

Evaporated fraction of thujopsene from *Thujopsis dolabrata* starves cancer cells via PKM2 Anti-tumor effect of the volatile components of *Thujopsis dolabrata*

Takuya Nagata (✉ takuya.nagata@med.toho-u.ac.jp)

Toyo University Medical Center Ohashi Hospital: Toho Daigaku Iryo Center Ohashi Byoin

<https://orcid.org/0000-0003-2437-646X>

Tadaaki Satou

International University of Health and Welfare: Kokusai Iryo Fukushi Daigaku

Aya Sasaki

Toyo University Medical Center Ohashi Hospital: Toho Daigaku Iryo Center Ohashi Byoin

Manabu Watanabe

Toho University Medical Center Ohashi Hospital: Toho Daigaku Iryo Center Ohashi Byoin

Yoshihisa Saida

Toyo University Medical Center Ohashi Hospital: Toho Daigaku Iryo Center Ohashi Byoin

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Abstract

Objective

Many cancer patients need for new treatment options with lower side effects. Herein, we report on the antitumor effect of thujopsene derived from the volatile components of *Thujopsis dolabrata*(asunaro).

Methods

The antitumor effect of the asunaro essential oil was analyzed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay and in vivo cancer metastasis model. Thujopsene was isolated from asunaro essential oil by liquid chromatography, and the tumor growth inhibitory mechanism of thujopsene was assessed using Western blotting and DARTS (Drug affinity responsive target stability) analysis.

Results

The volatile components of asunaro essential oil exhibited an antitumor effect on MCF7 and SKBR3 breast cancer cells as well as on MKN45 gastric and DLD1 colon cancer cells. Thujopsene was identified as an antitumor factor that tended to have a stronger tumor growth inhibitory effect. Pyruvate kinase M2 (PKM2) was found to be associated with thujopsene in cancer cells. The reaction of thujopsene with MKN45 cells reduced intracellular lactate production. These results indicate that thujopsene binds to PKM2 in cancer cells and inhibits the nutritional metabolic pathway, and causing apoptosis. In conclusion, thujopsene may suppress tumor growth and metastasis by inhibiting the trophic metabolism pathway of cancer cells.

Introduction

Cancer is the leading cause of death in Japan, and research has shown that it is diagnosed in one of every two people [1]. Today's multidisciplinary treatments for cancer are adequately combined with many diagnostic procedures and treatments, such as surgery, anticancer drugs, molecular targeted drugs, radiation therapy, genomic diagnosis, and immune checkpoint inhibitors that contribute to prolonging the prognosis of patients. In addition, genomic diagnosis has allowed for the selection and administration of highly effective anticancer drugs and molecular targeted drugs. However, anticancer drugs have various side effects, including hair loss, malaise, nausea, diarrhea, constipation, and febrile neutropenia, among others. Moreover, immune checkpoint inhibitors may also have life-threatening side effects, such as interstitial pneumonia and renal dysfunction [2], thereby requiring their careful administration.

In recent years, cancer treatment in geriatric patients has become a major problem. In general, compared with young people, older people have lower renal, cardiac, and respiratory functions. In addition, they often have various underlying diseases, such as hypertension and diabetes that render them at high risk for anticancer drugs. For these reasons, setting the type and dose of the anticancer drug to be used in a

geriatric patient can be difficult. Fortunately, trastuzumab, a molecular targeted drug for HER2 (human epidermal growth factor receptor 2)-positive breast cancer, has almost negligible side effects, such as hair loss and diarrhea, and is thus considered a relatively safe drug for geriatric patients. However, it can only be used by HER2-positive cancer patients, who account for only 20%~30% of all breast cancer patients. Therefore, not all patients are satisfied with the drugs for cancer treatment and the development of new drugs with higher therapeutic effects and fewer side effects is strongly warranted.

The refreshing and stress-reducing effects of forest bathing have been known for a long time. Among the volatile components released from forest trees is phytoncide, and its antibacterial, insect-repellent, and deodorant effects have been widely reported. Aromatherapy uses essential oils produced by collecting natural trees and flowers and applying several operations, such as distillation and compression. It exerts its healing effect via inhalation of the scent and having the oils imprinted on and massaged into the skin. It is also used for various purposes, such as stress reduction, restful sleep, and pain reduction. Essential oil constituents from plants are also used as alternative treatments for a wide range of illnesses, including cancer prevention and treatment [3]. We have previously reported that the essential oil of *Thujaopsis dolabrata* (asunaro) has an antitumor effect [4]. In the work described herein, we identified thujopsene as an antitumor factor contained in asunaro and clarified its antitumor mechanism.

Methods

Adjustment of asunaro essential oil. In this study, asunaro essential oil was mainly derived from Yuica (Takayama, Japan) and Kaga Lumber (Kanazawa, Japan). Asunaro was purified by steam distillation, and its components were evaluated by gas chromatography–mass spectrometry. The concentration of asunaro essential oil was adjusted to 10^{-2} g/mL with dimethyl sulfoxide, and the solution was used in subsequent experiments. Hinokitiol was purchased from Fujifilm Wako Chemicals (Osaka, Japan), dissolved in dimethyl sulfoxide to a concentration of 10^{-2} g/mL, and stored at -20°C .

Cell line and culture conditions. The gastric cancer cell line MKN45, breast cancer cell lines MCF7 and SKBR3, and colorectal cancer cell line DLD1 were purchased from American Type Culture Collection (Manassas, VA, USA). Cancer cells were cultured in Dulbecco's modified Eagle medium containing 5% fetal calf serum. Bactericide was added to all cultures. Further, 7 mL of peripheral blood samples was obtained from healthy volunteers, and mononuclear cells were isolated using Lymphoprep (StemCell Technologies, Vancouver, Canada) and cultured in OpTmizer (Life Technologies Japan, Tokyo, Japan).

Cell proliferation assay. The antitumor effect of the asunaro solution or volatile components of asunaro essential oil was quantified using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cancer or mononuclear cells were adjusted to 96-well plates at a rate of 1×10^4 /100 μL . The asunaro solution was administered to cultured cells at a concentration in the range of 0.01 to 10 $\mu\text{g/mL}$ and cultured at 37°C for 1 to 72 h. For the experiment on volatile components, 0.001 g/0.1 mL of asunaro essential oil was administered to 2 wells in the center of the plate and reacted at 37°C for 48 h. After the reaction, 10 μL of solution I from an MTT cell proliferation assay kit (Roche Diagnostics, Tokyo, Japan)

was added to each well. After a reaction at 37°C for 4 h, 100 µL of visualization solution was added to each well and reacted at 37°C overnight. Then, the absorbance at 595 nm was measured using a multifunction plate reader (Filter Max F5; Molecular Devices, Wokingham-Berkshire, UK).

Observation of morphology and evaluation of apoptosis reaction. Breast cancer cells (SKBR3 and MCF7) were stained with a cell stain double-staining kit containing calcein–acetoxymethyl (AM) and propidium iodide (PI) (Dojindo Laboratories, Tokyo, Japan). Breast cancer cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Animal experimentation. Four-week-old female nude mice were purchased from SLC (Hamamatsu, Japan) and used in the study. A tumor growth model was established by injecting 2×10^6 MKN45 cells under the skin on the back of 10 mice. MKN45 cells were also injected into the peritoneal cavity of 10 other mice in the amount of 2×10^6 /animal, establishing a model of gastric cancer peritoneal dissemination metastasis as previously reported [4]. Experimental (asunaro) and control groups were set up, with each group consisting of 5 animals and placed in individual cages. Asunaro essential oil (0.01 g in 1 mL) was naturally transpired into the cage for 24 h. Mice in the control group were bred indoors. Flooring tips were changed twice weekly in each cage. Mice were weighed once a week to assess body weight variability. All mice were euthanized by cervical sprain that was performed by the skilled professional under the anesthesia with isoflurane (Pfizer, NY, USA) after 4 weeks from the start of the experiment. Tumors were excised from the back of tumor growth model mice and the area of each tumor was measured. In addition, the number of metastases in peritoneal dissemination model mice was counted and compared with controls. This study was performed in accordance with relevant approval by the Animal experiment committee, Faculty of Medicine, University of Toyama (A2018MED-39). And this study was reported in accordance with ARRIVE guidelines

Isolation of antitumor factors from asunaro essential oil. Asunaro essential oil (1.05 g) was suspended in methanol (20 mL) and partitioned with hexane (20 mL $\times$ 4) to give hexane-soluble fraction (922 mg). A portion of hexane-soluble fraction (610 mg) was subjected to silica gel column chromatography (80 g, 38 $\times$ 3 cm, hexane-ethyl acetate = 9:1) to give thujopsene (282 mg). Isolated thujopsene showed a molecular ion peak at m/z 204 (EIMS) and was identified by comparing their ^1H and ^{13}C NMR data with those in the literature [5].

Analysis of the concentration of volatile components and transfer into brain. A total of 0.1 mL of Dulbecco's modified Eagle medium was administered to each well of a 96-well plate. Mono Trap RCC18 (GL Sciences, Tokyo, Japan) was placed at regular intervals from the center. A total of 0.001 g/0.1 mL of asunaro essential oil was administered to 2 wells in the center of the plate, and the mixture was allowed to stand at 37°C for 48 h. Next, each Mono Trap was placed in a glass container containing 2 mL of hexane that was subsequently sealed. The concentration of thujopsene in each hexane solution was measured using GC-MS-QP2010 (Shimadzu Corporation, Kyoto, Japan). A DB-5MS capillary column (30 m $\times$ 0.25 mm I.D., 0.25 µm, non-polar column; Agilent Technologies, Tokyo, Japan) was used for analysis. Helium (99.99995%, 1.82 mL/min) was used as the carrier gas. The inlet line temperature and source

temperature were set at 250°C. The temperature levels of the column oven were 40°C for 2 min, 40 to 200°C at 5°C/min, and then 200°C for 2 min.

The compound was identified by direct comparison with the standard thujopsene product (Sigma-Aldrich, Tokyo, Japan), and quantitative calculation was performed using the absolute calibration curve method. The transfer of thujopsene into the body was examined. A total of 100 µL of asunaro essential oil containing 68.3% thujopsene was diluted with 500 µL of olive oil and administered intraperitoneally to mice. Whole brains were collected 30 min after intraperitoneal administration, and hexane extraction was performed to examine the brain transfer of thujopsene.

Western blotting. After MKN45 cells were reacted with 1 µM of thujopsene or hinokitiol for 24 h individually, radioimmunoprecipitation assay buffer 1 mL + protease inhibitor 10 µL (Nakalai Tesque, Kyoto, Japan) was administered. After the reaction was left on ice for 5 min, it was centrifuged at 14,000g for 15 min. The supernatant was extracted and used as a protein solution. The protein concentration of the solution was measured using Takara BCA Protein Assay Kit (Takara Bio, Shiga, Japan). Each protein solution was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. Each solution was separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membrane uses the SNAP id2.0 protein detection system, mouse anti-p16INK4 antibody (Pharmingen, San Diego, CA, USA), mouse anti-p-21 (Pharmingen), rabbit anti-active caspase-3 (Abcam, UK), mouse anti–pro-caspase-3 (Santa Cruz Biotechnology, OR, USA), rabbit anti-cleaved caspase-9 (Abcam), and rabbit anti-PKM2 (Millipore, CA, USA). Blotting was performed on each antibody (Merck Millipore, Darmstadt, Germany). Samples were detected by chemiluminescence after 5 min of incubation with Luminata Forte Western HRP Substrate (Merck Millipore).

Drug affinity responsive target stability (DARTS) analysis. MKN45 cells were washed with phosphate-buffered saline and then lysed in M-PER (Thermo Fisher Scientific) containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The protein concentration of the solution was measured using Protein Assay Reagent (660 nm; Thermo Fisher Scientific). Thujopsene solution (10 mM) was added to the cell lysate and reacted at 25°C for 30 min. Thermolysin was added to this solution at a ratio of 1:0.1 and reacted at 37°C for 30 min to carry out the DARTS analysis. At the end of the reaction period, 0.5 M ethylenediaminetetraacetic acid (pH 8.0) was added to each sample at a 1:10 ratio on ice to stop proteolysis. Samples were incubated with NuPAGE LDS Sample Buffer (Life Technologies, Carlsbad, CA, USA) and 5% 2-mercaptoethanol at 95°C for 5 min. The samples were loaded onto 10% gradient polyacrylamide gels and electrophoresed. The gels were incubated in fixative solution (40% ethanol and 10% acetic acid in ultrapure water) at room temperature overnight. The proteins in the gels were silver stained for visualization using SilverQuest Kit (Invitrogen, Carlsbad, CA, USA).

One protein band (indicated by the arrow in Fig. 6a) was thinner in the sample treated with thujopsene than in the sample treated with vehicle solution. The band was excised from the gel, digested with trypsin, and then analyzed by mass spectrometry using a nano-liquid chromatography–tandem mass

spectrometry system (Japan Bio Services, Saitama, Japan). The candidate protein from the electrophoresis band was identified as pyruvate kinase M2 (PKM2) using the MASCOT database and spectrum data. Western blotting was performed after the DARTS reaction to confirm whether the candidate protein was PKM2.

Lactate assay test. Changes in lactate production in MKN45 cells that were reacted with thujopsene were analyzed using Lactate Assay Kit-WST (Dojindo, Kumamoto, Japan). MKN45 cells were adjusted to 96-well plates at a rate of $1 \times 10^4/100 \mu\text{L}$. Thujopsene was added to MKN45 cells and reacted at 37°C for 24 h. A total of 20 μL of the cell culture supernatant was collected and transferred to another well, to which 80 μL of working solution was next added; the mixture was reacted at 37°C for 30 min. Absorbance at a wavelength of 450 nm was measured using a multifunction plate reader.

Statistical analysis. All data are expressed as mean $\pm$ standard deviation. Two-tailed unpaired t-test, one-way analysis of variance, and post hoc Bonferroni's multiple comparisons test were used. The significance level was set at 5%. All statistical analyses were performed using JMP 15.0.

Results

Antitumor effect of the volatile components of asunaro essential oil. Asunaro essential oil was incubated with breast cancer cell lines (SKBR3 and MCF7) to evaluate its antitumor effect, and MTT assay was used to analyze its inhibitory effect on cancer cell growth. The results showed that asunaro essential oil significantly suppressed the growth of cancer cells in a dose-dependent manner, with half-maximal inhibitory concentrations of 2.9 and 1.4 $\mu\text{g/mL}$, respectively (Fig. 1a). This tumor growth inhibitory effect was confirmed in the gastric cancer cell line MKN45, colon cancer cell line DLD1, and breast cancer cell line SKBR3 (Fig. 1b). SKBR3 cells were emerged from the plate and their nuclei were shrunk to induce cell death (Fig. 1c). Next, the antitumor effect of the volatile components of asunaro essential oil was examined by incubating SKBR3 and MCF7 breast cancer cells in a 96-well plate, adding asunaro essential oil into 2 wells in the center of the plate, and incubating the mixture for 48 h; changes in tumor growth potential were compared using MTT assay. The results showed that the volatile components of asunaro essential oil suppressed the growth of breast cancer cells in a distance-dependent manner (Fig. 2a).

The concentration ratios of growing cells that were reacted with the essential oil in the proximal well (SKBR3: 10.5%, MCF7: 6.0%) were lower than those of growing cells that were reacted with the essential oil in the distal well (SKBR3: 85.5%, MCF7: 86.9%). On the other hand, less difference was observed in normal mononuclear cells (49.3% in the proximal well, 90.6% in the distal well) (Fig. 2b). Morphological changes of these breast cancer cells that were incubated with the volatile components of asunaro essential oil were analyzed after staining with calcein-AM and PI by fluorescence microscopy (Fig. 2c). In the proximal well, the number of living cells that were stained green by calcein-AM decreased, and the number of dead cells stained red by PI increased more than did the corresponding number in the distal well. And the apoptotic change as the shrinkage of nuclei was shown in the proximal well. These outcomes indicate that the volatile components of asunaro essential oil had an apoptosis inducing

activity on cancer cells, with said effect being stronger for proximal cells, but not for normal mononuclear cells. This finding suggests that the volatile components of asunaro essential oil are safe for the normal human body.

Suppression of peritoneal dissemination and metastasis by asunaro essential oil. A tumor growth suppression model was created by injecting MKN45 gastric cancer cells subcutaneously into the back of the 4-week-old mice (Fig. 3a). The model mice were divided into two groups, namely, the asunaro group and the control group. In the asunaro group, inhalation of the volatile components of asunaro essential oil was performed for 4 weeks. Body weight fluctuations were not significantly different between these groups. Mice in the asunaro group had a similar appearance to those in the control group and did not show possible changes in side effects, such as diarrhea, decreased appetite, and decreased body movement. After 4 weeks, the mice in both groups were killed, all tumors were resected, and the volume of each tumor was measured. The average volume of the tumor significantly shrunk in the asunaro group compared with the control group (Fig. 3b).

A gastric cancer peritoneal dissemination metastasis model was created by injecting MKN45 gastric cancer cells into the abdominal cavity of the 4-week-old mice to elucidate the tumor-suppressive activity of asunaro more clearly. The mice were also divided into the asunaro group and the control group. In the same manner as described above, evaporated asunaro was administered to the asunaro group for 4 weeks. After that, the mice in both groups were killed and the number of peritoneal disseminations was counted. The asunaro group showed a significant decrease in the number of peritoneal seeds disseminated compared with the control group (Fig. 3c). These results suggest that inhalation of the volatile components of asunaro essential oil tends to suppress the growth and metastasis of cancer in mice without obvious side effects.

Identification of thujopsene from asunaro essential oil. To identify antitumor factors in asunaro essential oil, activity guided isolation of asunaro essential oil was conducted. As a result, solvent partition and silica gel chromatography guided by MTT assay yielded thujopsene as active compound. Research has shown that hinokitiol has antibacterial and antitumor effects and that it constitutes ~1% of asunaro essential oil. Both hinokitiol and thujopsene have a small molecular weight and are easily transpired (Fig. 4a). Therefore, the antitumor effects of thujopsene and hinokitiol were compared in this study.

Hinokitiol, thujopsene, and asunaro essential oil were individually reacted with MKN45, and their antitumor effects were analyzed using MTT assay. The results showed that thujopsene significantly suppressed the proliferation of MKN45 cells more than did hinokitiol (Fig. 4b). They also revealed that asunaro had the strongest ability to suppress tumor growth. Next, the antitumor effects of the volatile components of thujopsene and hinokitiol were analyzed. Thujopsene or hinokitiol was added to the 2 center wells of a 96-well plate, and MKN45 cells deployed around the component were then reacted for 48 h. The growth inhibitory effects of tumor cells were compared and examined using MTT assay (Fig. 4c). The results showed that the volatile components of thujopsene and hinokitiol suppressed the growth of MKN45 cells in a distance-dependent manner. Compared with thujopsene, hinokitiol showed an antitumor

effect in a wider range. On the other hand, the antitumor effect of thujopsene was stronger than that of hinokitiol. These findings clarified that thujopsene has a strong antitumor effect in a narrow range and that hinokitiol has a relatively weak effect in a wide range.

Data on the relationship between the distance and concentration of the volatile components of thujopsene are shown in Fig. 4d. The results suggest that >100 pg/mL of thujopsene is necessary for inhibiting tumor cell growth. Moreover, the transfer of thujopsene into body organs was examined. The 100µl of asunaro essential oil, containing 68.3% of thujopsene, was administered intraperitoneally to mice and thujopsene was detected in the brain at a concentration of 2.2 µg/g.

Apoptosis-related factors of thujopsene and hinokitiol. Both thujopsene and hinokitiol could induce apoptosis in several types of cancer cells. Western blotting using an antibody related to cell death was performed to clarify the difference between the mechanisms of these two antitumor factors (Fig. 5). The results showed that caspase-9 was strongly expressed in thujopsene-treated tumor cells. The expression of pro-caspase-3 in hinokitiol was decreased compared with that in thujopsene, whereas the expression of active caspase-3 was enhanced in both. These findings indicate that thujopsene induces cell death via the caspase-3 and caspase-9 pathways and that hinokitiol induces cell death via caspase-3.

We also evaluated the expression levels of p53, p21, and p16. The results showed that the expression levels of p53 and p21 were enhanced in both thujopsene and hinokitiol but that those of p16 and p27 did not change. These outcomes indicate that both hinokitiol and thujopsene induce cell death via p53 and p21.

Antitumor mechanism of thujopsene. Clarifying the antitumor mechanism of thujopsene requires detecting the molecule with which thujopsene associates in tumor cells. A protein that binds to thujopsene was identified using the DARTS method. Thujopsene was added to the cell lysate of MKN45 and reacted. Thermolysin was added to this solution, which was incubated at 37°C for 30 min. After a DARTS reaction was carried out, analysis by SDS-PAGE was performed (Fig. 6a). Silver staining of the gel revealed numerous bands. Of these, a band that became thinner after reacting with thujopsene was excised and analyzed by nano-liquid chromatography–tandem mass spectrometry, which revealed PKM2 as a candidate binding protein of thujopsene (score: 1,256; coverage: 66%). Western blotting after the DARTS reaction confirmed that thujopsene made a thinner PKM2 band under proteolysis by thermolysin (Fig. 6b). These data strongly suggest that thujopsene binds to PKM2.

PKM2 is known to be a protein involved in glycolysis, especially in the Warburg effect. Lactic acid production was also evaluated to investigate how the glycolytic reaction was changed by reacting with thujopsene. The results showed that the production of lactic acid significantly decreased in MKN45 cells that were reacted with thujopsene (Fig. 6c). In addition, analysis by Western blotting revealed the expression of PKM2 and PKM4, which is a dimer of PKM2 and reported to promote anaerobic glycolysis. The results showed that the band of PKM2 was increased and the band of PKM4 was decreased in the incubation with thujopsene relative to hinokitiol (Fig. 6d). These findings suggest that thujopsene suppresses glycolytic reaction by binding to PKM2 and suppressing its metabolism.

Discussion

Plant-derived essential oils are generally concentrated and refined using steam distillation. They contain multiple types of hydrophobic small molecule compounds that are called terpenes. Each terpene has a peculiar odor and evaporates. More than 1,000 terpenes have been identified to date, and most of them are classified into one of three groups, namely, monoterpene, sesquiterpene, or diterpene, based on their structural characteristics [5]. The scent and functional characteristics of each essential oil are formed depending on the type and composition of the terpenes it contains.

Plant-derived essential oils are known to have antibacterial, antifungal, antiviral, and insect-repellent effects [6, 7]. In addition, aromatherapy using essential oils has been reported to have a wide range of effects, such as stress-suppressing and dementia-improving effects [8]. In recent years, plant-derived essential oils have become the focus of phytomedicine. Many studies have reported on the antioxidant and antitumor effects of essential oils [9]. Carvacrol is one of the monoterpenes extracted from *Origanum vulgare*, and it has been reported to induce cell death by stopping the cell cycle of multiple cancer cells, such as breast, gastric, and colorectal cancer cells [10]. In addition, β -elemene, a sesquiterpene contained in *Curcuma wenyujin*, which is a Chinese medicinal herb, has been found to up-regulate the effect of 5-fluorouracil treatment on triple-negative breast cancer cells and induce apoptosis [11].

T. dolabrata is used in essential oil preparations for aromatherapy. It is an evergreen conifer unique to Japan and has been shown to have relaxation [12], antibacterial [13, 14], and insect-repellent effects [6, 15]. We have previously reported that asunaro has an antitumor effect. In this study, we have identified thujopsene as an antitumor factor in the essential oil of *T. dolabrata*. Thujopsene is abundant in the essential oils of various conifers, especially the Canary Islands juniper and *T. dolabrata*, and it is reported to account for ~2% of the cores [16]. It is concentrated in 68.3% of asunaro essential oil purified by steam distillation and has the advantage of being efficiently used in an antitumor essential oil preparation. It is also seen in *Waldheimia glabra* and has been reported to have an antitumor effect on the breast cancer cell lines MDA-MB-231 and MCF-7 [7]. In addition, it is known to have antibacterial and antitumor effects [9]. Regarding the antitumor mechanism of thujopsene, previous studies have shown that mTOR (mechanistic target of rapamycin) and AKT (protein kinase B) expression and the growth of tumor cells are suppressed when thujopsene blocks the PI3K (phosphatidylinositol-3-kinase) pathway [5].

The mechanism by which the components of evaporated essential oil show pharmacological effects involves the transmission of signals to the hypothalamus through the sensory nerves by binding to the olfactory receptors in the nasal cavity and their subsequent control of the autonomic nervous system and hormones [17]. On the other hand, the transpiration component that reaches the lungs is considered to migrate into the peripheral blood and bind to the specific receptors of each organ cell to exert its effect. We measured the concentration of thujopsene in the asunaro transpiration component and analyzed it against the antitumor effect of asunaro. Our data revealed that the scattering range of thujopsene has a negative correlation with the concentration in the air and a negative correlation with the antitumor effect. At this point, we found that a concentration ≥ 100 pg/mL was required for thujopsene in the transpiration

component to show an antitumor effect. We also found that thujopsene administered intraperitoneally in mice accumulates in the brain. In other words, thujopsene was found to transfer to the brain and other parts of the body if it is transferred into the bloodstream. This finding is similar to the intracerebral results reported with inhalation and intraperitoneal administration of the volatile compounds α -pinene, linalool, limonene, and 1,8-cineole [18]. Taken together, these data indicate that thujopsene from asunaro essential oil taken up from the nasal mucosa and lungs moves into the blood, reaches the target organ, and has a direct effect on cancer cells.

Hinokitiol, which constitutes ~1% of asunaro essential oil, has been reported to suppress the growth of various cancers and induce apoptosis, in addition to demonstrating an antibacterial effect, and its clinical application is expected [19–21]. Regarding the antitumor mechanism of hinokitiol, its suppression of metalloproteinase-9/2 in tumor cells, suppression of the nuclear factor κ B/mitogen-activated protein kinase signaling pathway, and suppression of OH radical by increasing superoxide dismutase activity have been reported [22–24]. Similar to our results, those of another study found that hinokitiol induced apoptosis in tumor cells by activating p53 and caspase-3 [25]. Our results also showed that thujopsene had a stronger antitumor effect than hinokitiol. Thujopsene, similar to hinokitiol, was also found to have a strong antitumor effect via many mechanisms, such as caspase-3, caspase-9, p27, and p53. Furthermore, in contrast to hinokitiol, thujopsene was observed to exhibit an antitumor effect mediated by PKM2.

Because hinokitiol is a compound belonging to monoterpenes that is easily dissolved in the water and its molecular weight is smaller than that of thujopsene, the scattering range of the volatile components of hinokitiol is considered to be wider than that of the volatile components of thujopsene. Comparisons between said scattering distance and the antitumor effect revealed that thujopsene has a strong effect within a short range, whereas hinokitiol has a relatively mild and widespread effect (Fig. 4c). As the strongest effect was obtained in mixing oil, a strong antitumor effect is expected to be obtained using the whole asunaro essential oil.

Studies that used the DARTS method have shown that thujopsene associates with PKM2. PKM2, a pyruvate kinase, consists of a dimer with protein phosphorylation activity and a tetramer with glycolytic activity, and its high expression in tumor tissues has been reported [26]. Tumor cells are known to acquire adenosine triphosphate mainly through the glycolytic pathway. This phenomenon is called the Warburg effect, and it plays an important role in the growth of tumor cells [27, 28]. In tumor cells expressing PKM2, the influx of glucose-derived carbon into the citric acid cycle is restricted and the production of lactic acid is promoted [26]. In this study, the action of PKM2 was blocked by treatment with thujopsene, which suppressed lactic acid production (Fig. 7). At this point, the acidity of lactic acid was not suppressed by the action of hinokitiol. These findings indicate that thujopsene induces an antitumor effect by blocking the glycolytic pathway of cancer cells. Because no other antitumor preparations use thujopsene and an antitumor inhalation or application preparation is currently not available, this product is considered to be an extremely novel and highly original product.

Conclusion

Thujopsene from asunaro essential oil inhibits the trophic metabolism pathway of tumors and, together with hinokitiol, suppresses tumor growth and metastasis.

Declarations

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Author Contributions

T.N. and Y.S. designed the experiments. T.N. and M.W. characterized the thujopsene. T.N., A.S. and T.S. performed the experiments. Y.S. and M.W performed data analysis. T.N. and T.S. wrote the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Declaration of accordance with the guidelines and regulations

All methods were performed in accordance with the relevant guidelines and regulations by including the policies of the Nature Portfolio journals.

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Figures

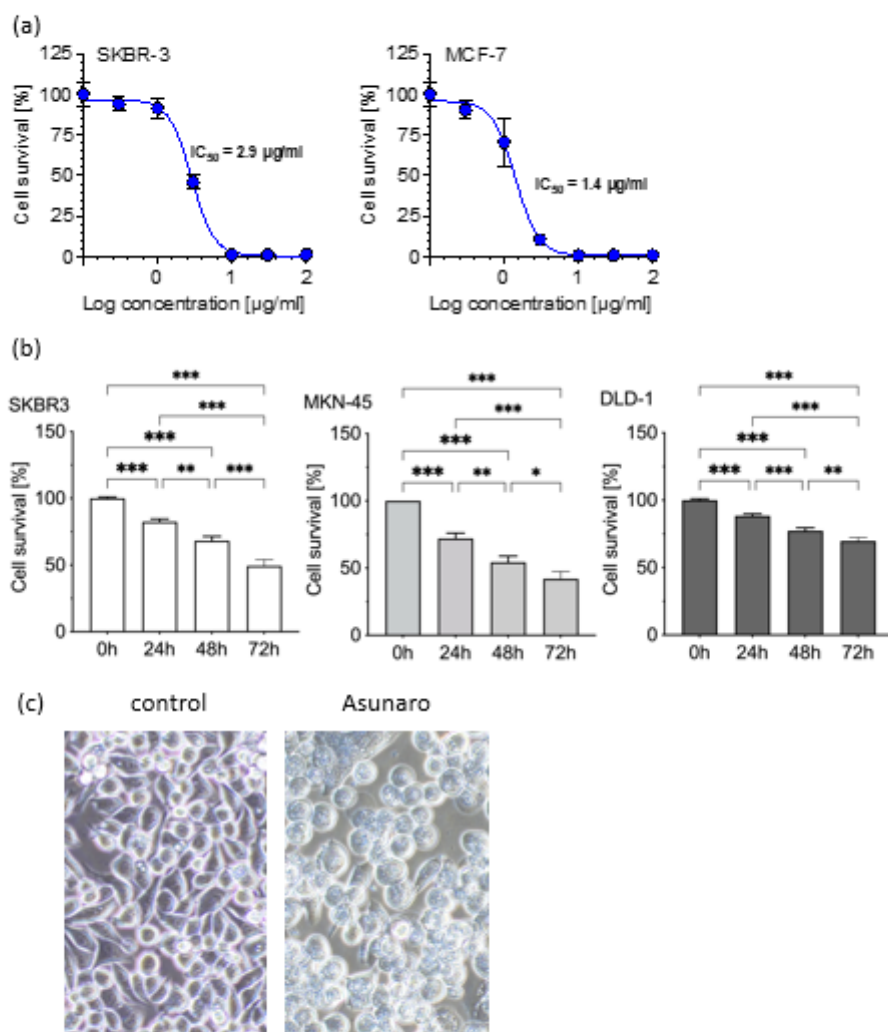


Figure 1

Anti-tumor effects of asunaro essential oil. **(a)** Each breast cancer cells (SKBR3, MCF7) were incubated with various dose of asunaro essential oil for 48hrs. MTT assay was performed and IC_{50} was analyzed respectively. **(b)** Breast cancer cell (SKBR3), gastric cancer cell (MKN45) and colon cancer cells (DLD1) were incubated in several time period and the MTT assay was performed. Each bar represents the mean $\pm$ SD of triplicate experiments (* P <0.05, ** P <0.01 and *** P <0.005). **(c)** The morphological change of SKBR3 cells by polarizing microscope (x400).

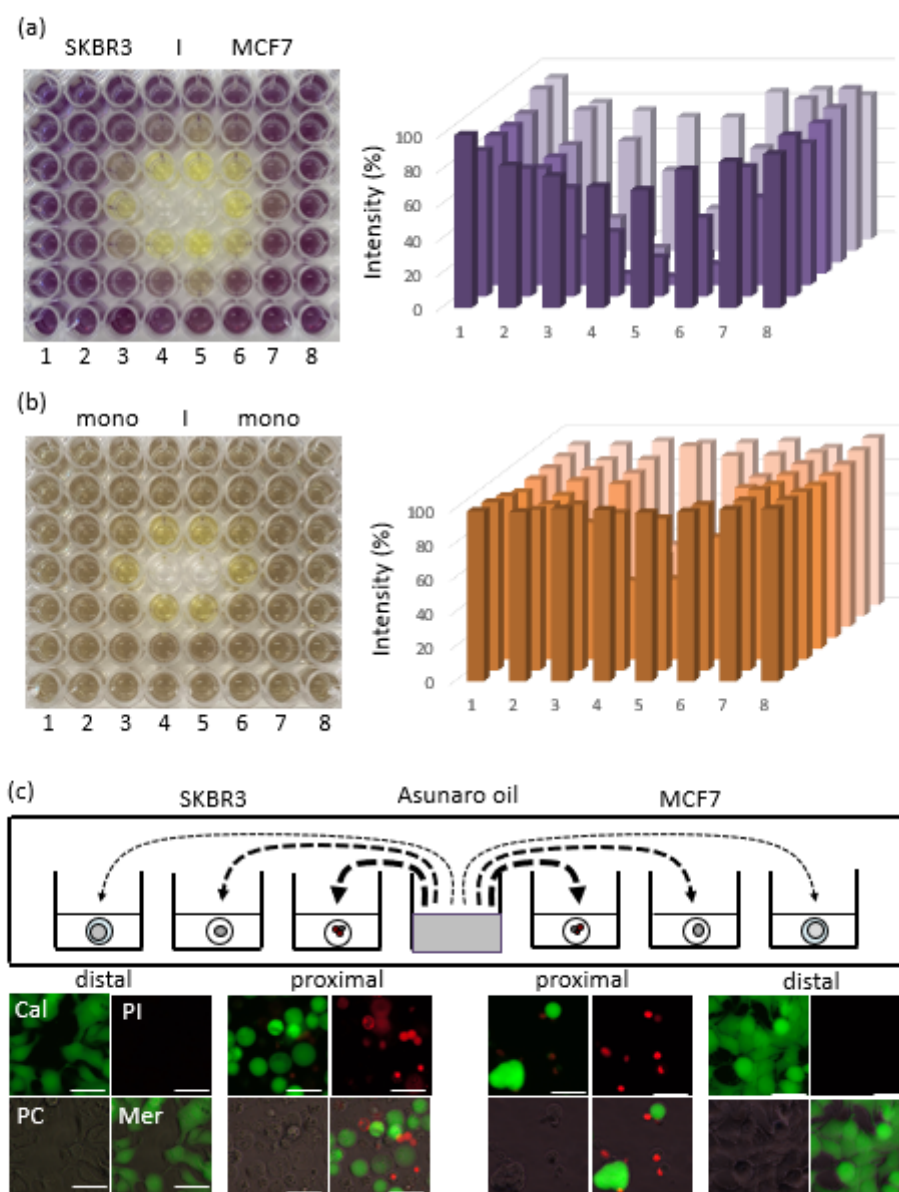


Figure 2

Antitumor effect of the volatile components of asunaro essential oil. **(a)** Two types of breast cancer cells (SKBR3, MCF7) were incubated in a 96-well plate, and asunaro essential oil was dropped into 2 center wells. After 48 hours, MTT assay was performed, and the result of the plate was shown (left). The intensity of each well was shown in the bar-graph (right). **(b)** The activity for normal Mono-nuclear cells were analyzed. **(c)** Morphological changes of breast cancer cell were analyzed by fluorescence microscope (x400) after staining with Calsein-AM (Cal) and Propidium Iodide (PI). Phase contrast modes (PC) and merged modes (Mer) were also shown. The white bar matched as 50mm.

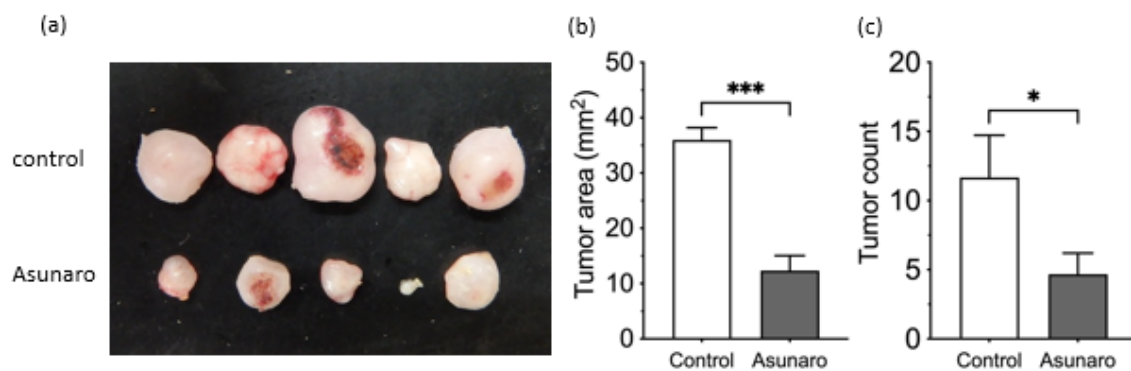


Figure 3

Suppression of peritoneal dissemination and tumor growth by asunaro essential oil. **(a)** Tumor growth suppression model mice were divided into asunaro group and control. Inhalation of volatile components of asunaro essential oil was performed for 4 weeks, and the subcutaneous tumor was resected. **(b)** The average volume of the tumor from Asunaro group was compared to the control. **(c)** The average number of intra peritoneal dissemination metastasis model of Asunaro group and control was compared. Each bar represents the mean $\pm$ SD ($P^* < 0.05$ and $*** < 0.005$).

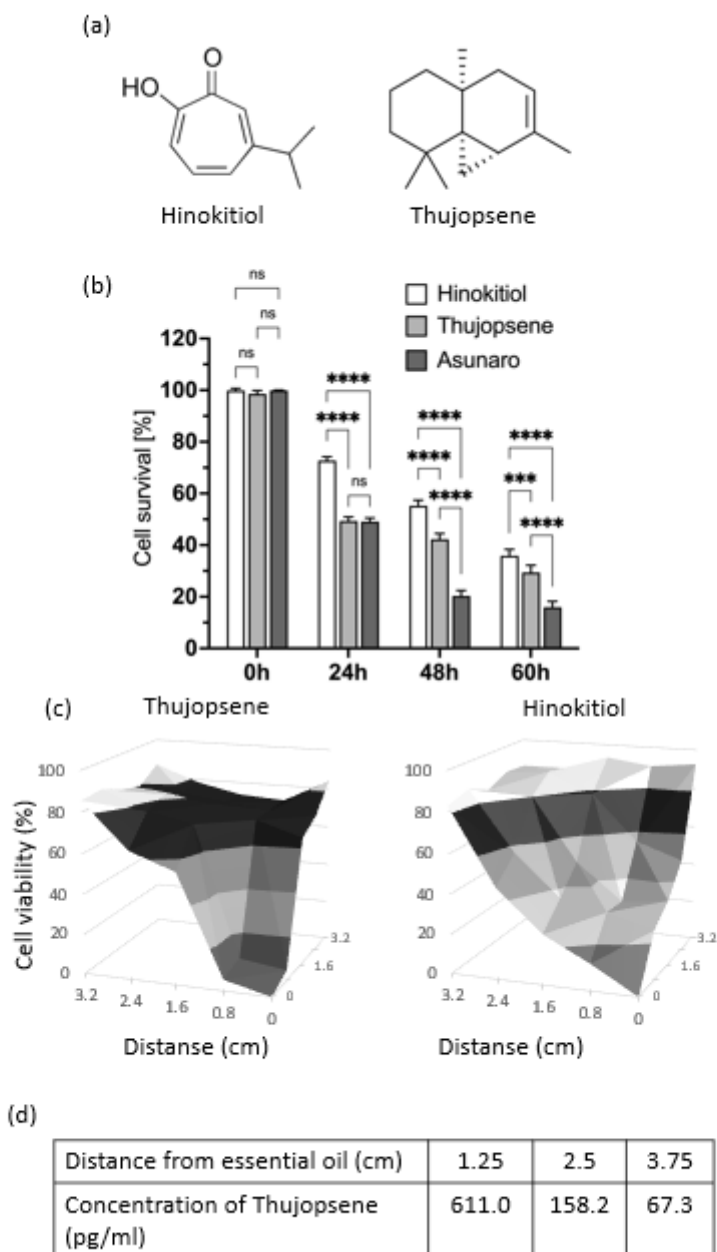


Figure 4

Antitumor factor of thujopsene and Hinokitiol. **(a)** The structure of Thujopsene and Hinokitiol. **(b)** Antitumor effects of thujopsene and hinokitiol. MKN45 was reacted with each of hinokitiol, thujopsene and asunaro essential oil in several time periods, and their antitumor effects were analyzed using MTT assay. Each bar represents the mean $\pm$ SD of triplicate experiments (*** P <0.005 and **** P <0.001). **(c)** Antitumor effects of the volatile component of thujopsene and hinokitiol were analyzed by MTT assay. **(d)** The distance from the essential oil (cm) and the concentration of volatile components of thujopsene (pg/ml) was analyzed by GC-MS.

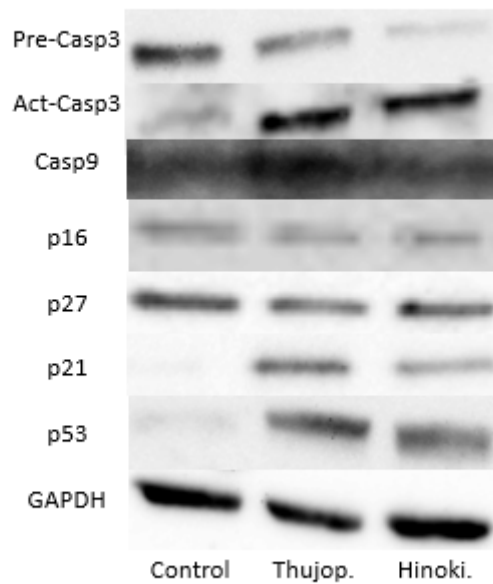


Figure 5

Apoptosis related factors of thujopsene and hinokitiol. Western blotting using antibodies (pre- and active-Caspase3, Caspase9, p16, p27, p21 and p53) was analyzed. GAPDH was used as control. (Thujop: Thujopsene, Hinoki: Hinokichiol)

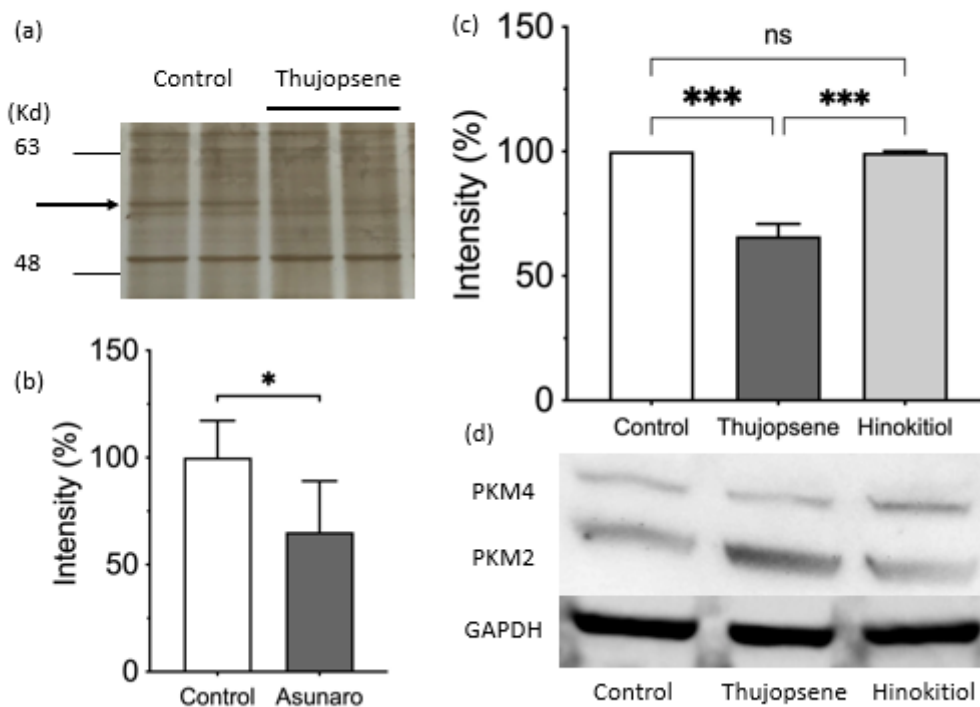


Figure 6

Antitumor mechanism of thujopsene. **(a)** The result of SDS-PAGE after the DARTS reaction. Bands of right two lanes for cell lysate that was incubated with thujopsene, were compared to left control bands. The arrow showed the point of difference. **(b)** Band intensity of PKM2 protein after DARTS reaction was shown. **(c)** The production of lactic acid in MKN45 cells reacted with thujopsene and Hinokichiol were analyzed, and the intensity was shown. **(d)** The result of western blotting for PKM2 and PKM4 protein existence after treatment of thujopsene and Hinokitiol. Each bar represents the mean $\pm$ SD of triplicate experiments (* $P < 0.05$ and *** $P < 0.005$).

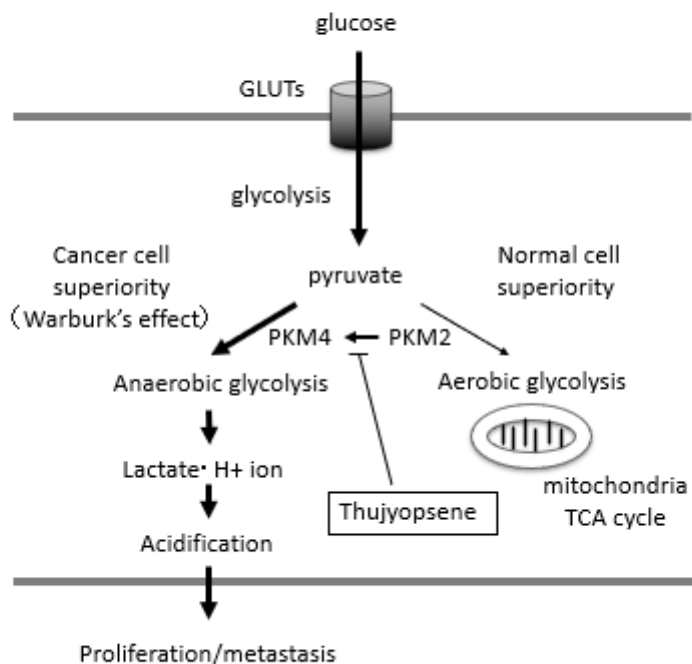


Figure 7

Schema of the thujopsene-mediated anti-tumor mechanism.

Supplementary Files

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