</td>
<td>0.00 (60)</td>
<td>0.00 (80)</td>
<td>14.5 (80)</td>
<td>62.7 (80)</td>
</tr>
<tr>
<td>Compound 11</td>
<td>14.8 (60)</td>
<td>39.0 (80)</td>
<td>71.5 (80)</td>
<td>94.6 (80)</td>
</tr>
<tr>
<td>Compound 12</td>
<td>0.00 (60)</td>
<td>24.2 (80)</td>
<td>65.1 (80)</td>
<td>88.3 (80)</td>
</tr>
<tr>
<td>Compound 13</td>
<td>0.00 (70)</td>
<td>22.6 (80)</td>
<td>72.0 (80)</td>
<td>93.7 (80)</td>
</tr>
<tr>
<td>Compound 14</td>
<td>0.00 (70)</td>
<td>0.00 (80)</td>
<td>39.6 (80)</td>
<td>88.0 (80)</td>
</tr>
<tr>
<td>Compound 15</td>
<td>0.00 (70)</td>
<td>34.7 (80)</td>
<td>66.5 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 16</td>
<td>26.7 (70)</td>
<td>54.9 (80)</td>
<td>78.1 (80)</td>
<td>100.0 (80)</td>
</tr>
<tr>
<td>Compound 17</td>
<td>0.00 (70)</td>
<td>27.5 (80)</td>
<td>66.3 (80)</td>
<td>87.5 (80)</td>
</tr>
</tbody>
</table>

---

**Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of 10 and Glycyrhetic Acid**

All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas, B: average number of papillomas per mouse.

- Control TPA alone; C, TPA + 85 nmol of 10; D, TPA + 85 nmol of glycyrhetic acid. At 10 and 15 weeks of promotion, the group treated with compound 10 was significantly different from the positive control group (p<0.05) in terms of papilloma bearers (%)(n=15), and at 10, 15 and 20 weeks of promotion, the group treated with compound 10 was different from the control group (p<0.05) in terms of papillomas per mouse (n=15 and at 20 weeks promotion, positive control group; 9.1±1.1, the group treated with glycyrhetic acid: 5.8±0.9, the group treated with 10: 4.3±0.4).


14) The activation percentages of the acetone extract of E. gallocha on EBV-EA were only 2.9% and 0% at the concentrations of 100 µg/ml and 10 µg/ml, respectively. And, the inhibitory percentages of this extract on EBV-EA activation were 89.6% and 19.9% at 100 µg/ml and 10 µg/ml, respectively.


16) A high viability of Raji cells is necessary for in vitro assay using an indirect immunofluorescence technique by antigen-antibody reaction and is beneficial for the following in vivo assay.


18) The relative ratio of EBV-EA activation with respect to the positive control (100%) in presence of glycyrrhetic acid was 15.6, 54.3, 100 and 100% at 1×10^3, 5×10^3, 1×10^5 and 1×10 mol ratio/TPA, respectively, and the viability percentage of Raji cells was more than 80% at each concentration; Mizutani K., Kuramoto T., Tamura K., Kozuka M., Tokuda H., Abstracts of Papers, the 113th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, March 1993, Part II, p. 206.