Bioorganic & Medicinal Chemistry 18 (2010) 3299–3306

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

In vitro TRPV1 activity of piperine derived amides

Edwin Andrés Correa [a], Edward D. Högestätt [b], Olov Sterner [c], Fernando Echeverri [a,*], Peter M. Zygmunt [b]

[a] Grupo de Química Orgánica de Productos Naturales, Instituto de Química, Universidad de Antioquia, Medellín, Colombia
[b] Division of Clinical Chemistry and Pharmacology, Lund University, Skåne University Hospital, SE-221 85 Lund, Sweden and Lund University Pain Research Centre, Sweden
[c] Division of Organic Chemistry, Lund University, PO Box 124, SE-221 00 Lund, Sweden

ARTICLE INFO

Article history:
Received 5 January 2010
Revised 4 March 2010
Accepted 6 March 2010
Available online 11 March 2010

Keywords:
Piperine
Piperine-derived amides
Transient receptor potential vanilloid 1
Fluorometric assays
Pain
Analgesic activity

ABSTRACT

A series of natural and synthetic piperine amides were evaluated for activity on the human TRPV1 expressed in HEK293 cells. The agonistic effect of piperine amides was mainly dependent on the length of the carbon chain. Structural changes of double bonds and stereochemistry in the aliphatic chain of these compounds did not change their potency or efficacy, indicating that increased rigidity or planarity of the piperine structure does not affect the activity. The opening of the methylenedioxy ring or changes in the heterocyclic ring of the piperine molecule reduced or abolished activity. Furthermore, inactive compounds did not display functional antagonistic activity.

1. Introduction

The *Piper* genus (Piperaceae) is a very important source of biologically active secondary metabolites.[1] It has been reported that molecules like alkaloids, lignans, isobutyl and piperidinic amides possess insecticidal, antiparasitic and enzyme inhibitory properties.[2] Other species of this genus are used in cooking for their spicy properties and pungency (*Piper nigrum, Piper longum*).[3] Piperine is contained within the seeds of *P. nigrum* (black pepper) and mainly responsible for the pungency of this spice.[4,5] This compound was found to be a strong activator of the non-selective cation channel transient receptor potential vanilloid 1 (TRPV1),[6] which in 1997 was identified as the receptor for capsaicin, the pungent ingredient in chili pepper.[7] In fact, piperine turned out to be a more effective activator than capsaicin of the human orthologue of TRPV1 (hTRPV1), which quickly desensitized in response to piperine but not capsaicin.[6]

TRPV1 is also activated by heat, protons, anandamide and other endovanilloids as well as indirectly by pro-inflammatory agents, such as bradykinin and prostaglandins, acting on G protein-coupled receptors.[8–10] The presence of TRPV1 on neurons involved in pain sensation and other tissue protective mechanisms has provided new avenues for development of drug treatment of pain, bladder hyperreflexia and airway hyperreactivity.[10–13] Not only antagonists, but also agonist of TRPV1 are presently being evaluated for treatment of pain and sensory hyperreactivity.[10,11,13] Interestingly, systemic administration of the TRPV1 agonists capsaicin, olvanil and SDZ 249-6654 has been shown to produce an acute antinociceptive effect in animals.[14–16] The initial excitation evoked by TRPV1 agonists, such as capsaicin and resiniferatoxin, is followed by a prolonged refractory period during which the neuron is unresponsive to stimulation.[11] This and other mechanisms may explain the analgesic effect of TRPV1 agonists, including the pain relief afforded by topical application of capsaicin in patients with neuropathic pain.[11]

Piperine is considered as an interesting chemical template for design of TRPV1 agonists with no or little pungency that can be developed into clinically useful drugs. However, very little information is available on the structure–activity relationship for piperine on hTRPV1. In this report we describe the isolation and pharmacology of piperine (5-(3′,4′-methylenedioxy phenyl)-2*E*,4*E*-pentadienoic acid piperidin amide) **1** and $\Delta^{\alpha,\beta}$-dihydropiperine (5-(3′,4′-methylenedioxy phenyl)-2*E*-pentenoic acid piperidin amide) **7** from *Piper tuberculatum*, and 5-(3′,4′-methylendioxy phenyl)-2*E*,4*E*-pentadienoic acid isobutyl amide **4** from *Piper holtonii*. The latter compound is also present in other *Piper* species.[17,18] In traditional medicine, these plants have been used as sedatives and for the treatment of toothaches and snake bites.[19] In addition, a series of piperine derivatives were prepared and their TRPV1 activities were evaluated in HEK293 cells expressing the hTRPV1,

* Corresponding author. Tel.: +57 42196595; fax: +57 42196565.
E-mail addresses: echeveri@udea.edu.co, echeveri@quimbaya.udea.edu.co, feche@une.net.co (F. Echeverri).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.03.013

using a fluorometric calcium assay. Compounds found inactive as agonists were further tested for functional antagonist activity.

2. Results and discussion

2.1. Chemistry

Several piperine derivatives were prepared, using a simple coupling method for amide synthesis, and tested for TRPV1 activity. Three main parts of the piperine structure **1** are shown in Figure 1; an aromatic ring moiety, an aliphatic chain and the amide substitution. Several amides were prepared with an appropriate acid, taking into account aromatic substitution as well as chain length and degree of saturation. The coupling to an amine was achieved according to the generic peptide synthesis methodology with dicyclohexylcarbodiimide or diisopropylcarbodiimide in presence of 1-hydroxybenzotriazol.[20–22] This methodology is simple and does not require multiple reaction steps and strong reagents, such as the classical amide formation reactions and thionyl chloride, respectively. To synthesize the 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*,4*E*-pentadienoic acid, a modified Knoevenagel reaction was used, because of its selectivity for *E* configurations in the formation of the new double bond.[23,24]

2.2. Biological evaluation

2.2.1. Fluorometric Ca^{2+} measurements in HEK 293 cells expressing hTRPV1

Piperine was characterized previously as a TRPV1 agonist using the whole cell configuration of the patch clamp technique.[6] Here, we isolated two known natural piperine analogues and prepared several derivatives (Fig. 2), some of which have previously been isolated from natural sources or synthesized.[25–28] An initial screening of the effect of piperine and its derivatives on hTRPV1 was carried out to detect active and inactive derivatives. A drug concentration of 100 μM was used in these tests. A full concentration–response curve was then constructed for compounds that evoked an agonist response above 50% in the initial screening.

Compounds **1**, **2**, **7**, **8**, and **9** displayed pronounced calcium responses in HEK293 cells induced to express hTRPV1, but were without effect in non-induced cells (Fig. 3). These compounds contain a heterocyclic ring and, except for compound **9**, possess a four carbon atoms chain between the aromatic ring and the amide group. Interestingly, compounds **3** and **4** were inactive although sharing the same chain length and aromatic substitution, possibly because of an isobutylamine or chlorobenzene present in the amide-linked group, respectively. The same seems to be the case when comparing compounds **9** and **10**, and thus structural changes in the amide linkage seem important for the agonistic activity. The lack of activity of compound **3** may indicate that the volume of the amide substituent and/or the charge density of the nitrogen atom in the amide are of importance for activity. Furthermore, a comparison of compounds having an aromatic methylene-dioxy group and a four carbon atoms middle chain with compound **5** having a *p*-hydroxy-3-methoxy group and the same chain length, suggest that substitution in the aromatic ring also affects agonistic activity (Fig. 3). This is further evident when comparing compound **9**–**11,** although these compounds have a shorter middle carbon chain (two carbon atoms).

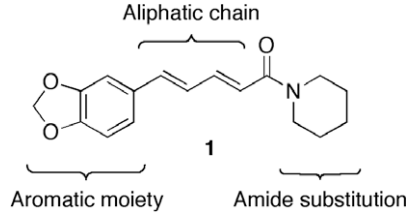

Figure 1. The piperine structure divided into three key regions.

Compound **11** together with other compounds having a two carbon atoms chain, such as compounds **12**–**14** (Fig. 2), were lacking agonistic activity (Fig. 3), once again indicating the importance of an intact aromatic methylene-dioxy group for activity. However, the middle chain length may also be of importance as a four carbon atoms chain rescued some of the activity as shown for compounds **5** and **6** compared to compounds **14** and **11**. The role of configuration and saturation of the double bonds located in the middle chain was also considered. As shown in the present study, $\Delta^{\alpha,\beta}$-dihydropiperine **7** and compound **8** were as active as piperine **1**, indicating that the presence of the conjugated diene (*E* configuration) is not necessary for TRPV1 agonistic activity. Experiments were carried out to determine the concentration–response relationship for compounds that exhibited an agonistic effect over 50% at 100 μM in the initial screening (Fig. 4A). Compounds **1, 2, 7 and 8** showed very similar EC_{50} and E_{max} values (Table 1). Therefore structural changes such as double bonds and stereochemistry in these compounds did not change the potency or efficacy and thus increased rigidity or planarity of the piperine structure does not seem to affect the activity. Compound **9**, with a shorter middle chain was less active than the others. Taking into account that the chain length is the only difference from piperine chain length is probably responsible for the decreased potency and efficacy (Table 1).

To explore the possibility that the inactive compounds **3, 4, 11**–**14** act as TRPV1 antagonists, HEK293 cells expressing hTRPV1 were incubated in the presence of these compounds at 100 μM for 30 min prior to exposure to various concentrations (10^{-11}–10^{-5} M) of capsaicin (Fig. 4C). The TRPV1 antagonist capsazepine (10 μM) was also tested to confirm that the ability to inhibit TRPV1 mediated responses could be detected. The above experiments did, however, not disclose any antagonistic properties of these compounds (Table 1), which thus seem to lack affinity for hTRPV1.

The region and amino acids of the TRPV1 ion channel protein that are required for activation of TRPV1 by piperine and some of its analogues remain to be identified. Gavva et al. recently described a model for the interaction of TRPV1 with vanilloids, such as capsaicin and resiniferatoxin.[29] Considering the structures of piperine and its analogues, it is not obvious how these compounds would fit into this model. In the present study, we found that the methylene-dioxy group is essential for activity of the piperine amides. The opening of this group, which creates a vanilloid structure, reduced or abolished the activity. Furthermore, capsaicin becomes inactive when its aromatic structure includes a methylene-dioxy group.[30] Radioligand binding studies with piperine showed no displacement of [^{3}H]-resiniferatoxin binding at concentrations 10 times higher than that causing maximal activation of hTRPV1 in the present study.[31] In line with a previous study,[6] we found that piperine as well as some of its active analogues displayed higher Hill coefficients compared to capsaicin for the activation of hTRPV1. This together with the synergistic effect between piperine and capsaicin[6] indicates that these compounds may activate hTRPV1 by different mechanisms. These differences in pharmacological profile between piperine amides and vanilloids suggest that piperine binds to TRPV1 at a site different from the vanilloid binding site.

In contrast to the study by McNamara et al.,[6] we found no difference in efficacy between piperine and capsaicin. Both agonists produced responses of similar magnitude and a subsequent application of ionomycin always produced a slightly larger response. The reason for this discrepancy is not known, but could

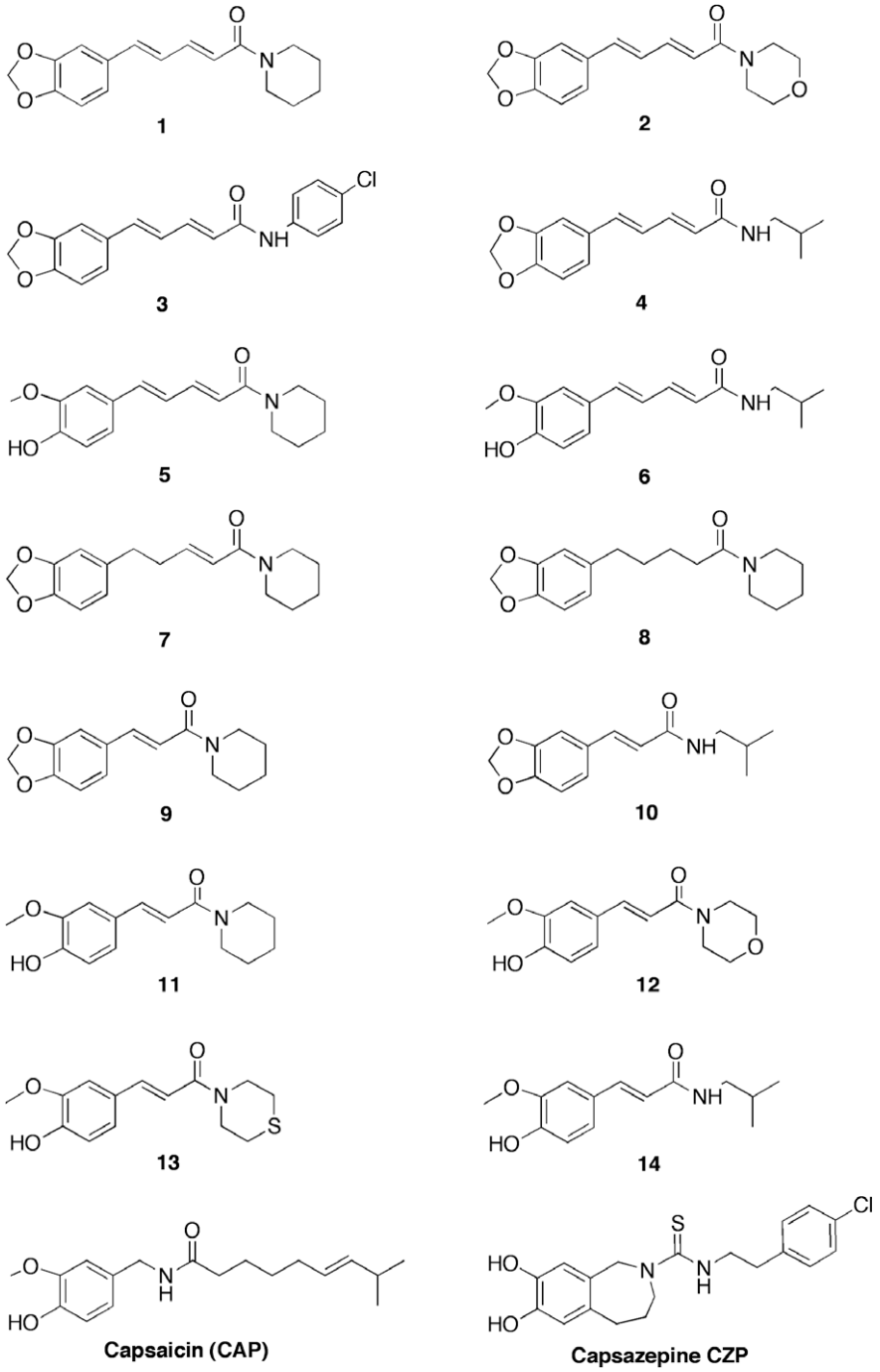

Figure 2. Chemical structures of natural piperine analogues, synthetic derivatives, capsaicin (**15**) and capsazepine (**16**). Compounds **1**, **7** were isolated from *P. tuberculatum* and **4** from *P. holtonii*.

be due to the different bioassays (calcium imaging or patch clamp electrophysiology) used to record TRPV1 activity. Notably, piperine was found to be a partial agonist of native rat TRPV1.[32] Nevertheless, the synergistic action between piperine and capsaicin, as shown by McNamara et al.,[6] would fit with separate binding sites for piperine and capsaicin on hTRPV1.

Interestingly, the finding that compound **9** is less effective than the other piperine analogues in activating hTRPV1 shows that small modifications of the piperine structure can be used to design partial agonists. Such partial agonists may have no or little pungency and thus may prove useful in TRPV1-based drug therapies for treatment of pain and sensory hyperreactivity.[6,33,34]

3. Experimental

3.1. Chemistry

3.1.1. General procedures

All reagents including starting materials were purchased from Sigma–Aldrich. ^{1}H NMR spectra were recorded with a Bruker DRX at 400 MHz and ^{13}C at 100 MHz and a Bruker AMX 300 MHz ^{1}H and 75 MHz ^{13}C, $CDCl_3$ and CD_3COCD_3 (Sigma–Aldrich) were used as solvents and TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in hertz. Melting points were measured in open capillaries (Mel-Temp®,

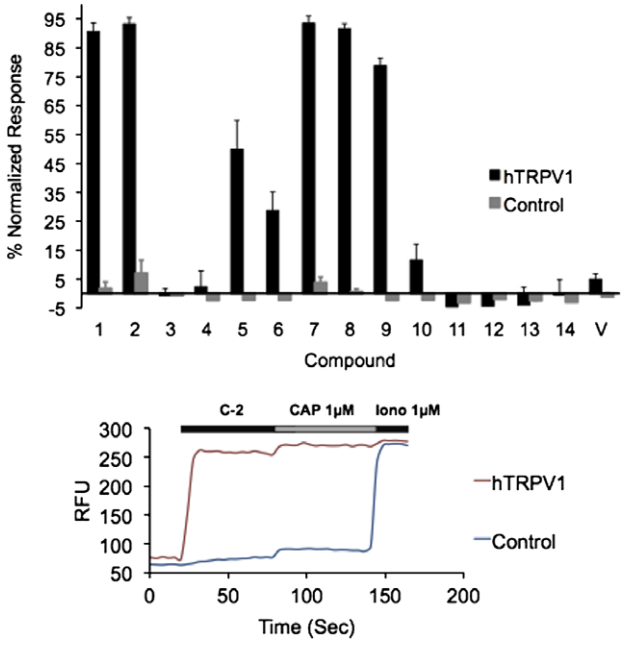

Figure 3. Effect of piperine derivatives on hTRPV1 expressed in HEK293 cells. The test concentration was 100 μM for all compounds except **3** which was tested at 10 μM due to poor solubility. The vehicle DMSO 0.1% (V) was also tested. The responses were normalized as a percentage of the 1 μM capsaicin response in cells expressing hTRPV1 and as a percentage of 1 μM ionomycin response in cells not expressing hTRPV1 (Control). Data are expressed as mean ± SEM (n = 4–6). The average traces (each line represents the mean peak response of three experiments) of compound **2** (100 μM) show the experimental design used to evaluate agonistic activity.

Table 1
Fluorometric determination of EC_{50}, E_{max} and Hill coefficient values for the most active compounds tested in HEK293 cells expressing hTRPV1

Compound	pEC_{50} [a]	E_{max} [b]	Hill coefficient	n
1	5.5 ± 0.04	91 ± 3	1.92 ± 0.36	4
2	5.6 ± 0.03	93 ± 7	1.72 ± 0.22	4
7	5.4 ± 0.09	88 ± 4	1.07 ± 0.24	4
8	5.2 ± 0.05	90 ± 2	1.47 ± 0.22	4
9	4.7 ± 0.04	66 ± 2	3.94 ± 0.73	3
Capsaicin + **3**	8.0 ± 0.07	94 ± 2		3
Capsaicin + **4**	8.2 ± 0.06	94 ± 3		3
Capsaicin + **11**	8.7 ± 0.07	93 ± 2		3
Capsaicin + **12**	8.6 ± 0.07	92 ± 2		3
Capsaicin + **13**	8.6 ± 0.10	93 ± 5		3
Capsaicin + **14**	8.2 ± 0.06	94 ± 2		3
Capsaicin + capsazepine	5.6 ± 0.03	92 ± 1		3
Capsaicin	8.6 ± 0.03	97 ± 1	1.12 ± 0.08	8

Responses of compounds **1, 2, 7, 8 and 9** were normalized as a percentage of a 1 μM capsaicin response. EC_{50} and E_{max} values for capsaicin in the presence of compounds without agonist activity and the TRPV1 antagonist capsazepine are also given. The capsaicin response was normalized as a percentage of the 1 μM ionomycin response. Data are expressed as mean ± SEM.

[a] The −log of the compound concentration eliciting half maximal response.

[b] The maximal increase in fluorescence as a percentage of 1 μM capsaicin or 1 μM ionomycin.

Electrothermal) and are uncorrected. IR was measured in KBr pellets in a FTS 6000 spectrometer (BioRad). ESI-TOF MS were recorded in a Waters Micromass Q-TOF spectrometer. Silica Gel 60 (200–300 mesh) and Silica Gel 60 F_{254} (Merck) were used for column and analytical thin layer chromatography (TLC, 0.25 mm).

3.1.2. Plant material

P. tuberculatum fruits were collected in San José del Nus (Antioquia-Colombia) in March 2006, voucher specimen (HUA 145354). *Piper holtonii* was collected in Armenia (Antioquia-Colombia) in October 2006, voucher specimen (HUA 168715), both were deposited in the herbarium of the University of Antioquia (Medellín—Colombia).

3.1.3. Extraction and isolation

3.1.3.1. Piperine (1) and $\Delta^{\alpha,\beta}$-dihydropiperine (7). Six-hundred grams of dry *P. Tuberculatum* fruits were extracted exhaustively with methanol (MeOH, 2 L), the solvent filtrated and evaporated giving 5.2 g of crude extract. One gram of crude extract was fractionated over silica gel using ethyl acetate (EtOAc)/petroleum ether (PE) 4:1 [500 mL], 3:1 [200 mL], 2:1 [150 mL], 1–1

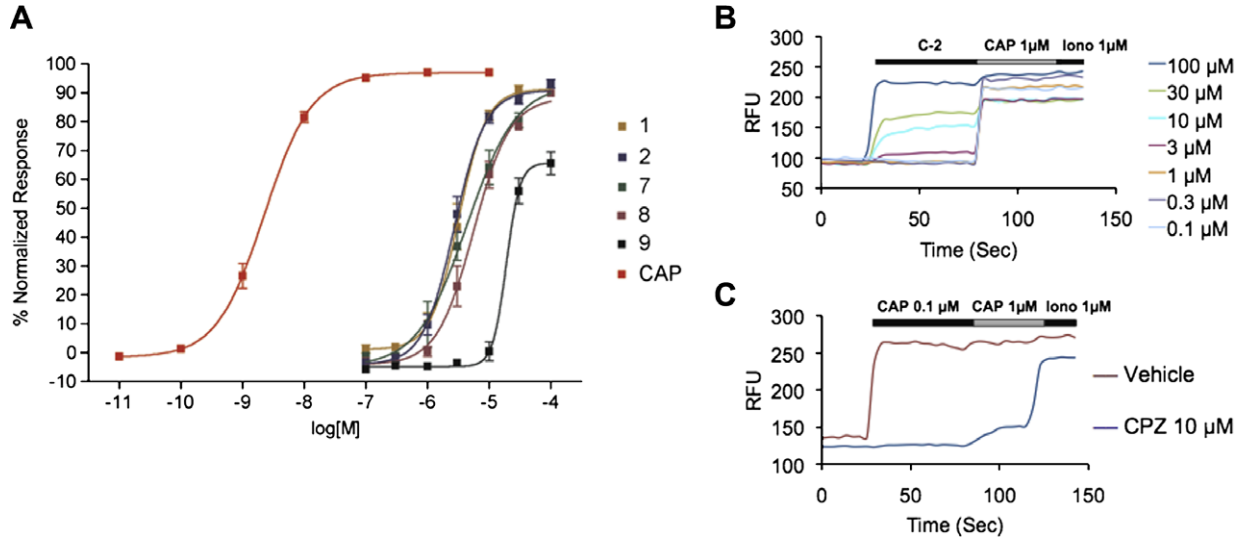

Figure 4. (A) Concentration–response curves for the hTRPV1 active compounds **1, 2, 7, 8, 9** and capsaicin (CAP). Data are expressed as mean ± SEM (n = 3–4). The responses were normalized as a percentage of a 1 μM capsaicin response. (B) Average traces of concentration-dependent responses for compound **2**, and the subsequent addition of capsaicin and ionomycin illustrates the experimental design used to evaluate agonistic activity. (C) Capsaicin responses in the presence of the TRPV1 antagonist capsazepine (CPZ) at 10 μM, showing the experimental design used to evaluate antagonistic activity of compounds **3, 4, 11–14**. The lines represent mean peak responses of three experiments.

[200 mL], the fractions 22–31 showed almost the same composition (TLC PE/EtOAc 3:1 and 1:1) and were subjected twice to a preparative TLC chromatography under the elution mixture: PE/acetone 9:1. Piperine (**1**) 11.9 mg and $\Delta^{\alpha,\beta}$-Dihydropiperine (**2**) 8.9 mg were obtained as yellow oils.

3.1.3.2. Isolation of piperine (1) from black pepper. Five-hundred grams of air dried black pepper seeds (commercially available) were extracted with ethanol and the solvent evaporated, the crude extract was fractionated over sephadex LH-20 and several fractions collected to afford 2 g of pale yellow crystals, identified by ^{1}H NMR as piperine.

3.1.3.3. Isolation of 5-(3′,4′-methylenedioxy phenyl)-2*E*,4*E*-pentadienoic acid isobutyl amide (4) from *Piper holtonii*. Eight-hundred and twenty grams of *Piper holtonii* air dried roots were extracted with 3 L of ethanol to gave 10 g of crude extract after filtration and evaporation of the solvent; 1.5 g of the extract was further fractionated over silica gel and by successive chromatographies using petroleum ether and acetone, 8.4 mg of compound **3** were obtained as a yellow oil. IR (KBr cm^{-1}): 2963, 1617, 1515, 1265, 1033, 979. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.95 (6H, d, J = 6.7 Hz, H-3″,4″), 1.83 (1H, m, H-2″), 3.19 (2H, t, J = 6.4 Hz, H-1″), 5.69 (1H, t, J = 6.3 Hz, –NH), 5.95 (1H, d, J = 14.8 Hz, H-2), 5.98 (2H, s, –O–CH_2–O–), 6.68 (1H, dd, J = 10.6, 15.4 Hz, H-4), 6.75 (1H, d, J = 15.6 Hz, H-5) 6.78 (1H, d, J = 8.0 Hz, H-5′), 6.89 (1H, dd, J = 1.7, 8.0 Hz, H-6′), 6.98 (1H, d, J = 1.7 Hz, H-2′), 7.37 (1H, dd, J = 10.5, 14.8 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 20.2 (C-3″-4), 28.6 (C-2″), 47.0 (C-1), 101.3 (–O–CH_2–O–), 105.7 (C-2′), 108.4 (C-5′), 122.6 (C-6′), 123.1 (C-2), 124.4 (C-4), 130.8 (C-1′), 138.8 (C-5), 141.1 (C-3), 148.2 (C3′-4′). ESI-TOF MS *m/z:* 274.1 [M+H] for $C_{17}H_{21}NO_3$.

3.1.4. Preparation of 5-(3′,4′-methylendioxy phenyl)-2*E*,4*E*-pentadienoic acid (piperic acid 15)

A solution of piperine in ethanol (500 mg [1.75 mmol] /30 mL) was refluxed for 16 h at 90 °C in presence of KOH (5 g), after this step the solvent was evaporated and the obtained solid was diluted with H_2O, 1 N hydrochloric acid (HCl) was added until no more precipitated was observed (pH 8) then the solution was extracted with EtOAc (3 × 200 mL), the organic layer was dried with anhydrous sodium sulfate, filtrated and the solvent evaporated giving 320 mg [1.46 mmol] of a pale yellow solid (83%). Mp 180–182 °C. IR (KBr cm^{-1}): 2921, 1675, 1420, 1256, 929. ^{1}H NMR (CD_3COCD_3, 300 MHz) δ (ppm) 5.98 (1H, d, J = 16.3, H-2), 6.03 (2H, s, –O–CH_2–O–), 6.83 (1H, dd, J = 5.3, 14.2, H-4), 6.86 (1H, d, J = 7.8, H-5′), 6.97 (1H, d, J = 5.3 Hz, H-5), 7.03 (1H, dd, J = 1.5, 8.2 Hz, H-6′), 7.17 (1H, s, H-2′), 7.36 (1H, dt, J = 5.6, 15.0, H-3). ^{13}C NMR (acetone, 75 MHz) δ (ppm) 107.2 (–O–CH_2–O–), 111.3 (C-2′), 113.9 (C-5′), 126.2 (C-2), 128.6 (C-6′), 130.4 (C-4), 136.5 (C-1′), 145.6 (C-5), 150.6 (C-3), 152.2 (C-3′), 152.5 (C-4′), 172.6 (–COOH).

3.1.5. Preparation of 5-(3′,4′-methylendioxy phenyl)-2*E*,4*E*-pentadienoic acid morpholine amide (2)

A stirred solution of piperic acid **(15)** (49.3 mg [0.22 mmol]) in CH_3CN–CH_2Cl_2 (1–1, 20 mL, 0 °C) DCC (1 equiv) was added, the mixture was allowed to reach the room temperature and morpholine (18.3 mg [0.22 mmol]) was added. The mixture was stirred 4 h and the reaction checked by TLC (PE/EtOAc 2:1) when the reaction was completed the solvent was evaporated and the residue redissolved in EtOAc and washed with $NaHCO_3$ solution (10%), water and citric acid solution (5%), the organic layer was dried over anhydrous sodium sulphate and filtrated; After dry the solvent 34.9 mg [0.12 mmol] of a white solid was obtained (54%). Mp 137–139 °C. IR (KBr cm^{-1}): 2926, 1635, 1489, 1444, 1251, 1038, 929. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 3.69 (8H, s, H-2″, H-3″, H-5″, H-6″), 5.97 (2H, s, –O–CH_2–O–), 6.34 (1H, d, J = 14.6, H-2), 6.74 (1H, dd, J = 9.7, 14.3, H-4), 6.78 (1H, d. J = 6.2, H-5), 6.79 (1H, d, J = 7.9 Hz, H-5′) 6.88 (1-H, d, J = 8.1, H-6′), 6.97 (1H, s, H-2′), 7.44 (1H, dd, J = 9.8, 14.5, H-3), NMR ($CDCl_3$, 75 MHz) δ (ppm) 42.8 (C-6″), 46.6 (C-2″), 67.2 (C-3″, C-5″), 101.7 (–O–CH_2–O–), 106.1 (C-2′), 108.9 (C-5′), 119.2 (C-2), 123.4 (C-6′), 125.4 (C-4), 131.2 (C-1′), 139.5 (C-5), 143.9 (C-3), 148.6 (C3′), 148.7 (C-4′), 166.2 (NH–C=O). ESI-TOF MS *m/z:* 288.1 [M+H] for $C_{16}H_{17}NO_4$.

3.1.6. Preparation of 5-(3′,4′-methylendioxy phenyl)-2*E*,4*E*-pentadienoic acid 4-chlorophenyl amide (3)

To piperic acid **(15)** (249 mg [1.1 mmol]) dissolved in DCM (30 mL) DIC (1 equiv) and HOBt (1 equiv) were added at room temperature and stirred for 10 min 140.3 mg [1.1 mmol] of *p*-chloro aniline was added to the stirred mixture, the reaction was monitored by TLC (petroleum ether/ethyl acetate 3:2) until the piperic acid was completely consumed, the solvent evaporated and the solid redissolved en EtOAc, filtrated and the residue purified by CC (petroleum ether/ethyl acetate 4:1–3:1) five fractions were collected and from fraction number five 27.5 mg [0.08 mmol] of the desired product were obtained as a yellow solid (7%). Mp 160–162 °C. IR (KBr cm^{-1}): 2923, 1781, 1598, 1446, 1254, 1074, 1035, 885. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 6.03 (2H, s, –O–CH_2–O–), 6.24 (1H, d, J = 15.2 Hz, H-2), 6.83 (1H, d, J = 8.0 Hz, H-5′), 6.85 (1H, dd, J = 11.6, 14.8 Hz, H-4), 6.98 (1H, dd, J = 1.6, 8.0 Hz, H-6′), 7.00 (1H, d, J = 15.6 Hz, H-5), 7.06 (1H, d, J = 1.73 Hz, H-2′), 7.43 (1H, d, J = 8.4 Hz, H-3″), 7.45 (1H, d, J = 8.0 Hz, H-5″), 7.54 (1H, d, J = 8.0 Hz, H-6″), 7.79 (1H, dd, J = 11.1, 15.2 Hz, H-3), 8.08 (1H, d, J = 8.4 Hz, H-2″). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 101.8 (–O–CH_2–O–), 106.3 (C-2′), 108.6 (C-5″), 108.9 (C-5′), 112.4 (C-2), 120.6 (C-2″), 123.7 (C-4), 124.4 (C-6′), 124.9 (C-3″), 128.8 (C-6″), 129.2 (C-1″), 129.9 (C-1′), 143.7 (C-4′), 144.8 (C-5), 148.7 (C-3′), 149.7 (C-4′), 151.4 (C-3), 163.3 (–NH–C=O). ESI-TOF MS *m/z:* 328.1 [M+H]; HRMS 327.0660 for $C_{18}H_{14}ClNO_3$.

3.1.7. Preparation of 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*-4*E*-pentadienoic acid (16)

To 356.4 mg [2 mmol] of 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenal (purchased from Sigma–Aldrich) in pyridine (30 mL), 468.3 mg [4.5 mmol] of malonic acid and piperidine (3 mL) were added; the mixture was refluxed for 1 h and then cooled to room temperature, neutralized (1 N HCl) and extracted with CH_3Cl, the organic layer dried over anhydrous sodium sulphate and the solvent evaporated, This product was used without further purification.

3.1.8. Preparation of 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*-4*E*-pentadien-piperidin amide (5)

The crude product of 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*-4*E*-pentadienoic acid (**16**) (72.8 mg, 0.33 mmol) was mixed with 44.2 mg of DIC and 47.3 mg of HOBt in DCM (25 mL) for 10 min at room temperature followed by the addition of 29.8 mg of piperidine, the resulting mixture was stirred until the starting product was completely consumed (TLC DCM/acetone 4:1), the crude product of the reaction was further purified using silica gel (DCM/acetone 100:0–50:50, to give 33.2 mg of a yellow oil (34%). IR (KBr cm^{-1}): 2934, 1653, 1558, 1457, 1255, 1021. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.58 (4H, m, H-3″-5″), 1.65 (2H, m, H-4″), 3.53–3.63 (4H, m, H-2″-6″), 3.91 (–OCH_3), 6.44 (1H, d, J = 14.6 Hz, H-2), 6.78 (2H, overlapped signal, H-4,5), 6.9 (1H, d, J = 8.2 Hz, H-5′), 6.95 (1H, d, J = 1.8 Hz, H-2′), 6.99 (dd, J = 1.9, 8.0 Hz, H-6′), 7.43 (dd, J = 5.1, 14.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 25.1 (C-4″), 26.0 (C-3″), 27.2 (C-5″), 43.7 (C-2″), 47.3 (C-6″), 56.3 (–OCH_3), 109.2 (C-2′), 115.1 (C-5′), 120.0 (C-2), 121.4 (C-6′), 125.3 (C-4), 129.5 (C-1′), 139.0 ((C-5), 143.2 (C-3), 146.8 (C-3′), 147.1 (C-4′), 165.9 (–NH–C=O). ESI-TOF MS *m/z:* 288.1 [M+H] for $C_{17}H_{21}NO_3$.

3.1.9. Preparation of 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*-4*E*-pentadien isobutyl amide (6)

130.3 mg [0.59 mmol] of 5-(4′-hydroxy-3′-methoxyphenyl)-2*E*-4*E*-pentadienoic acid (**16**) were mixed with DIC (1 equiv) and HOBt (1 equiv) in DCM (25 mL) and stirred for 10 min, 43.2 mg of isobutylamine were added at room temperature and when the reaction was completed the crude residue was purified with silica gel (DCM/acetone 100.0–50:50) and gave 145.3 mg [0.53 mmol] of a yellow solid. (89%). Mp 133–135 °C. IR (KBr cm^{-1}) 3358, 2926, 1652, 1514, 1279, 1147, 1033, 996. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.91 (6H, d, J = 6.7 Hz, H3″-4″), 1.81 (1H, m, H.2″), 3.16 (2H, t, J = 6.4, H-1″), 3.81 (3H, s, –OCH_3), 6.04 (1H, d, J = 14.9 Hz, H-2), 6.49 (1H, t, J = 6.08 Hz, –NH), 6.73 (1H, dd, J = 10.4, 15.6, H-4), 6.80 (1H, d, J = 15.2 Hz), 6.89 (1H, d, J = 8.3 Hz, H-5′), 6.93 (1H, d, J = 2.0 Hz, H-2′), 6.99 (1H, dd, J = 1.9, 8.3 Hz, H-6′), 7.38 (1H, dd, J = 10.4, 14.8 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 20.2 (C-3″-4″), 28.4 (C-2″), 47.2 (C-1″), 55.5 (–OCH_3), 109.2 (C-2′), 115.0 (C-5′), 120.9 (C-2), 122.9 (C-6′), 124.0 (C-4), 128.7 (C-1′), 139.3 (C-5), 141.2 (C-3), 146.9 (C-3′), 147.1 (C-4′), 166.8 (–NH–C═O). ESI-TOF MS *m/z:* 276.2 [M+H]; HRMS 275.1519 for $C_{16}H_{21}NO_3$.

3.1.10. Preparation of 5-(3′,4′-methylendioxy phenyl)-pentanoic acid piperidine amide (tetrahydro piperine 8)

To piperine (**1**) (100 mg, 0.35 mmol) in dichloromethane (DCM, 20 mL) was added a catalytic amount of Pd/C (5%) and hydrogen was bubbled over 12 h, giving 100.6 mg [0.34 mmol] of a colourless oil (97%). IR (KBr cm^{-1}): 2926, 1635, 1489, 1437, 1246, 1037, 936, 852. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 1.52–1.63 (10H, m, H-2″, H-3″, H-4″, H-3, H-4), 2.33 (2H, br s, H-2), 2.54 (2H, br s, H-5), 3.45 (4H, br s, H-1″-H-5″), 5.89 (2H, s, –O–CH_2–O–), 6.66 (3H, m, H-2′, H-5′, H-6′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm) 25.2 (C-2″, C-4″), 25.6 (C-3″), 32.2 (C-3), 34.1 (C-2), 36.2 (C-4, C-5), 101.4 (–O–CH_2–O–), 108.7 (C-5′), 109.5 (C-2′), 121.8 (C-6′), 136.8 (C-1′), 146.2 (C-4′), 148.2 (C-3′), 171.9 (–NH–C═O). ESI-TOF MS *m/z:* 290.1681 [M+H] for $C_{17}H_{23}NO_3$.

3.1.11. Preparation of 3-(3′,4′-methylenedioxyphenyl)-2*E*-propenoic acid piperidine amide (9)

To a stirred solution of 3-(3′,4′-methylendioxyphenyl)-2*E*-propenoic acid (Ferulic acid, purchased from Sigma–Aldrich) 192 mg [1 mmol], DIC (1 equiv) and HOBt (1 equiv) in DCM (25 mL), piperidine 85.2 mg [1 mmol] was added at room temperature, the reaction was checked by TLC (DCM/acetone, 9:1) when the starting material was consumed the reaction was stopped and the crude product purified using silica gel (DCM/acetone, 100:0–93:7), 244.2 mg [0.94 mmol] of an amorphous white solid were obtained (94%). Mp 88–90 °C. IR (KBr cm^{-1}): 2937, 1638, 1590, 1434, 1245, 1140, 1044, 819. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.59 (4H, m, H-3″-4″), 1.65 (2H, m, H-4″), 3.60 (4H, br s, H-2″-6″), 5.97 (2H, s, –O–CH_2–O–), 6.73 (1H, d, J = 15.4 Hz, H-2), 6.79 (1H, d, J = 8.0 Hz, H-5′), 6.99 (1H, dd, J = 1.7, 8.0 Hz, H.6′), 7.03 (1H, d, J = 1.7 Hz, H-2′), 7.56 (1H, d, J = 15.5 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 24.5 (C-4″), 25.4 (C-3″), 26.5 (C-5″), 43.1 C-2″), 46.6 (C-6″), 101.3 (–O–CH_2–O–), 106.1 (C-2′), 108.2 (C-5′), 115.5 (C-2), 123.3 (C-6′), 129.6 (C-1′), 141.6 (C-3), 147.9 (C-3′), 148.5 (C-4′), 165.2 (–NH–C═O). ESI-TOF MS *m/z:* 260.1 [M+H] for $C_{15}H_{17}NO_3$.

3.1.12. Preparation of 3-(3′,4′-methylendioxyphenyl)-2*E*-propenoic acid isobutyl amide (10)

With the same conditions for 3-(3′,4′-methylendioxyphenyl)-2*E*-propenoic acid piperidine amide **(9)** synthesis, 192 mg [1 mmol] of 3-(3′,4′-methylendioxyphenyl)-2*E*-propenoic acid was mixed with DIC (1 equiv) and HOBt (1 equiv) and isobutylamine 73.1 mg [1 mmol] was added to the stirred solution, the reaction was monitored by TLC (PE/EtOAc, 2:1) and the crude material purified with column chromatography (silica gel) petroleum ether/EtOAc, 4:1–2:1 and gave 215 mg [0.87 mmol] as a white solid (87%). Mp 116–118 °C. IR (KBr cm^{-1}): 3294, 2958, 1650, 1253, 1038, 931, 810. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.91 (6H, d, J = 6.7 Hz, H-3″-4″), 1.82 (1H, m, H-2″), 3.18 (2H, t, J = 6.5 Hz, H-1″), 5.91 (2H, s, –O–CH_2–O–), 6.38 (1H, d, J = 15.5 Hz, H-2), 6.64 (1H, t, J = 6.7 Hz, –NH), 6.69 (1H, d, J = 8.0 Hz, H-5′), 6.89 (dd, J = 1.7, 7.9 Hz, H-6′), 7.49 (1H, d, J = 15.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 20.2 (C-3″-4″), 28.7 (C-2″), 47.2 (C-1″), 101.4 (–O–CH_2–O–), 106.4 (C-2′), 108.4 (C-5′), 119.4 (C-2), 123.7 (C-6′), 129.4 (C-1′), 140.3 (C-3), 148.2 (C-3′), 148.9 (C-4′), 166.5 (–NH–C═O). ESI-TOF MS *m/z:* 248.1 [M+H] for $C_{14}H_{17}NO_3$.

3.1.13. Preparation of 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid piperidin amide (11)

Using the methodology for 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid morpholin amide **(2)** preparation, 151.6 mg [0.78 mmol] of ferulic acid and 66.4 mg [0.78 mmol] of piperidine were subjected to reaction and the crude was purified with column chromatography (Silica gel DCM/acetone 100:0–95:5) to give 159 mg [0.61 mmol] of yellow crystals (78%). Mp 125–127 °C. IR (KBr cm^{-1}): 3307, 2975, 1647, 1590, 1286, 1033, 833. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.56 (4H, m, H-3″-5″), 1.62 (2H, m, H-4″), 3.56–3.62 (4H, m, H2″-6″), 3.84 (3H, s, –O–CH_3), 6.72 (1H, d, J = 15.4 Hz, H-2), 6.89 (1H, d, J = 8.2 Hz, H-5′), 6.95 (1H, d, J = 1.9 Hz, H-2′), 7.02 (1H, dd, J = 1.8, 8.3 Hz, H-6″), 7.56 (1H, d, J = 15.4 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 24.3 (C-4″), 25.5 (C-3″), 26.5 (C-5″), 43.3 (C-2″), 47.0 (C-6″), 55.7 (–O–CH_3), 110.1 (C-2′), 114.5 (C-2), 115.0 (C-5′), 121.7 (C-6′), 127.4 (C-1′), 142.7 (C-3), 147.1 (C-3′), 147.7 (C-4′), 165.8 (–NH–C═O). ESI-TOF MS *m/z:* 262.1 [M+H] for $C_{15}H_{19}NO_3$.

3.1.14. Preparation of 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid morpholine amide (12)

To a solution of ferulic acid (purchased from Sigma–Aldrich) (500 mg [2.6 mmol]) in DCM (25 mL), DIC (1 equiv) and HOBt (1 equiv) were added and the mixture stirred for 10 min. Morpholine (226.5 mg [2.6 mmol]) was added to the mixture and allowed to react for 4 h. After the reaction was completed the solvent was evaporated and the residue dissolved in EtOAc washed with $NaHCO_3$ solution (10%), water and citric acid solution (5%), the organic layer was dried over anhydrous sodium sulphate, filtrated and the solvent evaporated, the crude product was further purified by column chromatography (DCM/EtOAc, 3:1, 2:1, 1:1, 1:2) giving 530 mg [2.02 mmol] of a white solid (78%). Mp 167–169 °C. IR (KBr cm^{-1}): 3150, 2963, 1644, 1585, 1515, 1270, 1111, 1037, 821. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 3.72 (8H, br s, H-2″, 3″, 5″, 6″), 3.93 (3H, s, –OCH_3), 6.64 (1H, d, J = 15.3 Hz, H-2), 6.86 (1H, d, J = 8.1 Hz, H-5′), 6.93 (1H, d, J = 2.2 Hz, H-2′), 6.98 (1H, dd, J = 1.9, 8.2 Hz, H-6′), 7.56 (1H, d, J = 15.2 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 42.4 (C-2″, C-6″), 55.9 (–OCH_3), 66.8 (C-3″, C-5″), 109.9 (C-2′), 113.7 (C-1), 114.8 (C-5′), 121.9 (C-6′), 127.5 (C-1′), 143.5 (C-2), 146.7 (C-3′), 147.5 (C-4′), 166.1 (–NH–C═O). ESI-TOF MS *m/z:* 264.1 [M+H] for $C_{14}H_{17}NO_4$.

3.1.15. Preparation of 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid thiomorpholine amide (13)

Ferulic acid (2.6 mmol) and thiomorpholine (2.6 mmol) were allowed to react under the same conditions and reagents as for 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid morpholin amide; the crude product was purified with silical gel (DCM/EtOAc, 3:1, 2:1, 1:1, 1:2) and gave 560.3 mg [2.00 mmol] of a pale yellow solid (77%). Mp 153–155 °C. IR (KBr cm^{-1}): 3100, 2918, 1634, 1514,

1454, 1285, 1189, 1032, 951, 823.. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.52 (4H, m, H-3″-5″), 3.82 (4H, br s, H-2″-6″), 6.62 (1H,d, J = 15.3 Hz, H-2), 6.81 (1H, d, J = 8.2 Hz, H-5′), 6.89 (1H, d, J = 1.8 Hz, H-2′), 6.93 (1H, dd, J = 1.9, 8.3 Hz, H-6′), 7.5 (1H, d, J = 15.3 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 27.3 (C-5″), 28.0 (C-3″), 45.0 (C-6″), 48.6 (C-2″), 55.7 (–O–CH_3), 110.2 (C-2′), 113.6 (C-2), 115.2 (C-5′), 122.1 (C-6′), 127.2 (C-1′), 143.7 (C-3), 147.2 (C-3′), 148.0 8 (C-4′), 166.1 (–NH–C=O). ESI-TOF MS *m/z:* 280.1 [M+H]; HRMS 279.0929 for $C_{14}H_{17}NO_3S$.

3.1.16. Preparation of 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid isobutyl amide (14)

Ferulic acid (2.6 mmol) and isobutylamine (2.6 mmol) reacted as before for 3-(4′-hydroxy-3′-methoxyphenyl)-2*E*-propenoic acid morpholin amide. The reaction mixture was purified using silica gel (DCM/EtOAc, 9:1) and 240.7 mg [0.92 mmol] of a yellow solid were obtained (35%). Mp 139–141 °C. IR (KBr cm^{-1}): 3338, 2945, 1646, 1462, 1273, 1024, 980, 839. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.93 (6H, d, J = 6.7 Hz, H3″-4″), 1.83 (1H, m, H-2″), 3.20 (2H, t, J = 6.9 Hz, H-1″), 3.82 (3H, s, –O–CH_3), 6.21 (1H, t, J = 5.9 Hz, –NH), 6.35 (1H, d, J = 15.5 Hz, H-2), 6.87 (1H, d, J = 8.2 Hz, H-5′), 6.94 (1H, d, J = 1.9 Hz, H-2′), 7.0 (1H, dd, J = 1.9, 8.2 Hz, H-6′), 7.54 (1H, d, J = 15.6 Hz, H-3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 20.1 (C-3″-4″), 28.6 (C-2″), 47.1 (C-1″), 55.8 (-O-CH_3), 109.8 (C-2′), 114.8 (C-2), 118.4 (C-5′), 121.9 (C-6′), 127.4 (C-1′), 140.9 (C-3), 147.0 (C-3′), 147.6 (C-4′), 166.7 (–NH–C=O). ESI-TOF MS *m/z:* 250.1 [M+H] for $C_{14}H_{19}NO_3$.

3.2. Pharmacology

3.2.1. General procedures

Capsaicin, capsazepine, ionomycin, probenecid and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich. Fluo-4/AM, lipofectamine and pluronic acid were obtained from Invitrogen.

3.2.2. Fluorometric Ca^{2+} imaging

HEK T-REX hTRPV1 inducible cells provided by AstraZeneca (Sweden) were grown in Dulbecco's modification eagle's medium 1X (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM Glutamine (Sigma–Aldrich), 5 μg/mL blasticidin S HCL (Invitrogen) and 350 μg/mL Zeocin (Invitrogen). Non-induced HEK293 cells used as controls were grown in Dulbecco's modification eagle's medium 1X (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM Glutamine (Sigma–Aldrich), and 1% penicillin/streptomycin (Invitrogen). The cells were maintained in a humidified incubator (5% CO_2 and 37 °C). hTRPV1 were induced with 0.1 μg/mL tetracycline (Invitrogen) 16–24 h prior the experiments.

For recording of intracellular calcium, cells plated in 96-well black-walled plates (Costar) were loaded with Fluo-4/AM 1 μM, probenecid 2 mM and pluronic acid 20% for 1 h at 37 °C. The cells were then washed with physiological buffer solution (PBS), containing (in mmol/l) 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 $CaCl_2$, and 1 $MgCl_2$ (pH 7.4), and allowed to equilibrate for a period of 30 min in the dark before the start of the experiments. The dye loading and the subsequent experiments were performed in PBS. The excitation wave length used was 488 nm and emission was measured at 520 nm before and at various times after compound addition in triplicate wells (22–24 °C) by using a Flexstation 3 (Molecular Devices). All compounds were dissolved in DMSO as stock solutions and the test solutions were further prepared in PBS buffer. The final concentration of DMSO did not exceed 0.1%. In antagonist experiments, the loaded cells were incubated with test compounds 30 min prior to capsaicin challenge.

3.3. Statistical analysis

Data were acquired with SoftMax® pro v5.2 (Molecular Devices) and processed with Prism 4.0c (GraphPad Software) software. Responses are calculated as a percentage of the response to either 1 μM capsaicin or ionomycin as indicated in the text. The −log of the agonist concentration eliciting half maximal response (pEC_{50}) was determined by nonlinear regression analysis. Data are presented as mean ± SEM and n indicates the number of separate experiments performed.

Acknowledgments

This work was supported by Universidad de Antioquia and COLCIENCIAS, the Swedish Medical Research Council (2007-3095) and the Medical Faculty of Lund. Edwin Correa was supported by a fellowship from Colciencias (Colombia) and Alfa Net EULADIV (EU). The authors thank 'AstraZeneca R&D CNS&P Local Discovery Södertälje' for providing the HEK T-REX hTRPV1 inducible cell line.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.013.

References and notes

1. Parmar, V. S.; Jain, S. C.; Bisht, K. S.; Jain, R.; Taneja, P.; Jha, A.; Tyagi, O. D.; Prasad, A. K.; Wengel, J.; Olsen, C. E.; Boll, P. M. *Phytochemistry* **1997**, *46*, 597.
2. Dyer, L.; Richards, J. D. Craig. In *Piper: A Model Genus for Studies of Phytochemistry, Ecology, and Evolution*; Dyer, L. A., Palmer, A. D., Eds.; Kluwer Academic/Plenum Publishers: New York, 2004; pp 117–134.
3. Zachariah, T. J.; Parthasarathy, V. A. In *Chemistry of Spices*; Parthasarathy, V. A., Chempakam, B., Zachariah, T. J., Eds.; CAB International: Oxfordshire, 2008; pp 21–40.
4. Vasques da Silva, R.; Debonsi Navickiene, H. M.; Kato, M. J.; Bolzani, V. S.; Meda, C. I.; Young, M. C. M.; Furlan, M. *Phytochemistry* **2002**, *59*, 521.
5. Navickiene, H. M. D.; Alecio, A. C.; Kato, M. J.; Bolzani, V. S.; Young, M. C. M.; Cavalheiro, A. J.; Furlan, M. *Phytochemistry* **2000**, *55*, 621.
6. Macnamara, F.; Randall, A.; Gunthorpe, M. *Br. J. Pharmacol.* **2005**, *144*, 781.
7. Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D. *Nature* **1997**, *389*, 816.
8. Tominaga, M.; Caterina, M. J.; Malmberg, A. B.; Rosen, T. A.; Gilbert, H.; Skinner, K.; Raumann, B. E.; Basbaum, A. I.; Julius, D. *Neuron* **1998**, *21*, 531.
9. Zygmunt, P. M.; Petersson, J.; Andersson, D. A.; Chuang, H.; Sorgard, M.; Di Marzo, V.; Julius, D.; Hogestatt, E. D. *Nature* **1999**, *400*, 452.
10. Patapoutian, A.; Tate, S.; Woolf, C. J. *Nat. Rev. Drug Disc.* **2009**, *8*, 55.
11. Knotkova, H.; Pappagallo, M.; Szallasi, A. *Clin. J. Pain* **2008**, *24*, 142.
12. Colsoul, B.; Nilius, B.; Vennekens, R. *Clin. Exp. Allergy* **2009**, *39*, 1456.
13. Birder, L. A. *Biochim. Biophys. Acta* **2007**, *1772*, 879.
14. Dray, A.; Dickenson, A. *Pain* **1991**, *47*, 79.
15. Perkins, M. N.; Campbell, E. A. *Br. J. Pharmacol.* **1992**, *107*, 329.
16. Urban, L.; Campbell, E. A.; Panesar, M.; Patel, S.; Chaudhry, N.; Kane, S.; Buchheit, K.; Sandells, B.; James, I. F. *Pain* **2000**, *89*, 65.
17. Gupta, O. P.; Atal, C. K.; Gaind, K. N. *Phytochemistry* **1972**, *11*. 2646–2646.
18. Chatterjee, A.; Dutta, C. P. *Tetrahedron Lett.* **1966**, *7*, 1797.
19. De Araujo-Junior, J. X.; Da-Cunha, E. V. L.; De, O.; Chaves, M. C.; Gray, A. I. *Phytochemistry* **1997**, *44*, 559.
20. Han, S.-Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447.
21. Crosignani, S.; Young, A. C.; Linclau, B. *Tetrahedron Lett.* **2004**, *45*, 9611.
22. Carpino, L. A.; El-Faham, A. *Tetrahedron* **1999**, *55*, 6813.
23. Aparicio, F. J. L.; Herrera, F. J. L. *Carbohydr. Res.* **1980**, *80*, C4.
24. Duarte, C. d. M.; Verli, H.; Araïjo-Jnior, J. o. X. d.; Medeiros, I. A. d.; Barreiro, E. J.; Fraga, C. A. M. *Eur. J. Pharm. Sci.* **2004**, *23*, 363.
25. Madhusudhan, P.; Vandana, K. L. *Biochem. Syst. Ecol.* **2001**, *29*, 537.
26. Koul, S.; Koul, J.; Taneja, S.; Dhar, K.; Jamwal, D.; Singh, K.; Reen, R.; Singh, J. *Bioorg. Med. Chem.* **2000**, *8*, 251.
27. Cotinguiba, F.; Regasini, L.; da Silva Bolzani, V.; Debonsi, H.; Duó Passerini, G.; Cicarelli, R.; Kato, M.; Furlan, M. *Med. Chem. Res* **2009**, *18*, 703.
28. Sangwan, P. L.; Koul, J. L.; Koul, S.; Reddy, M. V.; Thota, N.; Khan, I. A.; Kumar, A.; Kalia, N. P.; Qazi, G. N. *Bioorg. Med. Chem.* **2008**, *16*, 9847.
29. Gavva, N. R.; Klionsky, L.; Qu, Y.; Shi, L.; Tamir, R.; Edenson, S.; Zhang, T. J.; Viswanadhan, V. N.; Toth, A.; Pearce, L. V.; Vanderah, T. W.; Porreca, F.; Blumberg, P. M.; Lile, J.; Sun, Y.; Wild, K.; Louis, J. C.; Treanor, J. J. *J. Biol. Chem.* **2004**, *279*, 20283.

30. Walpole, C. S.; Wrigglesworth, R.; Bevan, S.; Campbell, E. A.; Dray, A.; James, I. F.; Perkins, M. N.; Reid, D. J.; Winter, J. *J. Med. Chem.* **1993**, *36*, 2362.
31. Szallasi, A.; Blumberg, P. M. *Brain Res.* **1991**, *547*, 335.
32. Liu, L.; Simon, S. A. *J. Neurophysiol.* **1996**, *76*, 1858.
33. Szallasi, A. *Trends Pharmacol. Sci.* **2005**, *26*, 437.
34. Gevaert, T.; Vandepitte, J.; Hutchings, G.; Vriens, J.; Nilius, B.; De Ridder, D. *Neurourol. Urodyn.* **2007**, *26*, 440. discussion 451–453.