Interaction of Vietnamese Medicinal Plant Extracts with Recombinantly Expressed Human Neurokinin-1 Receptor

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Abstract

A primary functional receptor screening assay was performed to examine the effects of methanolic extracts from ten Vietnamese medicinal plants on the human neurokinin-1 receptor expressed from Semliki Forest virus vectors in Chinese hamster ovary cells. Extracts from *Piper nigrum, Stephania cambodica* and *Styphnolobium japonicum* were found to exert inhibition on agonist-induced human neurokinin-1 receptor activity. Secondary assays and high performance liquid chromatography of the lead compounds addressed a possible association between pharmacological responses and these chemical compounds. Strong inhibition of human neurokinin-1 receptor was observed for extracts revealing the highest inhibitory potency for rotundine (tetrahydropalmatine) in *S. cambodica* extracts with IC_{50} of 0.88 µM, followed by piperine and capsaicin in *P. nigrum* extracts with IC_{50} values of approximately 2 µM, whereas rutin in *S. japonicum* extracts failed to inhibit hNK1R up to 100 µM.

Abbreviations

BHK:	baby hamster kidney
CHO:	Chinese hamster ovary
CNS:	central nervous system
EGFP:	enhanced green fluorescent protein
(h)NK1R:	(human) neurokinin-1 receptor
SFV:	Semliki Forest virus

Key words

Piper nigrum · *Stephania cambodica* · *Styphnolobium japonicum* · Piperaceae · Fabaceae · Menispermaceae · piperine · capsaicin · rotundine · rutin · neurokinin-1 receptor · functional assays

Vietnam possesses rich medicinal plant resources of more than 4000 species, which have been used for centuries in traditional medicine, but very little is known about their pharmacological mechanism and mode of action [1–3]. In this context, extracts and their compounds, prepared from ten Vietnamese plants, including *Cinchona officinalis* L. (Rubiaceae), *Codonopsis javanica* Blume (Campanulaceae), *Eleusine indica* (L.) Gaertn. (Poaceae), *Morinda officinalis* How (Rubiaceae), *Orthosiphon stamineus* Benth (Lamiaceae), *Panax bipinnatifidus* Seem. (Araliaceae), *Panax stipuleanatus* Tsai & Feng, *Piper nigrum* L. (Piperaceae), *Styphnolobium japonicum* (L.) Schott (Fabaceae), and *Stephania cambodica* Gagnep (Menispermaceae), have been applied for the treatment of various ailments and symptoms, such as pain relief, insomnia, nausea, hypertension, anxiety, depression, asthma and fever [4]. Since the hNK1R is known to be present in both the CNS and a number of other tissues and has been the target for development of drugs for migraine, emesis, anti-inflammation and psychiatric disorders [5,6], the potential interaction between plant extracts and the hNK1R is of significant interest. In this study, we employed the SFV system for overexpression of the hNK1R in CHO cells to investigate the effects of plant extracts on agonist-induced receptor activity. The methanolic extracts of the ten Vietnamese medicinal plants were subjected to a preliminary screening for their effects on receptors in presence of the agonist Substance P (SP). Extracts from three species, namely *P. nigrum, S. japonicum* and *S. cambodica,* were further selected and examined for probable associations between different geographical locations (accessions) with biological activity. Moreover, lead compounds of plant extracts from these three species were analyzed by HPLC and examined in functional receptor assays to elucidate whether extracts derived from different locations exert different activities in relation to their concentrations of lead compounds.

HPLC was applied to determine the amount of piperine and capsaicin in *P. nigrum*, rotundine in *S. cambodica*, and rutin in *S. japonicum* collected from five different geographical locations (**Fig. 1** and **Table 1**). The fruit extracts from *P. nigrum*, capsaicin and piperine showed the most consistent amounts of analyzed compounds ranging between 0.14 ± 0.01% and 0.24 ± 0.01% and between 1.51 ± 0.11% and 2.22 ± 0.08%, respectively. For tuber and root extracts from *S. cambodica*, the range of rotundine was between 0.98 ± 0.18% and 4.69 ± 0.15%, and moderately variable. In flower bud extracts from *S. japonicum*, rutin ranged from 0.17 ± 0.01% to 6.14 ± 0.18% showing the largest variation among analyzed compounds. The degree of variability within each species appeared to be influenced by not only the genotype, but also the plant parts examined. In this context, extracts of fruits (*P. nigrum*) showed the least variation, tuber and root extracts moderate variation (*S. cambodica*) and flower bud extracts the largest variation (*S. japonicum*). This variability in chemical properties of each medicinal plant species in relation to the plant parts examined should be considered when these medicinal plants are collected for the purpose of standardization of the herbal products.

The SFV system was employed for the overexpression of the hNK1R in CHO cells [7] to investigate the involvement of Vietnamese medicinal plants with known therapeutic potential receptor function. To confirm SFV-based heterologous gene expression in mammalian cells, BHK cells were infected with SFV-EGFP recombinant particles and visualized by fluorescence microscopy. High infection and expression rates were obtained 24 and 48 hours post-infection (**Fig. 2**). Hoechst 33342 staining demonstrated a good viability of infected cells after 24 hours. Prior to studies on pharmacological effects of plant extracts on the functional activity of hNK1R, CHO cells were infected with SFV-hNK1R particles and an hNK1R-specific band of approximately 46 kD was visualized by Western blotting (**Fig. 3**). In contrast, this band was absent in the control CHO cells.

In attempts to examine the effect of various plant extracts in relation to the functional activity of the hNK1R, K_d and K_i values were first determined for the reference NK1R agonist substance P (SP) and the antagonist aprepitant (AP), respectively (**Table 2** and **Fig. 4**). Next, intracellular calcium measurements were used to

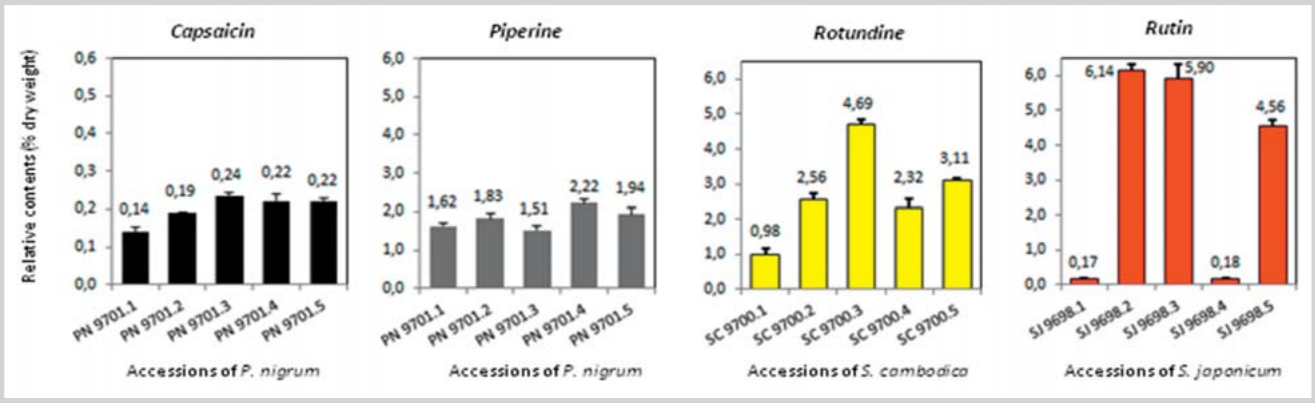

Fig. 1 HPLC quantification of extract compounds. Quantification of capsaicin and piperine in fruits of *P. nigrum*, of rotundine in mixture of tubers and roots of *S. cambodica* and of rutin in flower buds of *S. japonicum*.

Table 1 List of medical plant species and geographical locations where each species were collected for the study.

No.	Species (and parts used)	Accession localities (*district*, province)	Accession codes	Global Postitional Sites (GPS)
1	*Piper nigrum* L.	*Vinh Linh*, Quang Tri	PN 9701.1	17°02′13′′N – 106°57′03′′E
2	*Parts used:* Fruits	*Ngoc Hoi*, KonTum	PN 9701.2	14°47′28′′N – 107°34′50′′E
3	(pericarp removed)	*Dac To*, KonTum	PN 9701.3	14°38′45′′N – 107°51′40′′E
4		*Hoa Binh*, KonTum	PN 9701.4	14°15′08′′N – 108°58′37′′E
5		*Chu Se*, Gia Lai	PN 9701.5	13°29′36′′N – 108°10′56′′E
6	*Stephania cambodica* Gagnep	*My Duc*, Hanoi	SC 9700.1	20°35′29′′N – 105°46′36′′E
7	*Parts used:* Tubers and roots (periderm	*Kon Plong*, KonTum	SC 9700.2	14°39′54′′N – 108°17′45′′E
8	removed, tubers sliced before drying)	*Chu Pah*, Gia Lai	SC 9700.3	14°10′59′′N – 107°41′21′′E
9		*Lac Duong*, Lam Dong	SC 9700.4	12°08′21′′N – 108°26′03′′E
10		*Da Lat*, Lam Dong	SC 9700.5	11°55′34′′N – 108°27′04′′E
11	*Styphnolobium japonicum* (L.) Schott	Thuong Tin, Hanoi	SJ 9698.1	21°03′30′′N – 105°21′48′′E
12	*Parts used:* Flower buds	*Quynh Phu*, Thai Binh	SJ 9698.2	20°40′14′′N – 106°20′01′′E
13		*Dong Hung*, Thai Binh	SJ 9698.3	20°30′39′′N – 106°15′05′′E
14		*Tien Hai*, Thai Binh	SJ 9698.4	20°20′25′′N – 106°33′02′′E
15		Ho Chi Minh City	SJ 9698.5	10°56′55′′N – 106°34′50′′E

determine the IC_{50} values for the hNK1R in presence of methanolic plant extracts. Among the ten Vietnamese medicinal plant species examined, extracts from only three species showed a pharmacological effect. The effect was obtained for plant extracts from all five different geographical locations although variations in the level of inhibition occurred (**Table 2**). The most potent inhibition was observed for extracts from *S. cambodica* ranging from 4.74 to 53.09 µg/mL (**Fig. 5A**). A somewhat weaker inhibition was measured for extracts from *P. nigrum* ranging from 5.67 to 60.53 µg/mL (**Fig. 5B**). The weakest response was seen for extracts from *S. japonicum* with IC_{50} values between 15.38 and 140.60 µg/mL (**Fig. 5C**). The discrepancy in the *in vitro* pharmacological potencies of extracts from the three species collected from different geographical locations was up to 10-fold based on IC_{50} determination. Data correlation analysis indicated that the variation in IC_{50} values was random and not related to the geographical location of sample collection. For example, four of the five *P. nigrum* samples were collected within 100–200 km in central Vietnam, whereas the site for only one sample was 300 km further north and the variation was mainly within the samples from the same region. Interestingly, four of the *S. japonicum* samples were collected in the vicinity of Hanoi in northern Vietnam and only one sample was from the area of Ho Chi Minh City in the south (some 1160 km away). In this case, the 10-fold variation was observed among samples from the same region (Hanoi).

Remarkably, we found a significant correlation between the rotundine concentration in *S. cambodica* extracts and the IC_{50} values at an R^2 coefficient of 0.85, whereas concentrations of capsaicin and piperine in *P. nigrum* extracts and of rutin in *S. japonicum* did not correlate with the IC_{50} values of the extracts with corresponding R^2 coefficients of 0.10, 0.01 and 0.17, respectively (data not shown).

Interestingly, a further preliminary assay (n = 3 replicates from two different experiments) on isolated lead compounds (**Fig. 6**) concordantly showed the most potent inhibition for rotundine from *S. cambodica* with an IC_{50} value of 0.88 µM (**Fig. 6C**). Weaker receptor inhibition effects were observed for capsaicin and piperine from *P. nigrum* with corresponding IC_{50} values in the higher micromolar range, approximately 2.0 µM (**Fig. 6A, B**). In contrast, rutin from *S. japonicum* did not exhibit any apparent effect on receptor functional activity up to 100 µM (**Fig. 6D**). A relatively preserved pharmacological activity was observed for all five extracts of *S. cambodica* in concordance to their rotundine concentration. The strong inhibition preliminarily found for this compound indicated that NK1R might be one of

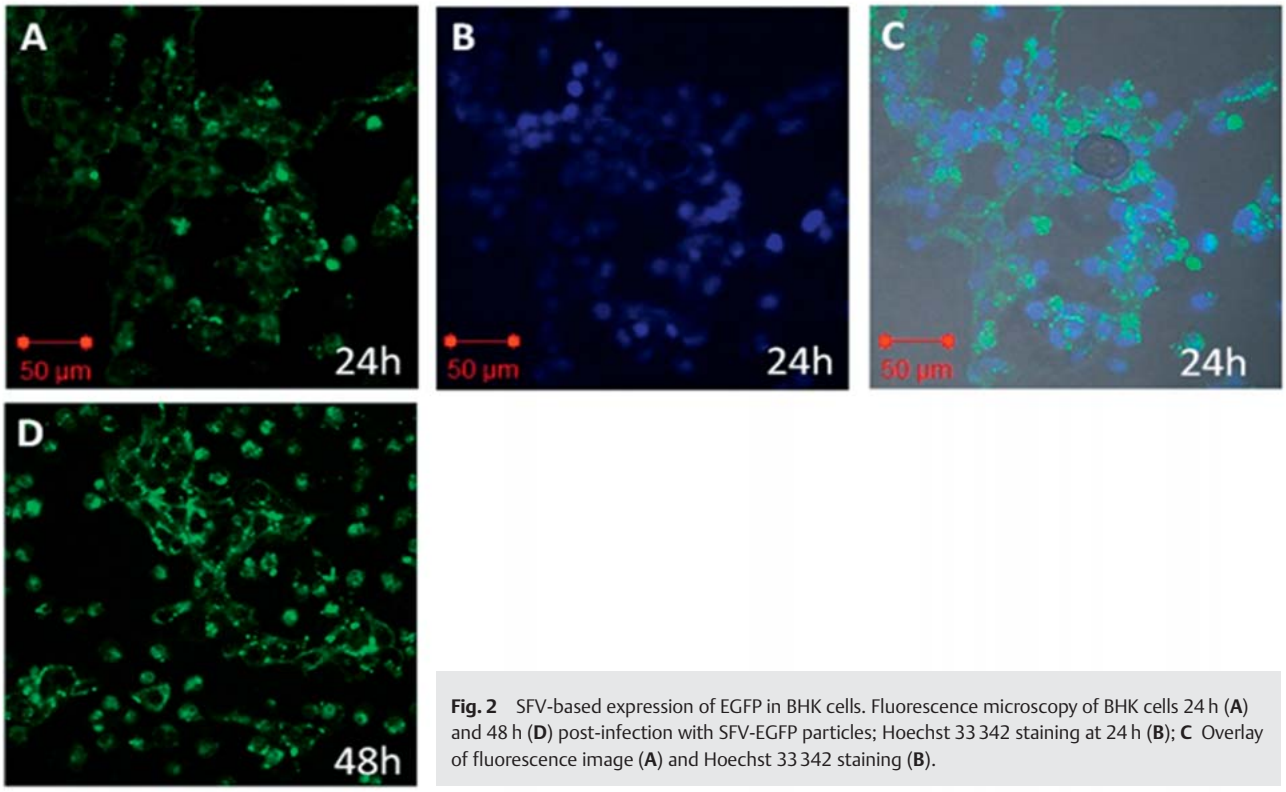

Fig. 2 SFV-based expression of EGFP in BHK cells. Fluorescence microscopy of BHK cells 24 h (**A**) and 48 h (**D**) post-infection with SFV-EGFP particles; Hoechst 33 342 staining at 24 h (**B**); **C** Overlay of fluorescence image (**A**) and Hoechst 33 342 staining (**B**).

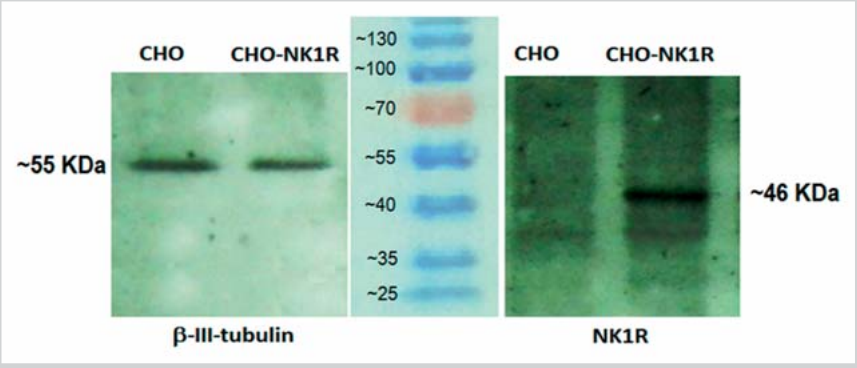

Fig. 3 Western blot of SFV-hNK1R infected CHO cells. Lysed CHO cells were subjected to Western blotting 24 h after infection with SFV-hNK1R particles. Left panel: treatment with tubulin β-III mouse mAb (Invitrogen); right panel: treatment with anti-NK1R antibody (Sigma).

the major targets involved in the beneficial therapeutic value of this medicinal plant and rotundine appeared to play a central role in its interaction with this receptor.

Consistent effects shown by extracts of *P. nigrum* also revealed that NK1R may present an important target for its known medicinal use. A moderate inhibitory activity exhibited by capsaicin and piperine indicated that these compounds might be involved in the etiology of this medicinal plant. Nevertheless, no obvious correlation between the compound contents and the inhibitory potencies of the extracts suggested that additional constituents might also be involved in the interaction of these extracts with the receptor.

Since rotundine failed to inhibit NK1R up to 100 µM despite all five extracts of *S. japonicum* showing persistent receptor inhibition, which indicated that this plant more likely contains other interactive compounds. It is worth to mention that some previous studies revealed that the pharmacological as well as therapeutic effects and chemical properties provided by plant extracts are not always straight forward. For instance, it has been well documented that extracts from St. John's wort [*Hypericum perforatum* L. (Hypericaceae)] target several CNS receptors and may generate an additive and synergistic effect of intrinsic constituents, which contribute to their beneficial antidepressant activity [8]. Further studies are therefore needed to elucidate which constituents of the *S. japonicum* extracts exert effects on NK1R.

Materials and Methods

Plant extracts: For each of the ten Vietnamese medicinal plants selected for this study, one specimen was randomly collected and subjected to a primary receptor screening in triplicates. Due to initial positive responses, extracts from *P. nigrum, S. japonicum* and *S. cambodica*, were studied more detailed. Five geographical locations for each species were included in the study (**Table 1**).

Table 2 Pharmacological effect of methanolic extracts derived from fruits of *P. nigrum*, from mixture of tubers and roots of *S. cambodica* and from flower buds of *S. japonicum* on hNK1R expressed by Semliki Forest virus vectors in CHO cells.

Species	Accessions	IC_{50} ± SEM (µg/mL)*
Piper nigrum L.	PN 9701.1	21.28 ± 1.09
	PN 9701.2	5.67 ± 0.41
	PN 9701.3	60.53 ± 2.77
	PN 9701.4	26.30 ± 1.26
	PN 9701.5	35.48 ± 2.10
Stephania cambodica Gagnep	SC 9700.1	4.74 ± 0.21
	SC 9700.2	7.24 ± 0.44
	SC 9700.3	53.09 ± 1.93
	SC 9700.4	9.44 ± 0.38
	SC 9700.5	25.94 ± 0.46
Styphnolobium japonicum (L.) Schott	SJ 9698.1	15.38 ± 0.27
	SJ 9698.2	63.53 ± 1.42
	SJ 9698.3	40.27 ± 0.46
	SJ 9698.4	140.60 ± 3.15
	SJ 9698.5	32.66 ± 1.31
K_d and K_i of reference substances in this study (nM)		
Agonist	Substance P (SP)	7.32 ± 1.24
Antagonist	Aprepitant (AP)	41.10 ± 9.90

* Data represent the means ± SEM of three replicates from three experiments with the same extracts or test compounds.

Fruits of *P. nigrum*, flower buds of *S. japonicum* and tubers (tuberous roots) and roots of *S. cambodica* collected from 5–6 year old plants according to a good agriculture and collecting procedure (GACP) were provided by botanists from the Vietnam National Institute of Medicinal Materials (VN-NIMM). Voucher specimens are registered in the herbarium of VN-NIMM in Hanoi with accession codes shown in **Table 1**.

For the preparation of methanolic extracts, plant materials were dried in a ventilating drier at 35 °C for 48 hours. The dried samples were pulverized and extracted twice with methanol in an ultrasonic bath for 15 minutes. The solvent was then evaporated to dryness and the residues were diluted in methanol to a final concentration of 50 mg/mL. The extracts were stored at − 20 °C until used for HPLC analysis and functional receptor assays. The final concentration of methanol in the functional receptor assays had only a small influence (< 5%) as revealed by control experiments.

HPLC analyses of plant extracts: Analyses of piperine and capsaicin (*P. nigrum*), rotundine (*S. cambodica*) and rutin (*S. japonicum*) were carried out on an analytical HypersilGold C18 column (3 µm, 150 × 2.1 mm) using a Shimadzu LC-10 A HPLC system (Shimazdu) coupled to an SPD 10Avp UV-vis detector. The samples were eluted with the mobile phase constituents and relevant compounds were quantified as previously described for piperine [9], capsaicin [10], rutin [11] and rotundine (DL-tetrahydropalmatine) [12]. Chromatographic standard compounds, including piperine, capsaicin and rutin were purchased from Extrasynthese S. A. with a purity ≥ 90% for piperine (#94–622), ≥ 95% for capsaicin (#404–86–4) and ≥ 99% for rutin (#153–18–4). Rotundine was obtained from ABcam Biochemicals with a purity ≥ 98% (#ab143 555). The identification of plant compounds of interest in the extracts was based on comparing the retention times and peak areas between the samples and the standard compounds. Piperine was detected at a wavelength of 340 nm, whereas capsaicin, rutin, and rotudine were measured at 222 nm, 259 nm and 280 nm, respectively. Each sample was separately extracted at least twice and analyzed by HPLC. Analytical determinations are given as means ± standard deviation.

Expression of human neurokinin-1 receptor: The hNK1R was expressed using the SFV system as described previously [7]. Briefly, *in vitro* RNA was transcribed with SP6 RNA polymerase (Thermo Scientific™, Life Technologies Inc.) from the pSFV2gen expression vector carrying the hNK1R gene (pSFV-hNK1R) and pSFV-Helper2 vector [13] and electroporated into BHK cells. After 24 hours, virus stocks were collected and recombinant virus particles activated by chymotrypsin treatment and aliquots stored at − 20 °C [14]. To evaluate the efficiency of host cell infection and recombinant protein expression, virus stocks of SFV-GFP particles were prepared in parallel. CHO cells (0.6×10^5 cells/well) cultured in the presence of plant methanolic extracts diluted 5-fold in 10 × HBSS buffer (Gibco®, Life Technologies Inc.) were infected with SFV-hNK1R virus stock on 96-well plates and subjected to functional assays 24 hours post-infection.

Functional assays for determining effect of plant extracts: The effect of plant extracts on hNK1R binding was assessed according to the NIMH-PDSP protocols [15] using substance P (SP) and aprepitant (AP) as reference agonist and antagonist, respectively. SP and AP with more than 95% purity were purchased correspondingly from Sigma-Aldrich (#S6883) and Toronto Research Chemicals Inc. (#A729 800). As our primary screening assays

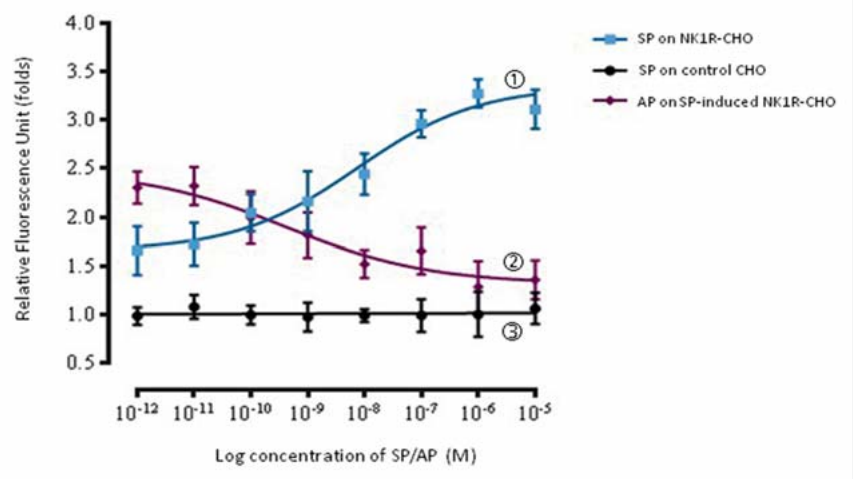

Fig. 4 Functional activity of recombinant hNK1R. Determination of the K_d value for the agonist substance P (SP) on SFV-hNK1R-infected CHO cells (NK1R-CHO) based on dose response curve of calcium mobilization assays (line ①), and the K_i value for the antagonist aprepitant (AP) in the presence of 10^{-7} M SP (line ②). Non-infected CHO cells (Control CHO) did not response to increased concentration of SP (line ③).

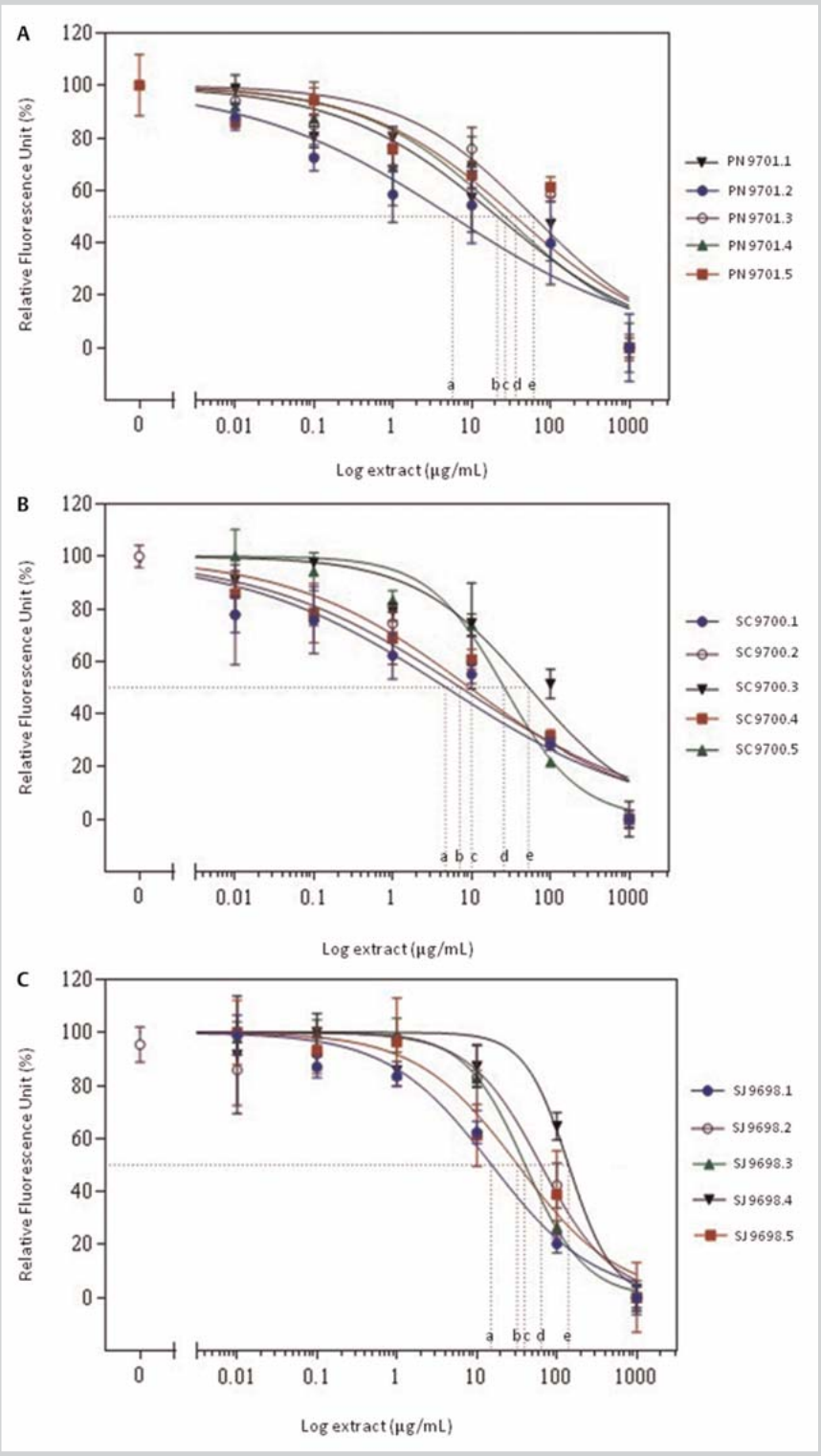

Fig. 5 Inhibition of agonist-induced hNK1R functional activity by *P. nigrum*, *S. cambodica*, and *S. Japonicum* extracts determined by intracellular calcium measurements. **A** *P. nigrum*; IC_{50} values of the following extracts were determined: PN9701.1 (b), PN9701.2 (a), PN9701.3 (e), PN9701.4 (c) and PN9701.5 (d). **B** *S. cambodica* IC_{50} values of the following extracts were determined: SC9700.1 (a), SC9700.2 (b), SC9700.3 (e), SC9700.4 (c) and SC9700.5 (d). **C** *S. japonicum*. IC_{50} values of the following extracts were determined: SJ9698.1 (b), SJ9698.2 (a), SJ9698.3 (e), SJ9698.4 (c) and SJ9698.5 (d).

showed that the plant extracts exhibited only potential antagonist activity, secondary functional assays were performed to determine IC_{50} values of extracts against an EC_{80} concentration of substance P (10^{-7} M) by plotting a full concentration-response curve, using the statistical and graphic program Sigmaplot® 12.0 (Systat Software Inc.). Intracellular calcium measurements were carried out as previously described [16] adapted for CHO cells. The Fura-2AM kit was purchased from Molecular Probes (Life Technologies Inc.) and assays were performed according to the manufacturer's instructions. Fluorometric determinations were carried out in a Hidex Sense Microplate Reader, employing kinetic measurement mode and Sense software (Hidex). Unless mentioned otherwise, the reported values represent the means ± SEM of three replicates from three experiments with the same extracts or test compounds.

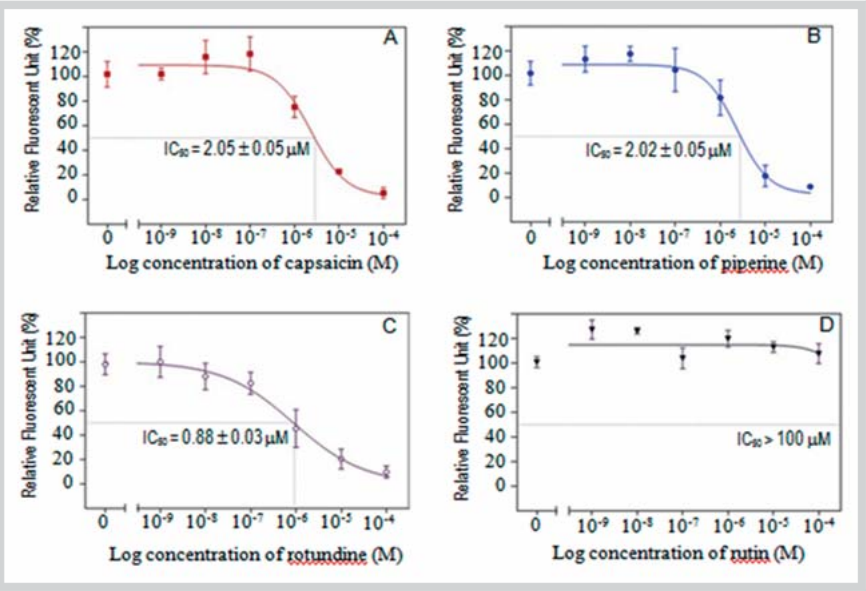

Fig. 6 Inhibition of agonist-induced hNK1R functional activity by lead compounds from plants. IC_{50} values of the following compounds were determined: capsaicin and piperine in fruits of *P. nigrum* (**A** and **B**, respectively), rotundine in tubers and roots of *S. cambodica* (**C**) and rutin in flower buds of *S. japonicum* (**D**).

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Conflict of Interest

The authors declare that there is no conflict of interest.

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