



# From medicinal plant extracts to defined chemical compounds targeting the histamine H<sub>4</sub> receptor: *Curcuma longa* in the treatment of inflammation

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Received: 27 April 2017/Revised: 2 June 2017/Accepted: 21 June 2017/Published online: 24 June 2017  
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## Abstract

**Objectives** The aim was to evaluate the activity of seven medicinal, anti-inflammatory plants at the hH<sub>4</sub>R with focus on defined chemical compounds from *Curcuma longa*.

**Materials** Activities were analyzed with membrane preparations from Sf9 cells, transiently expressing the hH<sub>4</sub>R, G<sub>αi2</sub> and G<sub>β1γ2</sub> subunits.

**Methods** From the methanolic extract of *C. longa* curcumin (**1**), demethoxycurcumin (**2**) and bis(4-hydroxycinnamoyl)methane (**3**) were isolated, purified with HPLC (elution-time 10.20, 9.66, 9.20 min, respectively) and together with six additional extracts, were characterized via radioligand binding studies at the hH<sub>4</sub>R.

**Results** Compounds from *C. longa* were the most potent ligands at the hH<sub>4</sub>R. They exhibited estimated  $K_i$  values of 4.26–6.26 μM (1.57–2.31 μg/mL) (**1**); 6.66–8.97 μM (2.26–3.04 μg/mL) (**2**) and 10.24–14.57 μM (3.16–4.49 μg/mL) (**3**) (95% CI). The estimated  $K_i$  value of the crude extract of curcuma was 0.50–0.81 μg/mL. Fractionated curcumin and the crude extract surpassed the effect of pure curcumin with a

$K_i$  value of 5.54 μM or 2.04 μg/mL [95% CI (4.47–6.86 μM), (1.65–2.53 μg/mL)].

**Conclusion** Within this study, defined compounds of *C. longa* were recognized as potential ligands and reasonable lead structures at the hH<sub>4</sub>R. The mode of anti-inflammatory action of curcumin was further elucidated and the role of extracts in traditional phytomedicine was strengthened.

**Keywords** *Curcuma longa* · Natural compounds · hH<sub>4</sub>R · Inflammatory diseases · Phenylpropanoids

## Introduction

The evaluation of the fourth human histamine receptor subtype (hH<sub>4</sub>R) revealed its crucial role in chemotaxis, inflammation and autoimmune disorders [1]. Drug research on the hH<sub>4</sub>R is highly interested in antagonists, due to their suggested potential in the treatment of inflammatory diseases [2].

Natural products have been a source for therapeutic agents for ages [3] and a high amount of currently used biologically active agents are natural product inspired compounds or their derivatives [4]. They are a well-studied source for pharmaceutical development and provide high hit rates in pharmacological screenings as lead-compounds [5]. Therefore, drug discovery and development are highly interested in novel drugs and bioactive lead-compounds out of nature's kitchen [6]. Plants and spices like curcuma, thyme, saffron, cumin, and ephedra species are well reported for their use in traditional medicine, especially against inflammatory events [7–11] and could meet the demands for drug development by supporting natural-based compounds for treating inflammatory diseases. A well investigated representative of these plants is *Curcuma*

Responsible Editor: Bernhard Gibbs.

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*longa* L., a perennial herb of the *Zingiberaceae* (ginger) family [12]. It has traditionally been used to treat various conditions [13] and extensive research in vitro and in vivo during the last few decades revealed that *C. longa* possesses anti-inflammatory effects [14]. Its inhibition of NF- $\kappa$ B (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), Akt-phosphorylation (protein kinase B), m-TOR activation (mechanistic target of rapamycin) and in contrast an activation of AMPK (5' AMP-activated protein kinase) in inflammatory events are well reported, but its mode of action is still not conclusively investigated [15].

The aim of the current study is to evaluate the activity of different plant extracts from traditional phytomedicine approaches at the hH<sub>4</sub>R with a focus on defined chemical structures from *C. longa*. All seven plants were investigated because of their traditional use against inflammatory events and could support the treatment of those. Especially curcuma's purified and isolated compounds display different structural features from any previously known ligands and may provide new directions for selective and specific scaffolds for the hH<sub>4</sub>R, opening new ways in treating inflammatory diseases. In doing so the anti-inflammatory mode of action of curcuma can be further elucidated, supporting its rational use in the adjuvant treatment of inflammatory diseases.

## Materials and methods

*Curcuma longa* L. for the first extract was purchased from Al Alim-Medicinal Herb Center (Zippori, Israel), all other herbs and *C. longa* for the repeated measurement were gathered by the Institute of the Qasemi Research Centre (Baka EL-Garbya, Israel). Pure curcumin was kindly provided by Prof. Dr. Gunter Eckert, Frankfurt/Germany, another batch as curcumin 2 was purchased from Sigma Aldrich.

### Plant extract preparation

Each extract stems from one plant sample, except for *C. longa*, that was extracted from two different samples. The respective plant parts were chosen, because of their most common use in traditional phytomedicine. 20 mL of methanol were added to 2 g of the dried plant material. Samples were sonicated for 120 min at 45 °C and left for 2 h. The liquid was made to pass through a 0.4  $\mu$ m filter and concentrated by a rotary vacuum evaporator.

### HPLC experiments

The analytical HPLC (High performance liquid chromatography) was a Waters Alliance (e2695 separations

module) with a 2998 Photodiode Array detector (PDA). The preparative HPLC system consisted of a 3535 quaternary gradient module with a 996 PDA detector. The analytical experiments were run on an ODS column of Waters (XBridge, 4.6 ID  $\times$  150 mm, 5  $\mu$ m, guard column of Xbridge ODS, 20 mm  $\times$  4.6 mm ID, 5  $\mu$ m). The mobile phase consisted of acidified water at pH 3 adjusted with phosphoric acid (A) and acetonitrile (B). Linear gradient started at 55% of (A) and 45% of (B) to reach 30% (A) and 70% (B) in 13 min then to 100% (B) in 1 min keeping it at 100% (B) for 5 min and back to 55% (A) in 1 min. PDA wavelengths ranged from 200 to 600 nm. The flow rate was set to 1 mL/min, the injection volume was 10  $\mu$ L and the column temperature was at room temperature. The three major fractions were reinjected to the prep-HPLC with an ODS column (Agilent PrepHT C18, 22.2  $\times$  250 mm, 10  $\mu$ m). The elution program was set as in the analytical mode, the flow rate was 20 mL/min and 1 mL injection volume. Fractionation of the methanolic crude extract of *C. longa* by preparative HPLC lead to isolation of three purified compounds (**1**, **2**, **3**) (purity >90%). The isolated compounds were further determined by HR-ESI-MS (High-resolution electrospray ionization mass spectrometry) in positive mode (LTQ-Orbitrap XL hybrid ion trap with a high resolution Orbitrap detection system, Thermo Scientific, USA).

### Membrane preparation

Cell culture and membrane preparation were performed according to Schneider and Seifert [16] to gain membrane preparations from Sf9 cells transiently expressing the hH<sub>4</sub>R, the G <sub>$\alpha$ i2</sub> and G <sub>$\beta$ 1 $\gamma$ 2</sub> subunits. Briefly, Sf9 cells were cultured in spinner flasks at 28 °C and 100 rpm in Sf9 medium [with 5% (v/v) fetal bovine serum and 10  $\mu$ L/mL penicillin/streptomycin].  $3 \times 10^6$  cells/mL was infected with a baculovirus solution (1:100), containing the human histamine H<sub>4</sub> receptor and the G <sub>$\alpha$ i2</sub> and G <sub>$\beta$ 1 $\gamma$ 2</sub> subunits. 48 h after infection cells were harvested and membrane preparation was performed at 4 °C. The infected Sf9 cells were centrifuged at 1000 rpm for 10 min. The resulting pellet was resuspended in phosphate buffered saline (PBS) and centrifuged again at 1000 rpm for 10 min. Afterwards the pellet was resuspended in 15 mL lysis buffer [80  $\mu$ M benzamidine, 20  $\mu$ M leupeptin, 200  $\mu$ M phenylmethyl sulfonyl fluoride (PMSF), in H<sub>4</sub> binding buffer (13 mM MgCl<sub>2</sub>, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4)] per 100 mL cell suspension. After homogenization in a hand potter the suspension was centrifuged at 500 rpm for 5 min and the supernatant was centrifuged for 20 min at 18,000 rpm. The resulting pellet was resuspended in lysis buffer, homogenized and centrifuged for 20 min at 18,000 rpm. The pellet was

resuspended in 12 mL H<sub>4</sub> binding buffer per 100 mL cell suspension, homogenized and stored as 1 mL aliquots at -70 °C.

**[<sup>3</sup>H]Histamine competition binding assay**

The assays were performed as published recently [17]. The extracts (A)–(F) were tested at fixed concentrations of 5 µg/mL in two independent experiments to allow a rough screening. The crude extract of curcuma and its three isolated compounds were tested at 10 µg/mL in three independent experiments, performed in triplicates for one-point measurements. For concentration–response curves, the curcuma extract and compounds (2)–(3) were tested at four concentrations ranging from 0.3 to 10 µg/mL allowing an estimation of the 95% confidence intervals of the K<sub>i</sub> values. Compound (1) and pure curcumin were tested at six and eight distinct concentrations, respectively (0.01 ng/mL–100 µg/mL). A second crude extract of curcuma was tested at eleven concentrations (0.01 ng/mL–100 µg/mL) and the testing of pure curcumin (curcumin 2) was repeated in ten concentrations (0.01–10 ng/mL), allowing determination of the K<sub>i</sub> values for the latter four substances. The used concentrations were chosen as standard logarithmic or half-logarithmic concentrations, limited by the solubility of the given compounds in the assay buffer. Bound radioligand was separated from free radioligand by filtration with a cell harvester (Inotech, Brandon, FL). The amount of bound radioactive ligand collected on the filtermats was determined by liquid scintillation counting with a Betacounter Trilux (Perkin Elmer, Waltham, MA, USA).

**Data**

HPLC Data acquisition used Empower 3 chromatography data software (Waters, Eschborn, Germany). Values of one-point measurements were calculated relative to specific binding. Concentration–response competition binding data were analyzed with Prism 6 (GraphPad

**Table 1** Composition of plant extracts tested at the hH4R

Plant	Part of the plant	Abbreviation
<i>Crocus sativus</i>	Flower	(A)
<i>Cuminum cyminum</i>	Seed	(B)
<i>Nigella sativa</i>	Seed	(C)
<i>Thymus serpyllum</i>	Leaf	(D)
<i>Cuminum karamani</i>	Seed	(E)
<i>Ephedra foeminea</i>	Stem	(F)
<i>Curcuma longa</i>	Rhizome	Crude extract

**Table 2** Percentual inhibition of radioligand binding at hH<sub>4</sub>R of extracts (A)–(F) (5 µg/mL), the extract of curcuma and the compounds (1)–(3) (10 µg/mL)

Inhibition (%)	Extract (A)	Extract (B)	Extract (C)	Extract (D)	Extract (E)	Extract (F)	Curcuma extract	Compound (1)	Compound (2)	Compound (3)
Mean	2.99	7.71	8.11	8.86	11.05	20.62	102.00	88.50	76.93	71.40
Lower limit to upper limit	-2.60 to 7.60	-2.60 to 26.67	-8.00 to 19.73	5.20 to 11.80	4.60 to 23.20	10.60 to 33.62	98.03 to 106.00	86.26 to 90.74	68.86 to 85.01	53.02 to 89.78
n	2	2	2	2	2	2	3	3	3	3

n = number of independent experiments performed with the same extract, each performed in triplicates

Software Inc., San Diego, CA, USA). The  $K_i$  values were calculated from the  $IC_{50}$  values using the Cheng–Prusoff equation [18]. Values of binding data obtained are means of three experiments, each performed as triplicates. Values of binding data obtained by eleven and ten point measurements are means of three independent experiments run in at least duplicates. Binding data is given as 95% confidence intervals and are complemented by means for six, eight, ten and eleven point measurements. One-point measurements are reported as means with the respective ranges.

## Results

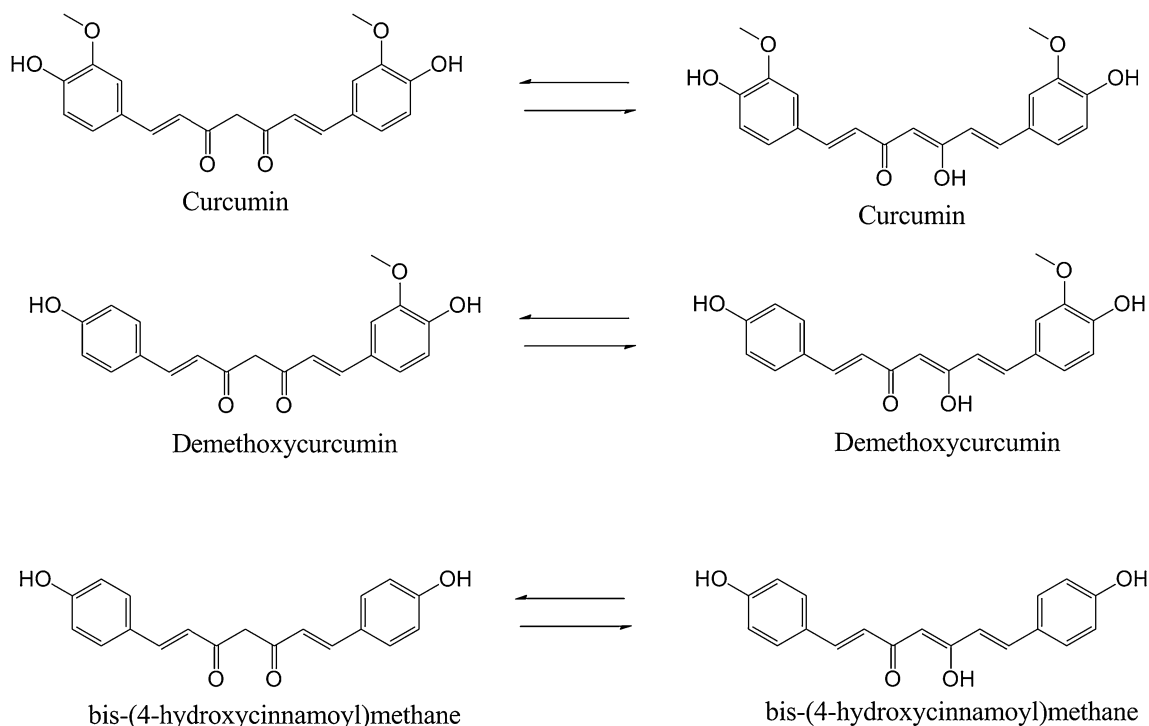
The methanolic extracts of *C. longa*, *Crocus sativus*, *Nigella sativa*, *Thymus serpyllum*, *Cuminum karamani* (*Carvon coptikum*), *Cuminum cyminum* and *Ephedra foeminea* (Table 1) were tested for their inhibition of radioligand binding at the  $hH_4R$  from purified membrane preparations.

The plant extracts displayed poor activity at the receptor (<50% inhibition at 5  $\mu\text{g/mL}$ ), with the exception of the methanolic extract of *C. longa* that showed strong activity (complete inhibition at 10  $\mu\text{g/mL}$ ) (Table 2). As extracts (A)–(F) showed low affinities, their further evaluation was discontinued after two independent experiments in this rough pre-screening.

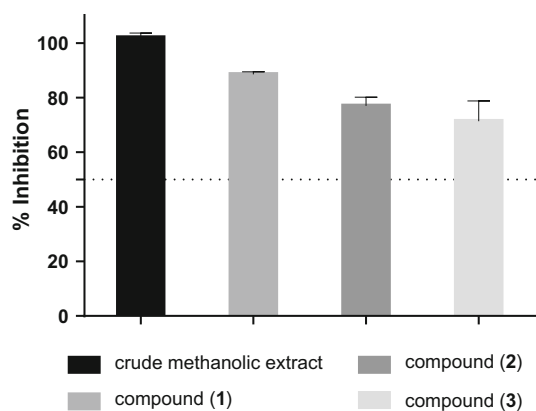
Due to its superior affinity the methanolic curcuma extract was further purified, which resulted in three chemically defined compounds (1–3). Curcumin (1), demethoxycurcumin (2), and bis-(4-hydroxy-cinnamoyl)methane (3), 1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (Fig. 1) were collected using a preparative HPLC, eluting at 10.20 (1), 9.66 (2) and 9.20 min (3), respectively (purity >90%), and showing UV–Vis spectra with  $\lambda_{\text{max}}$  at 425 nm (1), 421 nm (2) and 416 nm (3).

The compounds and the crude extract were able to inhibit radioligand binding at the  $hH_4R$  by more than 70% in measurements at 10  $\mu\text{g/mL}$ . Compound (1) showed highest inhibition of the three isolated compounds with 89% (1) >77% (2) >71% (3) inhibition, while the strongest activity was shown for the crude extract with a complete inhibition of radioligand binding (adjusted  $p$  value <0.05) (Fig. 2; Table 2). Statistical analysis showed, that the comparison of the crude extracts activity against the three compounds (1)–(3) was significant (adjusted  $p$  values <0.05), activity comparison of compound (1) against (2) was significant (adjusted  $p$  value = 0.03) and comparisons of activities from compound (1) against (3) and compound (3) against (2) were not significant.

The tested compounds and the crude extract showed clear concentration–response effects in radioligand binding studies. Competition binding assays resulted in 95% confidence intervals for the estimated  $K_i$  values ranging



**Fig. 1** Chemical structures of the fractionated compounds, curcumin, demethoxycurcumin, and bis-(4-hydroxy-cinnamoyl)methane



**Fig. 2** Percentual inhibition of radioligand binding at h<sub>4</sub>R of curcumas extract and fractions (1)–(3) (10 µg/mL)  $n = 3$  independent experiments each performed in triplicates. Data are given as mean with the respective ranges

between 1.57–2.31 µg/mL (1), 2.26–3.04 µg/mL (2) and 3.16–4.49 µg/mL (3). This led to molar values of 4.26–6.26 µM (1), 6.66–8.97 µM (2) and 10.24–14.57 µM (3), respectively (Table 3), for the purified compounds. Highest activity was shown for the crude extract with an estimated 95% confidence interval for the  $K_i$  value spanning 0.50–0.81 µg/mL, indicating a synergistic effect of all compounds.

It is noteworthy that the crude extract as well as the isolated curcumin (1) showed stronger binding to the h<sub>4</sub>R than pure curcumin with a  $K_i$  value of 5.54 µM [95% CI (4.47–6.86 µM)] or 2.04 µg/mL [95% CI (1.65–2.53 µg/mL)]. For verification, the evaluation was repeated with a second extract preparation from *Curcuma longa*. It displayed a  $K_i$  value of 9.36 µg/mL [95% CI (4.96–17.68 µg/mL)], while a second batch of pure curcumin (curcumin 2) confirmed the first measurement with a measured  $K_i$  value of 4.97 µM [95% CI (3.26–7.57 µM)] or 1.83 µg/mL [95% CI (1.20–2.79 µg/mL)] (Fig. 3; Table 3).

## Discussion

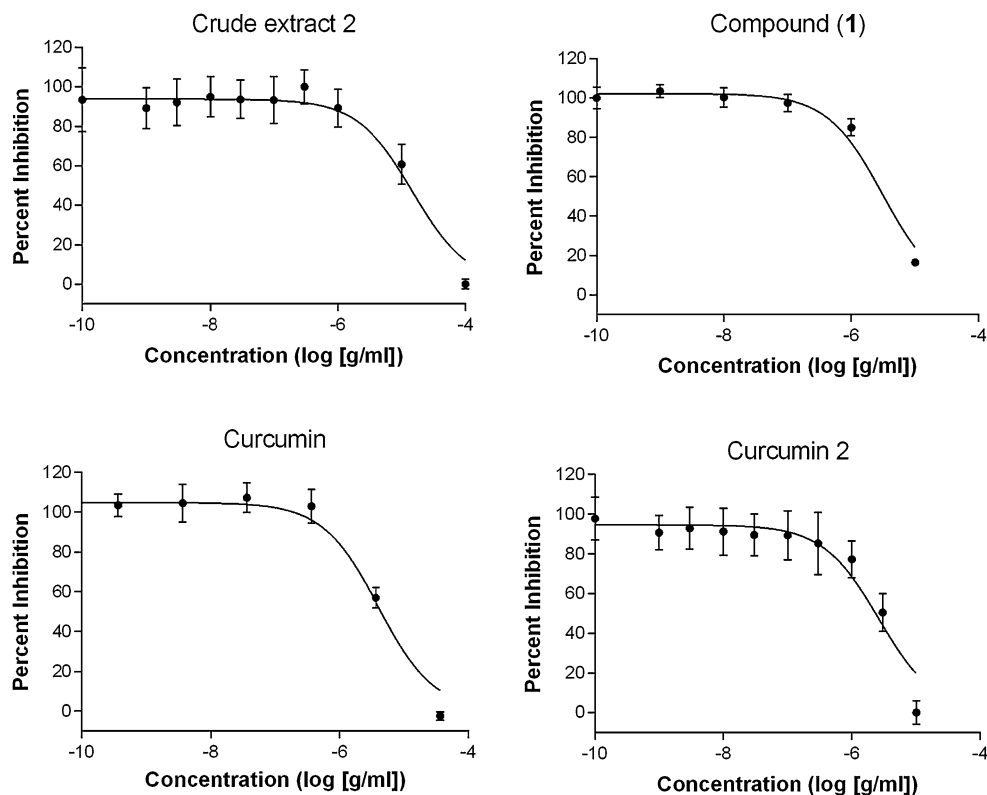
The study showed a superiority of *C. longa* crude extract and isolated curcumin in contrast to pure curcumin. This could be due to the presence of glycosides, complex carbohydrates or associated plant compounds like other

polyphenols, that are not detectable in the used detector system, but are known for inheriting anti-inflammatory effects [19]. Although not yet tested at the h<sub>4</sub>R, their attendance could result in synergistic effects in the in vitro testing-systems. The superiority of extracts in comparison to purified compounds is well reported [20] and could be one reason why clinical studies that used pure curcumin instead of extracts cannot confirm the in vitro activity often reported. The discrepancy in activity between different batches of the same extract may be caused by the natural deviation of compound composition in plants. The compounds concentration and composition strongly depends on the environmental conditions, gathering seasons and extraction conditions. Though a thorough standardization method is essential for the therapies success when using plant extracts. Although JNJ-7777120, a well-studied reference h<sub>4</sub>R antagonist, displays a  $K_i$  value of 22.26 nM, compounds showing activity in low micromolar concentrations could serve as potential lead structures for designing optimized and structurally novel h<sub>4</sub>R ligands [21]. Achievable plasma concentrations of curcumin in in vivo testings are in the same range as affinities in our in vitro H<sub>4</sub> studies of curcumins methanolic extracts and isolated compounds. Thus, an interaction with the H<sub>4</sub> receptor has to be considered when assessing anti-inflammatory effects of curcumin and its extracts. In humans peak plasma levels of curcumin after the oral dosing of 8 g were 1.77 µM [22]. The simultaneous application of piper alkaloids (2 g/kg curcumin + 20 mg/kg alkaloids) resulted in maximal serum concentrations of 0.18 µg/mL [23] and special formulations like nanocurcumin [24] or poly lactic-co-glycolic acid encapsulated curcumin [25] are on the rise to improve its bioavailability. Complex carbohydrates that are attendant in most extracts could further enhance bioavailability of the extracts [26]. At the same time curcumin displays very low toxicity even at high doses up to 12 g per day [22] allowing application of reasonable therapeutic doses in anti-inflammatory treatment. Although curcumin has traditionally been used against inflammatory diseases and in vitro as well as in vivo data supports the anti-inflammatory effects, the clinical effectiveness as well as the main molecular target still needs to be proven. This led to a wide discussion whether or not the pharmacological use of curcumin and its derivatives should be

**Table 3** Estimated 95% CI and  $K_i$  values of curcumas crude extract, the compounds (1)–(3) and pure curcumin

	Crude extract	Crude extract 2	Curcumin	Curcumin 2	Compound (1)	Compound (2)	Compound (3)
$K_i$	–	9.36	2.04	1.83	1.90	–	–
(95% CI) µg/mL	(0.50–0.81)	(4.96–17.68)	(1.65–2.53)	(1.20–2.79)	(1.57–2.31)	(2.26–3.04)	(3.16–4.49)
MW g/mol	–	–	368.39	368.39	368.39	338.36	308.33
$K_i$	–	–	5.54	4.97	5.17	–	–
(95% CI) µM	–	–	(4.47–6.86)	(3.26–7.57)	(4.26–6.26)	(6.66–8.97)	(10.24–14.57)

**Fig. 3** Concentration–response curves for the crude extract 2, compound (1), curcumin and curcumin 2. Data are given as mean with the respective 95% CI. All assays were performed in three experiments, each run at least in duplicates



recommended and how in vitro investigations may be interpreted [27]. Certainly pure curcumin will not be the miracle cure of inflammations, but considering the comprehensive scientific data of its activity against inflammatory targets [15] the adjuvant treatment of inflammation with curcumin can be considered. Thereby, the bioavailability of a given formulation, the composition of bioactive compounds in an extract or capsule and a potential affinity optimization of the lead-compounds could be taken into account.

In conclusion, our research reveals that among other inactive plant extracts, *C. longa* and three of its isolated compounds display affinity at the hH<sub>4</sub>R, despite not showing typical structure motifs [28]. They are not positively charged at physiological pH value and do not contain the aminergic structure of typical imidazole or non-imidazole hH<sub>4</sub>R ligands. Thus, dicinnamoyl methanes might depict reasonable lead structures for novel scaffolds in hH<sub>4</sub>R targeting. Further evaluation of different derivatives will be necessary to allow a thorough structure–activity relationship evaluation, as there are only minor structure differences between the three compounds.

Although the hH<sub>4</sub>R plays an important role in chemotaxis, inflammation and autoimmune disorders, at recent date there are no approved drugs on the market, leaving the development of new ligands at this receptor highly desired. We found

that the crude extract of *C. longa* displays higher potency than its purified compounds. This illustrates the advantages of using complex extracts in pharmaceutical therapy as they sometimes surpass their isolated compounds in therapeutic potency due to synergistic effects [29]. When using extracts in pharmacotherapy it is important to take care of the naturally deviating compound concentrations in plants and a thorough standardization method of extracts is essential for a phytotherapy's success. The conclusive data of in vitro as well as in vivo results of pharmacologic investigations can support the use of *C. longa*, especially extracts, in the adjuvant treatment of inflammatory diseases when combined with modern, orthodox medicine.

Within this study, we were able to evaluate seven different natural extracts at the hH<sub>4</sub>R and identified curcumin derivatives as potential lead for future drug development. Thereby, we further characterized curcumins mode of action on molecular targets in fighting inflammatory events and extended the lead structure database of biologically active compounds at the hH<sub>4</sub>R.

**Acknowledgements** This study was supported by the Al-Qasemi Research Foundation, the Ministry of Science, Space and Technology, BM0806, CM1204 and CA15125 COST Actions as well as DFG INST 208/664 and GRK2158. We declare that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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