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ORIGINAL PAPER

Anticarcinogenic compounds in the Uzbek medicinal plant, *Helichrysum maracandicum*

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Abstract An ethanol extract of *Helichrysum maracandicum* showed antiproliferative activity against cultured cells of SENCAR mouse in an in vitro assay, and activity-guided fractionation of the extract resulted in the isolation of isosalipurposide as an active substance. Naringenin chalcone, the aglycone of isosalipurposide, also showed strong antiproliferative activity. An in vivo assay of two-stage carcinogenesis on mouse skin revealed that epidermal application of isosalipurposide resulted in delayed formation of papillomas. Western blot analysis showed that the expression of p38 mitogen-activated protein kinase was suppressed by the administration of naringenin chalcone or isosalipurposide, which might be related to the anticarcinogenic activity.

Keywords *Helichrysum maracandicum* · Isosalipurposide · Anticarcinogenic activity · Naringenin chalcone · Two-stage carcinogenesis

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Introduction

Carcinogenic processes include two or more stages such as initiation and promotion. Prevention of the promotion stage has been a major target for the investigation on anticarcinogenic natural products [1], and an in vivo two-stage carcinogenesis test using the SENCAR mouse had been established as a model screening system; the assay starts with treatment of SENCAR mouse skin with 12-dimethylbenz[*a*]anthracene (DMBA) as an initiator followed by promoter treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) after a specific period of time [2]. In the process of carcinogenesis, it is known that some genes of growth-related signal transduction pathways are activated and some others are silenced. In particular, transcription factors such as activator protein 1 (AP1) and nuclear factor kappa B (NFκB) are involved in MAP kinase signaling pathways and are activated by tumor promoters [3].

In our in vitro screening of medicinal plants collected in Uzbekistan for antiproliferative activity against SENCAR mouse skin transformed cells (SST cell) and SENCAR mouse skin transformed tumor cells (SST-T cell), an extract of *Helichrysum maracandicum*, one of the original plants of a folk medicine helichrysum, showed strong activity and was studied for its active compound.

Helichrysum (*Helichrysum* sp., Compositae) is a common folk medicine for gall bladder disorders [4, 5] from

Turkey to Central Asia. Extracts of helichrysum were reported to contain flavonoids, phloroglucinol derivatives and diterpenes [4, 6–8] and to show choleretic [5], antimicrobial [9], antioxidative [10], antiinflammatory [11], diuretic and antihypertensive activities [12]. In this report, we show that a chalcone glycoside derived from *H. maracandicum*, as well as its aglycone, showed the antiproliferative activity.

Materials and methods

Plant materials

Aerial parts of *Allium motor* R. KAM. et LEVICHEV (Liliaceae) (no. ESM-C03051), leaves of *Betula tianschanica* RUPR. (Betulaceae) (no. ESM-C03054), whole plant of *Dracocephalum komarovi* LIPSKY (Labiatae) (no. ESM-C03055 and ESM-C03056), flowers of *H. maracandicum* M. POP. ex KIRP. (Compositae) (no. ESM-C03052), aerial parts of *Paeonia hybrida* PALL. (Paeoniaceae) (no. ESM-C03053) and fruits of *Rhamnus cathartica* L. (Rhamnaceae) (no. ESM-03057) were collected in Uzbekistan in 2003. Voucher specimens are deposited at the Graduate School of Pharmaceutical Sciences, Kyoto University. The ethanol extracts of these plants were used in the in vitro bioassay described below.

Cell culture and short-term in vitro bioassay for cell proliferation

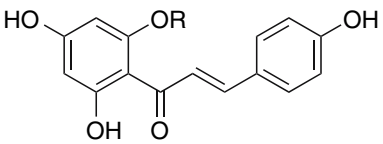
SENCAR mouse skin transformed (SST) cells are a cultured cell line of normal skin epithelium cells of SENCAR mouse. SENCAR mouse skin transformed tumor (SST-T) cells are a cell line made by applying peroxynitrite [13] to SST cells. The cells were incubated in 4-cm diameter dishes (approx. 5×10^5 cells/dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 35°C in an atmosphere of 5% CO_2/95% air. Plant extracts dissolved in 10 µl dimethylsulfoxide (DMSO) were added to the dishes. Ten microliters of DMSO and quercetin dissolved in DMSO were used as negative and positive controls, respectively. Cells were observed under a microscope 24 h after the sample addition, and the number of viable cells determined by a trypan blue exclusion test was counted with a cytometer.

Two-stage mouse skin carcinogenesis test

Fifteen SENCAR mice (female, 6 weeks old) were used for each test compound. Mice were housed in a polycarbonate cage in an SPF room with free access to food and water throughout the experiment. Back skin of the mouse was shaved 1 day before DMBA treatment, and 100 µg of DMBA (390 nmol) in 100 µl acetone was applied topically to the shaved back as an initiator. One week after the initiation treatment, a sample solution (50 µg/100 µl acetone) was applied to the initiated area, which was followed by an administration of TPA (1 µg, 1.7 nmol) in 100 µl acetone an hour later as a promoter treatment. Thereafter, a set of sample and TPA treatments was repeated twice a week for 20 weeks. The number of papillomas was counted weekly for 20 weeks. All animal experiments were performed according to the guidelines for animal experimentations of Kyoto Prefectural University of Medicine.

Isolation of isosalipurposide (**1**) and preparation of naringenin chalcone (**2**)

Flowers of *H. maracandicum* (1.4 kg) were extracted with ethanol overnight at room temperature three times to give an ethanol extract (111 g). Ten grams of the extract was dissolved in 30% MeOH in water and was extracted sequentially with hexane and then AcOEt to give hexane-soluble (1.2 g) and AcOEt-soluble (4.3 g) fractions. The aqueous layer was concentrated to dryness to give a water-soluble fraction (3.0 g). The AcOEt-soluble fraction was subjected to silica gel column chromatography with $CHCl_3$–MeOH (4:1) to give three fractions, fr-1 (0.56 g), fr-2 (2.99 g) and fr-3 (0.31 g). Three hundred milligrams of fr-2 was further fractionated by column chromatography with the same conditions as above to afford isosalipurposide (**1**) (11 mg). The compound was identified by comparing NMR data with those previously reported [14] and measuring HMQC and HMBC spectra. Naringenin chalcone (**2**) was prepared from naringenin according to the method of Le Bail [15].



R = β-D-Glc; isosalipurposide (**1**)
R = H; naringenin chalcone (**2**)

Western blot analysis

Cultured cells were washed with phosphate-buffered saline (PBS) and then lysed and homogenized in CelLytic M cell lysis buffer (Sigma). Mouse skin epithelia were collected under ice-cold conditions 1–10 days after applying 1 mg of sample in 100 µl acetone to partially shaved back skin of SENCAR mouse and homogenized in CelLytic MT cell

lysis buffer. Cytosolic fractions of the cultured cells and the epithelial cells were collected as the supernatant after centrifugation at 10,000 *g* for 5 min at 4°C. SDS-PAGE was performed on 4–20% gradient polyacrylamide gels and aliquots of the cytosolic fractions diluted with lysis buffer were loaded. Separated proteins were transferred to Immobilon transfer membranes (Millipore). After blocking with 5% low fat milk in PBS, membranes were incubated for 1–3 days at 4°C in PBS containing 3% bovine serum albumin (BSA) and H-Ras, Raf-1, MEK-2 or p38 MAP kinase antibodies (Santa Cruz), followed by 30–40 min incubation with peroxidase-labeled second antibodies (Amersham). The signals were detected using ECL Chemi-Lumi One (Nacalai Tesque) according to the manufacturer's instructions.

Results and discussion

Ethanol extracts of six species of medicinal plants collected in Uzbekistan were tested for inhibitory activity against cell proliferation (Table 1). The extract of *H. maracandicum* was found to be the most effective among them especially on SST cells. The stronger activity of the extract on SST cells compared to SST-T cells might suggest that its activity was anticarcinogenic rather than antitumoral. Fractionation of the ethanol extract into hexane-, AcOEt- and water-soluble portions revealed that the activity was soluble in organic solvents. Treatment of SST cells with the hexane-soluble fraction caused vacuolization and swelling of the cytoplasm, whereas treatment with the ethanol extract caused nucleic and cytoplasmic metamorphosis (Fig. 1). The hexane-soluble fraction was separated by column chromatography into several fractions; however, every fraction showed similar activity. This suggests that necrosis-like cellular vacuolization was due to compounds commonly distributed among the separated fractions. Antiproliferation activity-guided fractionation of the AcOEt-soluble fraction resulted in isolation of a chalcone glycoside, isosalipurposide (**1**), as an active compound. Generally, glycosides are hydrolyzed into aglycones and sugars in the gastrointestinal tract after oral administration. Naringenin chalcone (**2**), the aglycone of **1**, was prepared. Both **1** and **2** showed stronger inhibitory effects than quercetin [16] to SST cells (Table 1).

Table 1 IC_{50} of EtOH extracts of Uzbek plants, isolated compounds and quercetin (positive control) against SST and SST-T cells

	SST (μg/ml)	SST-T (μg/ml)
EtOH extracts		
Dracocephalum komarovi	>100	>100
Allium motor	>100	>100
Betula tianschanica	>100	>100
Helichrysum maracandicum	25	62
Paeonia hybrida	>100	>100
Rhamnus cathartica	>100	>100
Isosalipurposide (**1**)	138	115
Naringenin chalcone (**2**)	92	184
Quercetin	248	331

In vivo activity of the fractions and compounds was examined using a two-stage mouse skin carcinogenesis test. Papillomas were observed on all mice in the control group 10 weeks after TPA treatment (Fig. 2a). Administration of the ethanol extract, **1** or **2**, reduced the percentage of mice with papillomas to 0, 20 and 11% at 10 weeks and 60, 80 and 67% at 20 weeks, respectively. Anticarcinogenic activity was also demonstrated by counting the number of papilloma per mouse (Fig. 2b). At 20 weeks, the average numbers of papillomas per mouse treated with the ethanol extract, **1** or **2** were less than that of the control group at 11, 44 and 22%, respectively. However, the hexane-soluble fraction showed no anticarcinogenic activity.

In order to understand the mechanism of their tumor preventive activity, the expression of some proteins related to the MAP kinase pathway was analyzed. The MAP kinase pathway is suggested to be involved in carcinogenesis [3], and its related proteins, namely, H-Ras, Raf-1, MEK-2 and p38 MAP kinase, were observed for their expression in SST and SST-T cells treated with the ethanol extract of *H. maracandicum*. It was found that p38 MAP kinase was suppressed in a time-dependent manner in SST cells, but not in SST-T cells (Fig. 3a). Expression of the kinase was also suppressed by the ethanol extract, **1** and **2** in two-stage mouse skin carcinogenesis tests, but not by the hexane-soluble fraction (Fig. 3b). These results were consistent with the antiproliferative activity on SST cells and the decrease in the percentage of mice with papillomas and

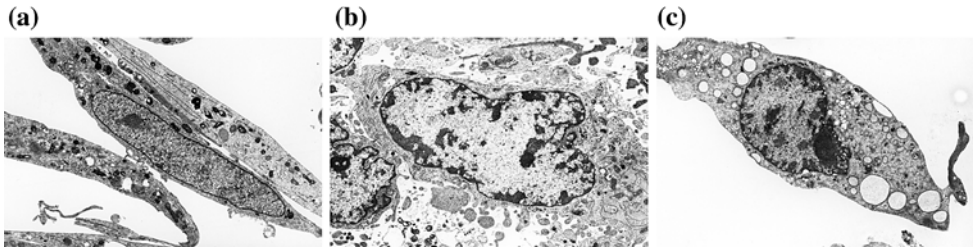


Fig. 1 Electron microscopic pictures of SST cells incubated with **a** negative control, **b** ethanol extract (100 μg/ml) and **c** hexane-soluble fraction of *H. maracandicum* (100 μg/ml; 2,000×; 15 h after sample addition)

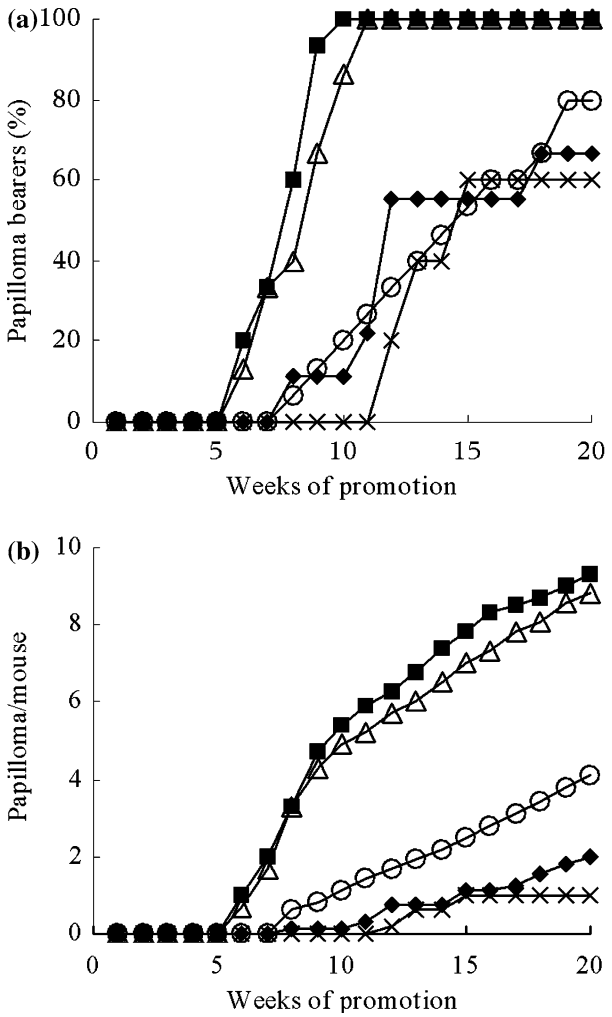


Fig. 2 Inhibitory effects of *H. maracandicum* extracts and test compounds on TPA-induced mouse skin carcinogenesis. **a** Percentage of mice with papillomas; **b** average number of papillomas per mouse ($n = 15$ for each group) [*filled square*, control, TPA alone; *cross symbol*, TPA + ethanol extract (50 μg); *open triangle*, TPA + hexane-soluble fraction (50 μg); *open circle*, TPA + isosalipurposide (**1**) (50 μg, 115 nmol); *filled diamond*, TPA + naringenin chalcone (**2**) (50 μg, 183 nmol)]

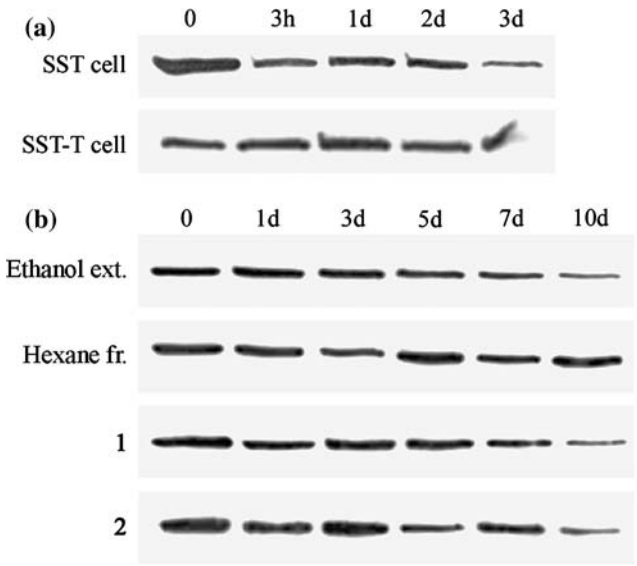


Fig. 3 p38 MAP kinase protein expression. **a** SST and SST-T cells after administration of ethanol extract of *H. maracandicum* (100 μg/ml); **b** SENCAR mouse skin after administration of the ethanol extract (1.0 mg), hexane-soluble fraction (1.0 mg), isosalipurposide (**1**) (1.0 mg, 2.30 μmol), or naringenin chalcone (**2**) (1.0 mg, 3.67 μmol)

average number of papillomas per mouse, suggesting that the suppression of p38 MAP kinase expression was involved in the prevention of tumorigenesis.

The present study elucidated that compound **1** is the active anticarcinogenic component in the ethanol extract of *H. maracandicum*. Compound **2**, the aglycone of **1**, was also shown to be an effective compound; however, the efficacy of these isolated compounds (50 μg) was less than that of the original ethanol extract (50 μg) in two-stage mouse skin carcinogenesis tests. There may be other potent components and/or synergistic effect among constituents in the extract.

Compounds **1** and **2** were reported to have antioxidative activity [17, 18]. Antioxidation would play a key role in the prevention of cancers, since reactive oxygen species cause cell damage, which can be followed by carcinogenesis. Antioxidants have been shown to attenuate MAP kinase signals [19, 20], and the ethanol extract of *H. maracandicum* as well as compounds **1** and **2** were shown to suppress the expression of p38 MAP kinase. Inhibition of p38 MAP kinase is known to reduce cyclooxygenase-2 (COX-2) gene expression [21], and inhibition of COX-2 is assumed to be important in cancer prevention [21, 22] as well as in antiinflammation. These factors may be involved with the anticarcinogenic effect of the ethanol extract and compounds **1** and **2**.

In this study, we revealed the anticarcinogenic compounds of *H. maracandicum*, one of the original plants of helichrysum that have been used across a wide region of Eurasia as a common tea herb for cholagogue [4, 5]. Traditional folk medicines have high potential as resources in the development of new drugs. Modern scientific techniques can clarify the active components of folk medicines and give explanations for their usefulness, although the active compounds may not have novel structures. We hope our results here can serve as an example of these cases.

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