

Activation of the Chemosensory Ion Channels TRPA1 and TRPV1 by Hydroalcohol Extract of *Kalopanax pictus* Leaves

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Abstract

TRPA1 and TRPV1 are members of the TRP superfamily of structurally related, nonselective cation channels. TRPA1 and TRPV1 are often co-expressed in sensory neurons and play an important role in somatosense such as cold, pain, and irritants. The first leaves of *Kalopanax pictus* Nakai (Araliaceae) have long been used as a culinary ingredient in Korea because of their unique chemesthetic flavor. In this study, we observed the intracellular Ca²⁺ response to cultured cells expressing human TRPA1 (hTRPA1) and human TRPV1 (hTRPV1) by Ca²⁺ imaging analysis to investigate the ability of the first leaves of *K. pictus* to activate the hTRPA1 and hTRPV1. An 80% ethanol extract of *K. pictus* (KPEX) increased intracellular Ca²⁺ influx in a response time- and concentration-dependent manner via either hTRPA1 or hTRPV1. KPEX-induced response to hTRPA1 was markedly attenuated by ruthenium red, a general blocker of TRP channels, and HC-030031, a specific antagonist of TRPA1. In addition, the intracellular Ca²⁺ influx attained with KPEX to hTRPV1 was mostly blocked by ruthenium red, and capsazepine, a specific antagonist of TRPV1. These results indicate that KPEX selectively activates both hTRPA1 and hTRPV1, which may provide evidence that the first leaves of *K. pictus* primarily activate TRPA1 and TRPV1 to induce their unique chemesthetic sense.

Key Words: *Kalopanax pictus*, TRPA1, TRPV1, Ca²⁺ imaging, Chemesthesis

INTRODUCTION

Transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1) are members of the TRP superfamily of structurally related, nonselective cation channels and are primarily expressed in sensory neurons (Damann *et al.*, 2008). TRPA1 is often co-expressed with TRPV1 (Tominaga, 2007), and plays an important role in somatosensation such as temperature (>43°C for TRPV1 and <17°C for TRPA1) (Caterina *et al.*, 1997, Macpherson *et al.*, 2005; 2007), pain (Bevan and Andersson, 2009), and irritants (Bessac and Jordt, 2008). In recent studies, TRPA1 and TRPV1 were reported as key chemosensory ion channels that are activated by the pungent molecules found in commonly consumed foods and plants used in traditional medicines in several countries (Gerhold and Bautista, 2009). The most commonly used TRPA1 activators are allyl isothiocyanate (AITC) (Bandell *et al.*, 2004, Jordt *et al.*, 2004), allicin (Bautista *et al.*, 2005), and cinnamaldehyde (Bandell *et al.*, 2004), which are present in mustard oil, garlic, and cinnamon oil, respectively. TRPV1 was initially identified as the receptor

for capsaicin, a pungent ingredient in hot chili peppers. TRPA1 and TRPV1 have a diverse tissue distribution, with high expression in sensory neurons.

Kalopanax pictus Nakai (Araliaceae), commonly known as the prickly castor oil tree, is mainly distributed across East Asia. Its bark and stem have long been used in Asian countries to treat rheumatoid arthritis, neurotic pain, and diabetes mellitus (Lee *et al.*, 2001, Choi *et al.*, 2002). However, the first leaves of *K. pictus*, which sprout in early spring (Fig. 1), are used in Korean cuisine for the unique tingling, numbing, and burning sensation they produce (Nam *et al.*, 2006). Given their traditional culinary uses in Korea, it is very likely that the first leaves of *K. pictus* function as activators of chemosensory ion channels. However, no conclusive scientific data have yet shown that the first leaves of *K. pictus* contain activators of TRPA1 or TRPV1. Here we examined the effects of the first leaves of *K. pictus* on the both of chemosensory ion channels TRPA1 and TRPV1. A criterion for evaluating extract of the first leaves of *K. pictus* is changes on intracellular Ca²⁺ influx of cultured cells expressing human TRPA1 (hTRPA1) and human TRPV1 (hTRPV1). The changes on intracellular

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Fig. 1. Characteristics of *Kalopanax pictus*. (A) Stem and leaves. (B) First leaves. (C) *Kalopanax pictus*. Source for A: Korea Botanical Garden; source for B and C: <http://wildgreen.co.kr/plant/204060>.

Ca²⁺ influx are monitored using fluorescent dyes, fura-2/AM.

MATERIALS AND METHODS

Materials

Capsaicin (Cap), ruthenium red (RR), capsazepine (CPZ) and HC-030031 were purchased from Sigma (St. Luis, MO, USA), allyl isothiocyanate (AITC) from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). All cell culture media was obtained from Life Technologies, Inc. (Grand Island, NY, USA).

Plant extracts

The first leaves of *Kalopanax pictus* Nakai (Araliaceae) was obtained from Sanche-Gol (151 Daei-ri, Hwacheong-gun, Gangwon-do, Korea) and were botanically verified by Young Chul Kim (Director of Rare and Endangered Plants Research Center, Korea Botanic Garden; 405-2 Byungnaeri, Pyeongchang-gun, Gangwon-do, Korea). The leaves were freeze-dried and milled with a commercial food mixer. Milled leaves of *K. pictus* was extracted by distilled water, and 80% ethanol using homogenizer and evaporated under reduced pressure at low temperatures (37-40°C) and then lyophilized to a powder (*KPWx* and *KPEx*, respectively). The solids were stored at -80°C until use. *KPWx* and *KPEx* were dissolved in dimethyl sulfoxide (DMSO) to give 100 mg/ml solutions. The samples further diluted in assay buffer for the bioassay on the day of the experiment to give final concentrations of 0.001-0.1 mg/ml in the well. Voucher specimen nos. KP001 and KP002 have been deposited at Korea Food Research Institute, Gyeonggi-do, Korea. A HPLC fingerprint of *KPEx* was developed by dissolving in 40% MeOH. After centrifuging 16,853 g for 15 min, the supernatant was filtered through a 0.22 μm filter prior to analysis. HPLC was carried out on a JASCO Model 900 Series (JASCO Co., Tokyo, Japan) equipped with a UV detector, an on-line degasser and an autosampler. An YMC-Pack ODS AM (25 cm × 4.6 mm, 5 μm ID) was used for chromatographic separations. Column temperature was held constant at 35°C in a JASCO thermal chamber controller. Elution was carried out at a flow rate of 1 ml/min with the following solvent system: A: 0.1% acetic acid in deionized water, B: 0.1% acetic in 75% acetonitrile. After injection of 20 μl of

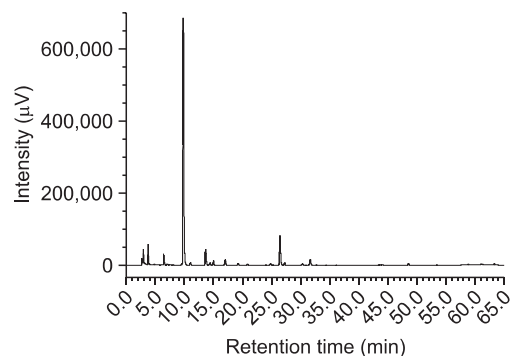


Fig. 2. HPLC profile of *KPEx* at 285 nm. Chromatographic measurements were done at 35°C with an eluent flow rate of 1.0 ml/min with a gradient consisting of 0.1% acetic acid and acetonitrile. Column: YMC-Pack ODS-AM (250×4.6 mm, 5 μm ID)

sample, the system was maintained at 88% A for 18 min, then decreased to 0% in 58 min and held for 2 min, and then increased to 88% within 3 min and held for 2 min for a total run of 65 min per sample. The product sample was monitored with UV at 285 nm (Fig. 2).

Cell culture and transfection

Flp-In 293 cells stably expressing the human TRPA1 (hTRPA1) were constructed as previously reported (Hata *et al.*, 2012). hTRPA1-expressing cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich), containing 10% fetal bovine serum (FBS, Invitrogen) and 0.02% hygromycin B (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. hTRPA1-expressing cells were cultured and used for the measurement of Ca²⁺ responses to the extracts of the first leaves *K. pictus*.

Constructs used for transfection were Human TRPV1 (hTRPV1, NCBI accession number: NG_029716.1) cloned in a pEAK10 vector (Edge Biosystems, Gaithersburg, MD, USA). The hTRPV1 was constructed using the PCR, and were confirmed by sequencing with an ABI 3130 DNA genetic analyzer (Applied Biosystems, Foster City, CA, USA). hTRPV1 expressing-cells were prepared by the transient transfection. HEK293T cells were cultured at 37°C in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded onto 100 mm dish and transiently transfected with the hTRPV1 expression plasmid using Lipofectamine 2000 reagent (Invitrogen). Cells were used for measuring the cellular responses 24 hr after transfection.

Measurement of the cellular responses of hTRPA1- or hTRPV1-expressing cells by Ca²⁺ imaging analysis

Cells were seeded in a 96-well black-wall imaging plates (BD Falcon Labware, Franklin Lakes, NJ, USA) 18-26 h prior to their use in an experiment. The cells were washed with the assay buffer (130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, pH 7.4), and loaded with 5 μM of the calcium indicator dye, fura-2 AM (Invitrogen), that was diluted with the assay buffer. The incubation was continued for another 30 min at 27°C. The cells were rinsed and incubated in 100 μl of the assay buffer for more than 10 min, prior to adding 100 μl each of the 2× ligand solution by pipetting. The intensities of fura-2 fluorescence emis-

sions resulting from excitation at 340 and 380 nm were measured at 510 nm using a computer-controlled filter changer (Lambda DG4; Sutter, San Rafael, CA, USA), a Andor Luca CCD camera (Andor Technology, Belfast, Northern Ireland) and an inverted fluorescence microscope (IX-71; Olympus, Tokyo, Japan). Images were recorded at 3-s intervals and analyzed using the MetaFluor software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

The statistical significance was analyzed by one-way ANOVA and the Tukey's multiple comparison test was used to evaluate between groups. Each datum represents the mean ± SD; the symbols *, **, and *** indicates $p < 0.05$, 0.01, and 0.001, respectively.

RESULTS

Effects of the extract of the first leaves of *K. pictus* on hTRPA1

To test the potency of the first leaves of *K. pictus* in activating TRPA1, we generated cell lines that stably expressed human TRPA1 (hTRPA1) as previously reported (Hata *et al.*, 2012). The effects of a water extract (KPWx) and an 80% ethanol extract (KPEx) of the first leaves of *K. pictus* on Ca²⁺ influx in fura-2-loaded cells stably expressing hTRPA1 were examined. AITC, the most potent TRPA1 agonist among all natural products present in mustard oil, was used as a selective activator of TRPA1. AITC (10 μM) significantly increased Ca²⁺ influx in a time-dependent manner in cells expressing hTRPA1. This response was blocked by pretreatment with ru-

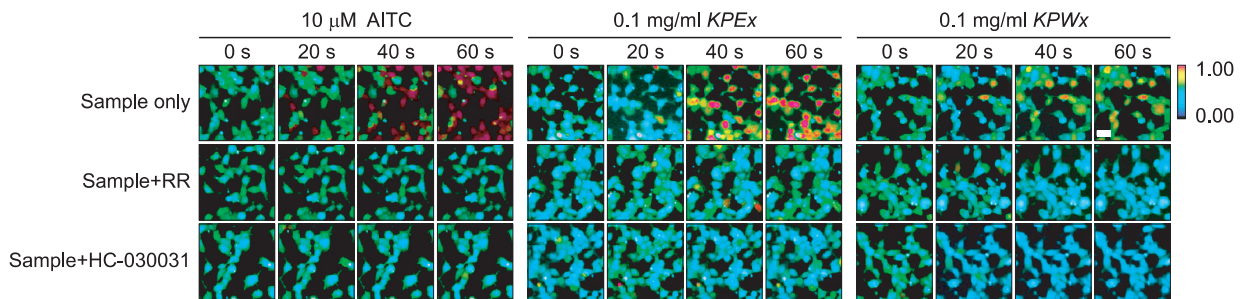


Fig. 3. Response to *K. pictus* extract of cells expressing hTRPA1. Representative ratiometric images of fura-2-loaded hTRPA1-expressing cells, after sample application, are shown. *K. pictus* extract was used to determine Ca²⁺ responses in the later part of the experimental work. The cation ion channel blocker RR was added at a concentration of 30 μM (Sample+RR), and the TRPA1-specific antagonist HC-030031 was used at a concentration of 100 μM (Sample+HC-030031). KPEx was extracted in 80% EtOH. KPWx: water extract. Scale bar: 11.04 μm.

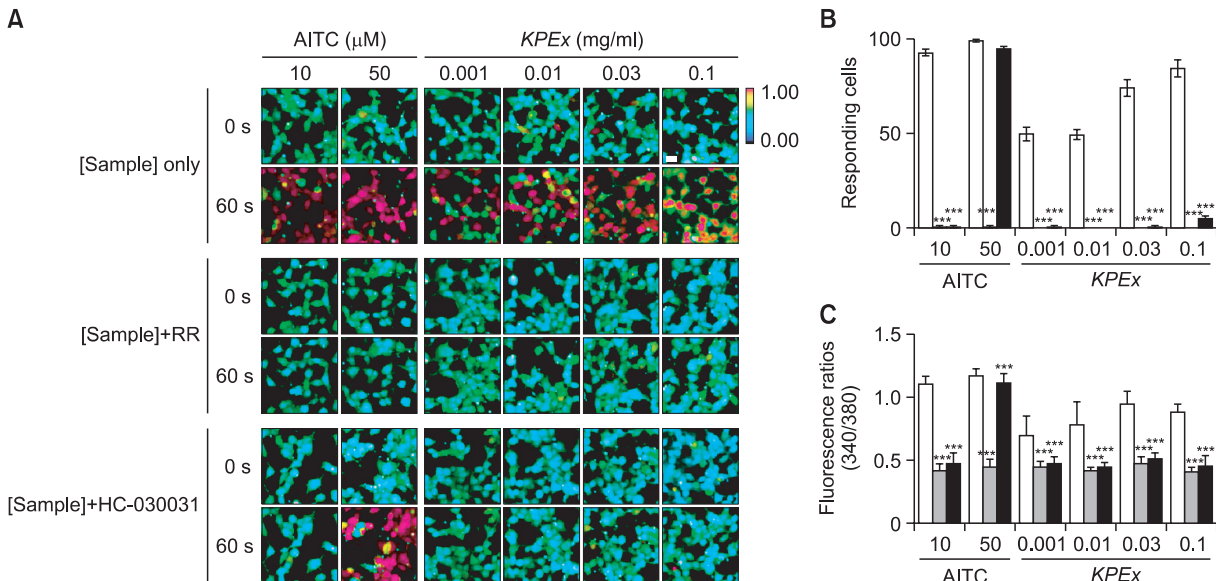


Fig. 4. Dose-response effect of KPEx on cells expressing hTRPA1. (A) Responses to KPEx of cells expressing hTRPA1. The upper and lower columns indicate the images obtained 0 and 60 sec after stimulation, respectively. Scale bar: 11.04 μm. (B) Counts of cells responding to KPEx. Responsive cells were counted among approximately 100 cells observed in the microscopic field, and cells were defined as responding positively when the F340/F380 ratio increased above 0.7 after the addition of the sample. AITC (10 or 50 μM) was used as a positive control to increase the Ca²⁺ influx. Data represent the mean ± standard error of the mean of three independent experiments. (C) Response of stable cell lines expressing hTRPA1 to KPEx in the absence (white bars) or presence (gray or black bars) of RR or HC-030031. Data represent the mean ± SD (n = 3). *** $p < 0.001$ (one-way ANOVA with Tukey's multiple comparison test).

thienium red (RR; 30 μ M), a general blocker of TRP channels, and HC-030031 (100 μ M), a specific antagonist of TRPA1. Similarly, *KPEx* (0.1 mg/ml) increased the Ca^{2+} influx in a time-dependent manner, a response that was attenuated by RR (30 μ M) and HC-030031 (100 μ M). The potency of *KPEx* was not superior to that of AITC (Fig. 3). In the same manner, *KPWx* selectively activated hTRPA1, albeit far more weakly than *KPEx* (Fig. 3), implying a role for alcohol-soluble pungent molecules in activating hTRPA1 in the first leaves of *K. pictus*. The possible involvement of TRPA1-active molecules in *KPEx* was confirmed with various concentrations of *KPEx* (0.001-0.1 mg/ml), which caused the selective, concentration-dependent activation of hTRPA1 (Fig. 4). Although AITC activated hTRPA1, the effect of a higher concentration of AITC (50 μ M) was not significantly inhibited by HC-030031 (100 μ M), a specific

antagonist of TRPA1 (Fig. 4). In agreement with this, recent studies suggest that AITC may have effects that are independent of TRPA1 (Everaerts *et al.*, 2011; Capasso *et al.*, 2012).

Effects of the extract of the first leaves of *K. pictus* on TRPV1

Because TRPV1 is often co-expressed with TRPA1 in sensory neurons, we also examined the effects of *KPEx* and *KPWx* on hTRPV1. *KPEx* (0.1 mg/ml) activated TRPV1; *KPWx* (0.1 mg/ml; Fig. 5) or a lower concentration of *KPEx* (0.001-0.03 mg/ml; Fig. 6) induced minimal activation. The Ca^{2+} influx induced by 0.1 mg/ml *KPEx* was eliminated by the co-application of the general TRP channel blocker RR (30 μ M) and the selective TRPV1 antagonist capsazepine (5 μ M; Fig. 5). These results imply that *KPEx* contains a nonselective

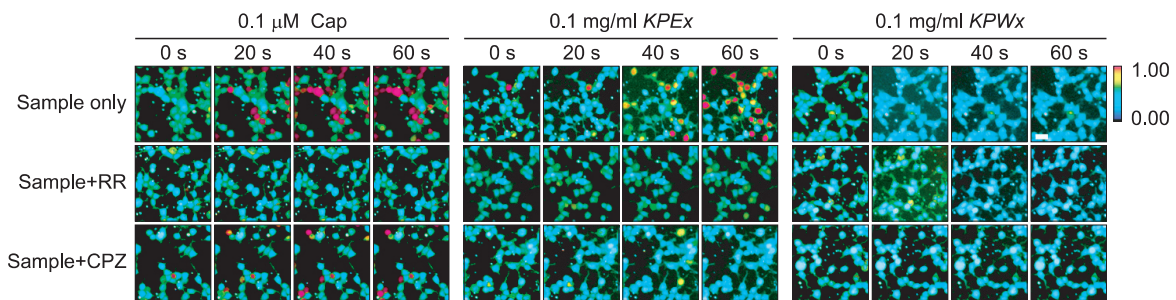


Fig. 5. Response to *K. pictus* extract of cells expressing hTRPV1. Representative ratiometric images of fura-2-loaded hTRPV1-expressing cells, after application of the sample, are shown. *K. pictus* extract was used to determine Ca^{2+} responses in the later part of the experimental work. The cation ion channel blocker RR was added at a concentration of 30 μ M (Sample+RR), and the TRPV1-specific antagonist capsazepine (CPZ) was used at a concentration of 5 μ M (Sample+CPZ). Scale bar: 11.04 μ m.

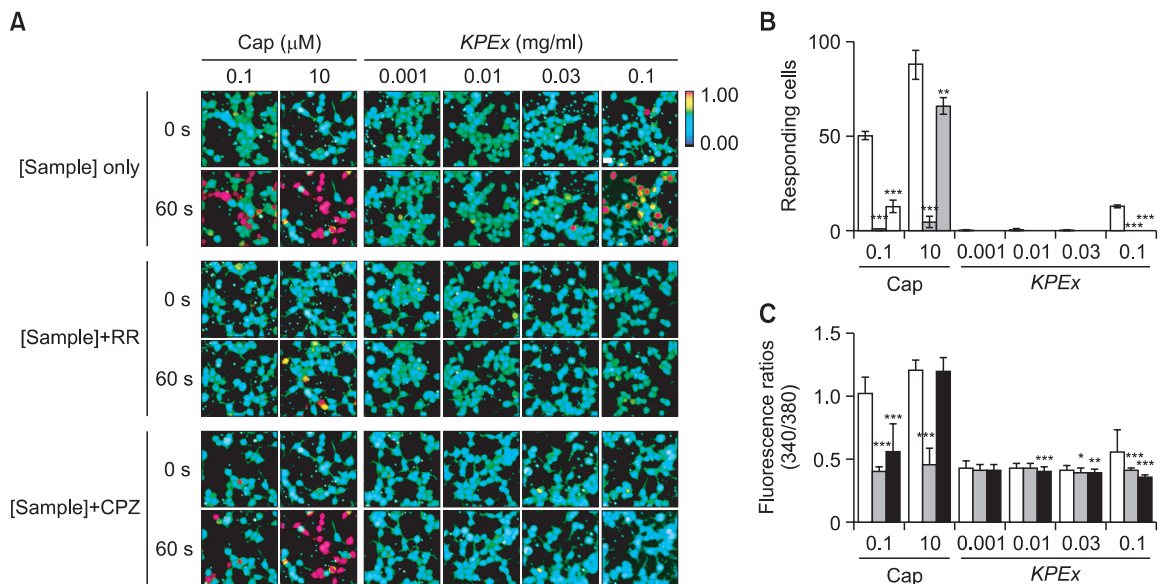


Fig. 6. Dose-response effect of *KPEx* on cells expressing hTRPV1. (A) Representative ratiometric images of fura-2-loaded hTRPV1-expressing cells. The upper and lower columns indicate the images obtained 0 and 60 sec after stimulation, respectively. Scale bar: 11.04 μ m. (B) Counts of cells responding to *KPEx*. Responsive cells were counted among approximately 100 cells observed in the microscopic field, and cells were defined as responding positively when the F340/F380 ratio increased above 0.7 after the addition of the sample. Cap (0.1 or 10 μ M) was used as a positive control to increase Ca^{2+} influx. Data represent the mean \pm standard error of the mean of three independent experiments. (C) Response of stable cell lines expressing hTRPA1 to *KPEx* in the absence (white bars) or presence (gray or black bars) of RR or CPZ. Data represent the mean \pm SD (n=3). * p <0.05, ** p <0.01, and *** p <0.001 (one-way ANOVA with Tukey's multiple comparison test).

hTRPV1-activating molecule, although the quantity or activity is lower or weaker than that of hTRPA1.

DISCUSSION

These results demonstrate, for the first time, that the first leaves of *K. pictus* possess selective activity to nonselective chemosensory cation channels hTRPA1 and hTRPV1. The sensory neurons are heterogeneous, so the distribution of both channels are not identical in every sensory neuron cell line. 97% of sensory neurons expressing TRPA1 co-express TRPV1, whereas 30% of sensory neurons expressing TRPV1 co-express TRPA1 (Story *et al.*, 2003). A variety of plant-derived pungent compounds found in wasabi, yellow mustard, Brussels sprouts, nasturtium seeds (Jordt *et al.*, 2004), perilla leaf (Bassoli *et al.*, 2009), celery (Zhong *et al.*, 2011), hot peppers (Riera *et al.*, 2009), garlic (Koizumi *et al.*, 2009), turmeric (Yeon *et al.*, 2010), ginger (Dedov *et al.*, 2002), and ginseng (Jung *et al.*, 2001) activate TRPA1 and/or TRPV1.

Based on their traditional culinary uses in Korea, it is very likely that the first leaves of *K. pictus* function as activators of chemosensory ion channels. Therefore, we examined the effects of the first leaves of *K. pictus* on the both of chemosensory ion channels TRPA1 and TRPV1 in fura-2-loaded cultured cells expressing hTRPA1 and hTRPV1.

KPEx selectively activated both hTRPA1 and hTRPV1, and *KPWx* also showed similar activation, albeit far more weakly than *KPEx*, implying a role for hydroalcohol-soluble pungent molecules in activating hTRPA1 and hTRPV1 in the first leaves of *K. pictus*, although the quantity or activity to hTRPV1 is lower or weaker than that of hTRPA1. Although the potency of *KPEx* to induce intracellular Ca^{2+} influx far more weaker than AITC, the most potent TRPA1 agonist among all natural products, the selectivity to hTRPA1 and hTRPV1 indicate that the first leaves of *K. pictus* primarily activate TRPA1 and TRPV1 to induce their unique chemesthetic sense. The first leaves of *K. pictus* have for centuries been appreciated for their culinary use, which likely reflects their chemesthetic effects on sensory systems. Here we have focused on understanding the molecular basis of the somatosensory actions of *K. pictus* that contribute to its unique chemesthesis. Specifically, we have shown that *KPEx* directly activates TRPA1 and TRPV1 in cultured cells stably expressing hTRPA1 and hTRPV1, which may contribute to an understanding of the chemesthesis of the first leaves of *K. pictus*. The identification of the active ingredient(s) of *KPEx* is underway.

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