Ichthyotoxic and Anticarcinogenic Effects of Triterpenoids from *Sandoricum koetjape* Bark

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After bioassay-guided fractionation of the extract from *Sandoricum koetjape* bark, which exhibited significant toxicity to killifish (*Oryzias latipes*), two ichthyotoxic triterpenoids were isolated and characterized as koetjapic acid and 3-oxo-olean-12-en-29-oic acid. These constituents, along with non-toxic katiconic acid, had a remarkable inhibitory effect on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which is a preliminary *in vitro* screening method for possible anti-tumor-promoting agents. Of the triterpenoids active *in vitro*, koetjapic acid appears to be a promising cancer chemopreventive agent, since it significantly delayed tumor promotion in two-stage mouse skin carcinogenesis induced by 7,12-dimethylbenz(a)anthracene and promoted by TPA.

Key words ichthyotoxic activity; Epstein-Barr virus early antigen (EBV-EA); koetjapic acid; *Sandoricum koetjape*; cancer chemoprevention

Naturally occurring ichthyotoxic substances often have a variety of other biological activities, including insecticidal,¹⁾ anti-tumor,²⁾ tumor-promoting,³⁾ and anti-ulcer activities.⁴⁾ Many have been reported to be cytotoxic against tumor cell lines.^{2,5)} Therefore, the ichthyotoxic assay is a preliminary screening method used to search for new biologically active compounds in nature.⁵⁾ In our ongoing survey of bioactive substances using the ichthyotoxic assay with killifish (*Oryzias latipes*; Japanese name, medaka), a hexane extract prepared from the bark of *Sandoricum koetjape* (BURM. f.) MERR. (syn=*Sandoricum indica* CAV.) was found to show remarkable ichthyotoxic activity. *S. koetjape* is a large tree species belonging to the Meliaceae that grows in India, Malaysia, and other tropical countries in Asia. Its bark is used as a folk medicine for treating colic and leucorrhea.⁶⁾ Phytochemical studies of this plant have previously been reported; triterpenoids and limonoids have been isolated from its heartwood, bark, and seeds,^{7–10)} and recently, new trijugin-type limonoids, sandrapins A, B, and C, were obtained from its leaves.¹¹⁾ Bioassay-guided fractionation of the hexane extract led to the isolation of three triterpenoids (1–3). In the hope of exploring their function as chemopreventive agents in chemical carcinogenesis, their *in vitro* and *in vivo* anti-tumor-promoting effects were evaluated. Here, we describe the characterization of the ichthyotoxic components of the bark extract and their possible cancer chemopreventive effect.

MATERIALS AND METHODS

Isolation and Characterization of Ichthyotoxic Compounds An aqueous acetone extract of dried bark (240 g) purchased in Malaysia was divided into hexane-, EtOAc-, and water-soluble portions. The hexane-soluble portion that was ichthyotoxic to medaka (lethal dose 40 ppm after 1 h) was subjected to column chromatography on silica gel with hexane–EtOAc, with increasing amounts of EtOAc by monitoring the toxicity to medaka. Compounds 1–3 isolated as crystals were identified as koetjapic acid (1) (mp 295–298 °C, 197.6 mg), 3-oxo-olean-12-en-29-oic acid (2) (mp

253–256 °C, 26.0 mg), and katiconic acid (3) (mp 258–260 °C, 85.6 mg), by comparing their spectral data with reported values.⁷⁾

Chemicals and Test Compounds The cell culture reagents, *n*-butyric acid, and other reagents used in the bioassay were purchased from Nacalai Tesque, Inc. (Japan). TPA and 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Epstein-Barr virus early antigen (EBV-EA)-positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University, Japan.

Ichthyotoxic Assay A test solution was prepared by adding an acetone solution (0.5 ml) of the test compounds at known concentrations to aerated water (100 ml). The median tolerance limit (TL_m) after 24 h was evaluated for five fish/group by straight-line graphical interpolation. A control experiment was carried out under the same conditions, adding acetone (0.5 ml) alone.

Short-Term *in Vitro* Bioassay Using the Inhibition of EBV-EA Activation The inhibition of EBV-EA activation was assayed according to the method previously reported.¹²⁾ Raji cells were incubated at 37 °C for 48 h in a medium (1 ml) containing *n*-butyric acid (4 mM), TPA [20 ng (32 pM) in DMSO 2 μ l] as an inducer, and a known amount of test compound in 5 μ l DMSO. Smears were made from the cell suspensions, and the activated cells that were stained with EBV-EA-positive serum were detected using an indirect immunofluorescence technique.¹³⁾ In each assay, at least 500 cells were counted, and the number of stained cells was

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recorded. Triplicate assays were carried out for each compound. The average EBV-EA induction of the test compound was expressed as a relative ratio to a positive control (100%), with *n*-butyric acid plus TPA in which EA induction was ordinarily 35%.

Two-Stage Carcinogenesis Test on Mouse Skin The animals (6-week-old female ICR mice) were housed five per polycarbonate cage in a temperature-controlled room at $24 \pm 2^\circ\text{C}$, and given water and food *ad libitum* throughout the experiment. The animals were divided into three groups of 15 mice each. The assay was performed as previously described.¹⁴ Namely, the back of each mouse was shaved with surgical clippers one day before initiation, and the mice were topically treated with DMBA (100 μg , 390 nm) in acetone (0.1 ml) for initiation. One week after initiation, papilloma formation was promoted by applying TPA (1 μg , 1.7 nmol) in acetone (0.1 ml) to the skin twice weekly. One hour before each TPA treatment, the mice were treated with the sample (85 nmol) in acetone (0.1 ml). The incidence of papilloma was examined weekly over 20 weeks.

RESULTS AND DISCUSSION

Koetjapic acid (**1**) (TL_m 1.8 ppm after 24 h) and 3-oxo-olean-12-en-29-oic acid (**2**) (TL_m 1.9 ppm) showed ichthy-

otoxic activity, while katonic acid (**3**) was non-toxic to killifish. The activities of **1** and **2** were comparable to previously reported values for piscicides, buddlelin A (TL_m 1.2 ppm),¹⁵ and aspidins (TL_m 1.2–1.4 ppm).¹² The ichthyotoxic compounds **1** and **2** were previously reported to exhibit significant cytotoxicity toward cultured tumor cell lines⁷ and to inhibit DNA polymerase β .¹⁶ Our study provided additional evidence of the effectiveness of the ichthyotoxic assay in the search for bioactive natural products, although relationships between the ichthyotoxic activity and the other activities are not clear. The remarkable anti-tumor promoting effect of aspidin BB¹² prompted us to examine the anti-tumor effects of compounds **1** and **2** *in vitro* and *in vivo*.

First, the inhibitory effects of **1**–**3** on EBV-EA activation induced by TPA, which is a short-term *in vitro* screening method frequently used to survey possible anti-tumor promoters in nature, were assessed. As shown in Table 1, compounds **1**, **2**, and **3** inhibited EBV-EA activation by 65.4, 70.1, and 64%, respectively, at a concentration of 500 mol ratio/TPA, preserving high viability of the cells. These potencies were comparable to those reported for oleanolic acid¹⁷ and the well-known anti-tumor promoting agent EGCG^{12,18} (Table 1). Although many types of triterpenoids such as taraxastane-, multiflorane-, cucurbitane-, and oleanane-type have been reported to show an inhibitory effect on EBV-EA

Table 1. Relative EBV-EA Activation Caused by Hexane Extract and Triterpenoids of *S. koetjape* Relative to the Positive Control (100%)

	Relative EBV-EA activation ^{a)}			
	100 μg	10 μg	1 μg	
Bark (Hexane extract)	8.4 $\pm$ 0.6 (60) ^{b)}	44.9 $\pm$ 1.4 (>80)	91.7 $\pm$ 0.4 (>80)	
	Concentration (mol ratio/TPA) ^{c)}			
	1000	500	100	10
Compound				
1	6.5 $\pm$ 0.4 (70)	34.6 $\pm$ 1.4 (>80)	63.8 $\pm$ 1.9 (>80)	96.5 $\pm$ 0.6 (>80)
2	0 $\pm$ 0.3 (70)	29.9 $\pm$ 1.4 (>80)	69.2 $\pm$ 1.8 (>80)	89.3 $\pm$ 0.7 (>80)
3	4.1 $\pm$ 0.4 (70)	36.0 $\pm$ 1.5 (>80)	72.6 $\pm$ 2.0 (>80)	92.4 $\pm$ 0.4 (>80)
Reference				
EGCG ¹²⁾	6.4 $\pm$ 0.3 (70)	34.9 $\pm$ 1.3 (>80)	68.1 $\pm$ 1.7 (>80)	87.7 $\pm$ 0.3 (>80)
Oleanolic acid ¹⁷⁾	12.7 $\pm$ 0.5 (>70)	30.0 $\pm$ 2.0 (>80)	80.0 $\pm$ 1.4 (>80)	100 $\pm$ 0.1 (>80)

a) The values are percentages relative to the positive control (100%). b) The values in parentheses are the percentage viability of Raji cells, which was assayed by the Trypan Blue staining method. c) The TPA concentration was 20 ng (32 pmol).

Fig. 1. Suppressive Effect of Koetjapic Acid (**1**) on Two-Stage Carcinogenesis on Mouse Skin

(A) Percentage of mice with papillomas. (B) Average number of papillomas per mouse. ●, control (DMBA/TPA alone); □, DMBA/TPA + **1** (85 nmol).

activation,^{17,19–22)} the effect of the oleanane-type related triterpenoids,^{17,20–22)} including compounds **1** and **2**, appears to be more potent than that of the multiflorane- and cucurbitane-type tripterpenoids.^{19,22)}

Since the inhibitory effects on EBV-EA activation induced by TPA have been documented to correlate well with *in vivo* anti-tumor promoting activity,^{15,17,20)} the *in vivo* effect of **1**, which is the most abundant ichthyotoxic substance, was then estimated using the two-stage carcinogenesis bioassay on mouse skin using DMBA as an initiator and TPA as a promoter. As seen in Fig. 1A, all the control animals had papillomas 9 weeks after promotion, whereas treatment with **1** reduced the percentage of mice with tumors to 13.3% at 9 weeks, 60% at 15 weeks, and 80% at 20 weeks. In the experimental groups, the average number of papillomas per mouse was also reduced to 18, 27, and 41% relative to the control group at 9, 15 and 20 weeks, respectively (Fig. 1B). Koetjapic acid (**1**) significantly delayed two-stage carcinogenesis on mouse-skin and was more effective than glycyrrhetic acid, an anti-tumor promoter reported from the Chinese drug licorice.²³⁾

Although the anti-tumor promoting mechanism of **1** is not yet clear, *S. koetjape* bark should be further studied as a source for possible cancer chemopreventive agents.

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