PHYTOTHERAPY RESEARCH
*Phytother. Res.* **29**: 1806–1813 (2015)
Published online 11 September 2015 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5448

# Mechanisms of Vasorelaxation Induced by Hexahydrocurcuminin Isolated Rat Thoracic Aorta

**Aida Moohammadaree,[1] Chatchawan Changtam,[2] Piyawadee Wicha,[1] Apichart Suksamrarn,[3] Jiraporn Tocharus[4] and Chainarong Tocharus[1]***

[1]Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Faculty of Science and Technology, Huachiew Chalermprakiet University, Samutprakarn 10540, Thailand
[3]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand
[4]Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

**This study was designed to examine the vasorelaxant effects of hexahydrocurcumin (HHC), one of the major natural metabolites of curcumin from *Curcuma longa*, on rat isolated aortic rings, and the underlying mechanisms. Isometric tension of the aortic rings was recorded using organ bath system. HHC (1 nM to 1 mM) relaxed the endothelium-intact aortic rings pre-contracted with PE and KCl in a concentration-dependent manner. Removal of the endothelium did not alter the effect of HHC-induced relaxation. In $Ca^{2+}$-free Krebs solution, HHC significantly inhibited the $CaCl_2$-induced contraction in high $K^+$ depolarized rings and suppressed the transient contraction induced by PE and caffeine in a concentration-dependent manner. HHC was also observed to relax phobal-12-myristate-13-acetate (PMA), an activator of protein kinase C (PKC), precontracted aortic rings in a concentration-dependent manner with $EC_{50}$ values equivalent to 93.36 ± 1.03 μM. In addition, pre-incubation with propranolol (a β-adrenergic receptor blocker) significantly attenuated the HHC-induced vasorelaxation. These results suggest that the vasorelaxant effect of HHC is mediated by the endothelium-independent pathway, probably because of the inhibition of extracellular $Ca^{2+}$ influx through voltage-operated $Ca^{2+}$ channels and receptor-operated $Ca^{2+}$ channels, the inhibition of $Ca^{2+}$mobilization from intracellular stores, as well as inhibition of PKC-mediated $Ca^{2+}$-independent contraction. Moreover, HHC produces vasorelaxant effects probably by stimulating the β-adrenergic receptor. Copyright © 2015 John Wiley & Sons, Ltd.**

*Keywords:* hexahydrocurcumin (HHC); vasorelaxation; endothelium independent; aorta; hypertension.

*Abbreviations:* AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; HHC, hexahydrocurcumin; $IP_3R$, $IP_3$ receptor; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROCC, receptor-operated $Ca^{2+}$channels; RyR, ryanodine receptors; SMC, smooth muscle cell; SR, sarcoplasmic reticulum; VOCC, voltage-operated $Ca^{2+}$ channels

## INTRODUCTION

Curcumin (diferuloylmethane, Fig. 1A) is the major active constituent of the rhizome from *Curcuma longa* L. (Zingiberaceae), commonly known as 'turmeric'. It is found throughout South and Southeast Asia, and has been used for a long time as a spice in cooking and as a traditional medicine. Extensive researches have been done on curcumin in both *in vitro* and *in vivo* studies for a wide variety of medicinal uses such as for its antiinflammatory (Morsy and El-Moselhy, 2013), anti-viral (Zhang *et al*., 2011), anti-fungal (Martins *et al*., 2009), antioxidant (Bhullar *et al*., 2013), anticancer (Lim *et al*., 2014), and neuroprotective properties (Belviranli *et al*., 2013). Aside from that, curcumin has been shown to possess potent vasodilation abilities on rat mesenteric and porcine coronary arteries by inhibiting the extracellular $Ca^{2+}$ influx, and increasing the production of nitric oxide and cyclic guanosine monophosphate, respectively (Xu *et al*., 2007; Adaramoye *et al*., 2009). Moreover, curcumin is also observed to have a hypotensive effect on L-NAME induced hypertensive rats (Hlavackova *et al*., 2011). Curcumin has poor aqueous solubility and is poorly absorbed in the gastrointestinal tract; also, it is chemically unstable and metabolizes rapidly in human blood (Anand *et al*., 2007; Dulbecco and Savarino, 2013; Metzler *et al*., 2013). These properties make it problematic as regards pharmaceutical development. The reduced analogues of the curcumin, tetrahydrocurcumin and hexahydrocurcumin (HHC, Fig. 1B), have also been found as minor constituents in some plant species (Matsuda *et al*., 2004). HHC has been found as curcumin metabolite in mice and human (Holder *et al*., 1978; Pan *et al*., 1999; Ireson *et al*., 2001; Ireson *et al*., 2002).

In this study, we are interested in HHC, which could be obtained in large quantity by catalytic hydrogenation reaction of curcumin. Previous studies have revealed that HHC shows stronger antioxidant activity, even greater than that of parent curcumin (Somparn *et al*., 2007; Deters *et al*., 2008). Furthermore, HHC exhibits much higher

* Correspondence to: Correspondence to: Chainarong Tocharus, Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: chainarongt@hotmail.com

*Received 12 January 2015*
*Revised 01 August 2015*
*Accepted 15 August 2015*

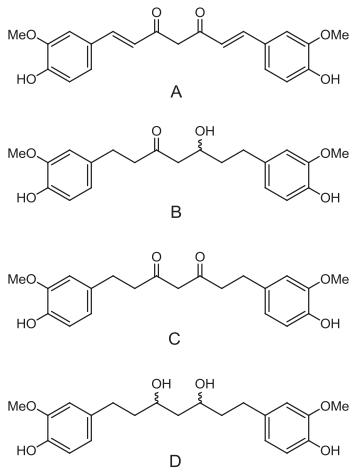


**Figure 1.** The chemical structures of curcumin (A), hexahydrocurcumin (HHC, B), tetrahydrocurcumin (THC, C), and octahydrocurcumin (OHC, D).

chemical stability as well as higher biodisponibility than parent curcumin (Dempe *et al*., 2013). HHC also exhibits anti-carcinogenesis by down regulating the expression of cyclooxygenase-2 (COX-2), an important enzyme in carcinogenesis (Srimuangwong *et al*., 2012a). The synergistic effect of using 5-fluorouracil, a conventional chemotherapy drug, in combination with curcumin to reduce aberrant crypt foci (ACF) numbers has already been demonstrated (Srimuangwong *et al*., 2012b). However, the medicinal properties of HHC against cardiovascular diseases, especially hypertension, have not yet been reported and require further study. Therefore, the present study was undertaken to assess the vasorelaxant effect of HHC in isolated rat aortic rings and to gain an insight into the mechanisms involved in these actions.

## MATERIALS AND METHODS

**Chemicals and drugs.** Acetylcholine (ACh), 4-aminopyridine (4-AP), barium chloride ($BaCl_2$), caffeine, dimethyl sulfoxide (DMSO), ethylene glycoltetraacetic acid (EGTA), glibenclamide, nifedipine, phenylephrine (PE), potassium chloride (KCl), phorbol-12-myristate-13-acetate (PMA), propranolol, and tetraethylammonium chloride (TEA) were purchased from Sigma (St. Louis, MO, USA).

**Animals.** Male Wistar rats, weighing 200–300 g, were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. The animals were housed in individual cages under standard laboratory conditions (12:12 h light–dark cycle conditions, at 24 ± 1 °C) and were allowed free access to rodent diet and tap water provided by the Animal Care Facility Center, Chiang Mai University, Thailand. In this study, all the experimental protocols were approved by the Animal Ethical Committee of Chiang Mai University, Chiang Mai, Thailand.

**Preparation of HHC.** The HHC was prepared from *C. longa*, as described previously (Changtam *et al*., 2010). Briefly, the curcuminoid mixture obtained from the rhizomes of *C. longa* was subjected to silica gel column chromatography, using *n*-hexane–dichloromethane, dichloromethane, and dichloromethane–methanol as eluents to afford curcumin as a major component. Then, recrystallization was accomplished by dissolving the evaporated eluate with a small quantity of dichloromethane, and ethanol was then added. Curcumin crystallized out as yellow needles, with a melting point 181–183 °C. Afterward, HHC was synthesized from curcumin by catalytic hydrogenation reaction in ethanol for 5 h, with palladium on charcoal as the catalyst. HHC was isolated from tetrahydrocurcumin (THC) and octahydrocurcumin (OHC) using silica gel column chromatography. Recrystallization by addition of dichloromethane–*n*-hexane gave 45% yield of HHC. THC and OHC were obtained in 28% and 11% yields, respectively.

Tetrahydrocurcumin (THC): crystals ($CH_2Cl_2$–*n*-hexane), mp 93–94 °C; IR (KBr): $\nu_{max}$ 3418, 3066, 2934, 2845, 1694, 1602, 1517, 1459, 1432, 1437, 1373, 1235, 1119, 1033, 922, 814, 799 $cm^{-1}$; $^1$H-NMR (400 MHz, $CDCl_3$): δ 2.52 (t, $J$ = 7.7 Hz, 4H, H-2 and H-6), 2.82 (t, $J$ = 7.7, 4H, H-1 and H-7), 3.83 (s, 6H, 2 × $OCH_3$), 5.40 (s, 1H, H-4), 5.49 (br s, 2H, 2 × OH), 6.64 (partially overlapping signal, 2H, H-5′ and H-5″), 6.65 (br s, 2H, H-2′ and H-2″), 6.80 (d, $J$ = 7.9 Hz, 2H, H-6′ and H-6″); EIMS: $m/z$ (% rel. abund.) 372 $[M]^+$ (25), 358 (3), 137 (100), 151 (8), 150 (8).

Hexahydrocurcumin (HHC): white amorphous solid ($CH_2Cl_2$–*n*-hexane), mp 81–82 °C; IR (KBr): $\nu_{max}$ 3405, 3013, 2859, 1604, 1515, 1452, 1430, 1368, 1271, 1234, 1152, 1034, 755 $cm^{-1}$; $^1$H-NMR (400 MHz, $CDCl_3$): δ 1.62 and 1.74 (each m, 2H, H-6a and H-6b), 2.51 (m, 2H, H-7), 2.57 (m, 1H, H-4a), 2.67–2.72 (m, 3H, H-4b and H-2), 2.80 (t, $J$ = 7.4 Hz, 2H, H-1), 3.83 and 3.84 (each s, 6H, 2 × $OCH_3$), 4.01 (m, 1H, H-5), 5.47 (br s, 2H, 2 × OH), 6.62–2.67 (m, 4H, H-2′, H-2″, H-5′, H-5″), 6.80 (d, $J$ = 7.9 Hz, 2H, H-6′ and H-6″); EIMS: $m/z$ (% rel. abund.) 374 $[M]^+$ (12), 219 (33), 137 (100).

Octahydrocurcumin (OHC): colorless sticky solid; IR (KBr): $\nu_{max}$ 3405, 3013, 2940, 2859, 1604, 1515, 1452, 1430, 1271, 1152, 1124, 1034, 935, 755 $cm^{-1}$; $^1$H-NMR (400 MHz, $CDCl_3$ + 10 drops of $CD_3OD$): δ 1.50 (m, 2H) and 1.67 (m, 4H) (H-2, H-4, H-6), 2.50 and 2.59 (each m, each 2H, H-1 and H-7), 3.77 (m, 2H, H-3 and H-5), 3.78 (s, 6H, 2 × $OCH_3$), 6.59 (d, $J$ = 8.0 Hz, 2H, H-5′ and H-5″), 6.63 (s, 2H, H-2′ and H-2″), 6.72 (d, $J$ = 8.0 Hz, 2H, H-6′ and H-6″); EIMS: $m/z$ (% rel. abund.) 376 $[M]^+$ (16), 358 (54), 340 (8), 190 (58), 137 (100).

The spectroscopic data of the above compounds were consistent with those reported previously (Venkateswarlu *et al*., 2005; Ishida *et al*., 2002; Lee *et al*., 2005). Throughout the experiments, the solution of HHC was dissolved in distilled water and DMSO, and the final concentration of DMSO did not exceed 0.1%.

**Preparation of thoracic aortic rings.** The rats were anesthetized with sodium pentobarbital 50 mg/kg by intraperitoneal route. The descending thoracic aorta was removed and carefully cleaned off the surrounding connective tissue and fat. The isolated aorta was then cut transversely into rings about 3–4 mm long. The aortic ring was suspended between a pair of tungsten wire hooks and immersed into a 2-ml organ bath chamber

which contained Krebs solution in the following composition (in mM): NaCl, 122; KCl, 5; [N-(2-hydroxyethyl) piperazine N′-(2-ethanesulfonic acid)] (HEPES), 10; $KH_2PO_4$, 0.5; $NaH_2PO_4$, 0.5; $MgCl_2$, 1; glucose, 11; and $CaCl_2$, 1.8 (pH 7.4). All of the chambers were maintained at 37 °C and were continuously gassed with 95% $O_2$ and 5% $CO_2$. Every aortic ring was placed with the initial resting tone at 1 g, according to the method followed by Brito *et al*. (2013), and was allowed at least 1 h for the equilibration of the vessel before testing in each experimental protocol. During this equilibrium period, the Krebs solution was changed every 15 min. The changes in the vascular tone tension were recorded using an isometric force transducer (Iworx System, Inc., NH 03820, USA) which was connected to a Power Lab system (ADInstrument, Sydney, Australia).

In some of the experiments, the endothelial layer was removed by gently rubbing the luminal surface of the vessel rings with a fine pair of small forceps. The presence of endothelium was verified by the ability of ACh (10 μM) to induce more than 90% relaxation of the aortic rings pre-contracted with PE (10 μM), whereas a relaxation of less than 10% was regarded as indicating endothelial denudation.

### Experimental protocols in isolated arteries

*Vasorelaxant effects of HHC on rat aortic rings pre-contracted with PE or KCl.* In order to determine whether the vasorelaxation effects of HHC were because of the endothelium-dependent and/or the endothelium-independent mechanisms, experiments were performed in both endothelium-intact and endothelium-denuded rings. After steady contraction was evoked by PE (10 μM) or KCl (80 mM) in both endothelium-intact and endothelium-denuded rings, the HHC (1 nM to 1 mM) was added cumulatively. The HHC-induced vasorelaxation in the aortic rings was calculated as a percentage of the relaxation in response to PE or KCl in the presence or absence of endothelium.

**Role of $\alpha_1$-receptor in inhibitory vasoconstriction effect of HHC.** To evaluate whether HHC acts as an $\alpha_1$-receptor blockade, endothelium-denuded rings were incubated with the HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) for 30 min before exposing them to PE (0.1 nM to 10 μM), which was added cumulatively. The obtained results are shown as percentages of contraction, and a comparison was done between the results obtained in the absence (control) and the presence of the HHC.

**Effect of HHC on extracellular $Ca^{2+}$-induced contraction activated by KCl.** To determine whether the vasorelaxation of HHC was because of the inhibition of extracellular $Ca^{2+}$ influx to smooth muscle cells (SMC), the experimental protocol was performed on endothelium-denuded aortic rings. Briefly, the aortic rings were stabilized in $Ca^{2+}$-free Krebs solution for 30 min. The rings were then replaced with $Ca^{2+}$-free 80 mM KCl solution to produce depolarization in SMC for opening voltage-operated $Ca^{2+}$ channels (VOCC). Next, the $CaCl_2$ was cumulatively added to the bath chamber (10 μM to 10 mM) to induce contraction. After the maximal response was sustained, the aortic rings were washed with $Ca^{2+}$-free Krebs solution for 30 min. When the tension went down to the basal, the rings were depolarized with the $Ca^{2+}$-free 80 mM KCl solution again. The four concentrations of the HHC (0.1 μM, 1 μM, 10 μM, and 100 μM), the vehicle, or 1 μM of nifedipine (L-type VOCC blocker) were incubated for 30 min before adding $CaCl_2$ cumulatively to the bath to generate the concentration–response curve again. The percentages of contraction induced by $CaCl_2$ were compared with regard to the percentages obtained in the absence (control) of HHC and those obtained in the presence of HHC.

**Effect of HHC on intracellular $Ca^{2+}$ release from intracellular stores sensitive to PE and caffeine.** To investigate whether the relaxation induced by HHC was related to the inhibition of intracellular $Ca^{2+}$ release from the sarcoplasmic reticulum (SR), the experiment was carried out on endothelium-denuded aortic rings. After verifying the endothelium, KCl (80 mM) was added to stimulate the SMC contraction by providing $Ca^{2+}$ loading into SR, mediated by opening the VOCC. The rings were washed with the $Ca^{2+}$-free Krebs solution containing EGTA (1 mM) for the complete removal of the extracellular $Ca^{2+}$, followed by the activation of transient contraction by PE (10 μM) or caffeine (20 mM) in the $Ca^{2+}$-free Krebs solution containing EGTA (1 mM) before and after the pre-incubation with the HHC (0.1 μM, 1 μM, 10 μM, and 100 μM). The results are expressed as percentages of contraction obtained in the absence of HHC and in the presence of HHC.

**Effect of HHC on $Ca^{2+}$ sensitivity of contractile proteins and on eliciting $Ca^{2+}$-independent vasoconstriction.** In order to determine the possibility that HHC may induce vasorelaxation through a $Ca^{2+}$-independent mechanism, PMA, an activator of PKC, was employed to evoke SMC contraction in the absence of extracellular $Ca^{2+}$. In this protocol, the experiment was carried out on endothelium-denuded aortic rings. After verifying the endothelium, the aortic rings were washed and stabilized in $Ca^{2+}$-free Krebs solution. Later, the endothelium-denuded aortic rings were inducted into the sustained contraction with PMA (1 μM) in the $Ca^{2+}$-free Krebs solution for 1 h. Next, cumulative concentration–response curves were constructed by adding cumulative doses of the HHC (1 nM to 1 mM) to the bath. The data are shown as the percentages of the maximal contraction that was induced by PMA in the $Ca^{2+}$-free Krebs solution, and the results obtained in the absence (control) and presence of HHC were compared.

**Role of $K^+$-channel blockers in HHC-induced vasorelaxation.** To further investigate whether the insightful mechanisms of the endothelium-independent relaxation of HHC were because of the activation of various types of $K^+$-channel blockers, the endothelium-denuded rings were pre-incubated with non-selective $BK_{Ca}$ channel inhibitor TEA (5 mM), $K_v$ channel inhibitor 4-AP (1 mM), $K_{ATP}$ channel inhibitor glibenclamide (10 μM), or $K_{IR}$ channel inhibitor $BaCl_2$ (1 mM) for 30 min before the contraction generated by PE (10 μM). After that, the HHC

(1 nM to 1 mM) was added cumulatively. The relaxant data are shown as the percentages of relaxation as compared between the results obtained in the absence (control) and in the presence of four types of $K^+$channel blockers.

**Involvement of β-adrenergic receptor in HHC-induced vascular relaxation.** To assess whether the vasorelaxation induced by HHC is associated with *β*-adrenoreceptor, the endothelium-denuded aortic rings were pre-treated with 1 μM of propranolol (a nonselective blocker of β-adrenergic receptor) for 30 min before the addition of 10 μM of PE. The HHC at concentrations in the range of 1 nM to 1 mM was then added cumulatively. The relaxant data are shown as the percentages of relaxation as compared between the results obtained in the absence (control) of propranolol and in the presence of propranolol.

**Statistical analysis.** The experimental data are presented as the mean ± S.E.M., and *n* refers to the number of aortic rings studied. The degree of relaxation was determined as the percentage of reduction in the maximal arterial tension of the aortic rings after adding PE, or KCl, or PMA. The statistical analysis was performed using unpaired Student's *t*-test between two groups, and one-way analysis of variance (one-way ANOVA) was used for multiple comparisons between the experimental groups, followed by the Post hoc Dunnett's test (SPSS-software version 17). A value of $p$ less than 0.05 is considered significant in all the statistical evaluations. Cumulative concentration–response curves were plotted and adjusted using a nonlinear curve-fitting program (GraphPad Prism 5).

## RESULTS

### Vasorelaxant effects of HHC on rat aortic rings pre-contracted with PE or KCl

HHC at concentrations ranging from 1 nM to 1 mM was observed to significantly attenuate the sustained contraction induced by both PE and KCl in a concentration-dependent manner (Fig. 2). The vascular relaxation induced by HHC was not modified by the removal of the endothelium of the aortic rings. HHC caused concentration-dependent vasorelaxant effect on PE-induced contraction in both endothelium-intact and endothelium-denuded arteries, with $E_{max}$ values of 111.32 ± 5.76 and 111.93 ± 3.55%, respectively (vs. control group $E_{max}$ values of −2.57 ± 2.16%, $p < 0.001$). Similarly, in the KCl pre-contracted rings, the HHC-induced relaxation in the endothelium-intact arteries was not significantly different from that found in the endothelium-denuded rings, with $E_{max}$ values of 112.21 ± 4.68 and 113.16 ± 4.07%, respectively (vs. control group $E_{max}$ values of 5.61 ± 0.85%, $p < 0.001$). The $EC_{50}$ values of the relaxing effect of HHC for the endothelium-intact arteries and the endothelium-denuded arteries were 3.87 ± 0.87 μM, 1.13 ± 0.07 μM on PE-induced contraction and were 95.12 ± 1.04 μM, 90.90 ± 0.91 μM on KCl-induced contraction, respectively.

### Role of $\alpha_1$-adrenergic receptor in inhibitory vasoconstriction effect of HHC

HHC was able to inhibit the contraction provoked by PE (10 μM) in a concentration-dependent manner. Pre-incubation with various concentrations of HHC, that is, 1 μM, 10 μM, and 100 μM, was found to significantly inhibit the concentration–response contraction by PE, and suppress its maximal contraction ($E_{max}$) to 87.45 ± 3.65%, 79.91 ± 4.08%, and 43.32 ± 4.96%, respectively (vs. control group $E_{max}$ = 103.81 ± 6.21%). However, pre-treatment with HHC at the concentration of 0.1 μM was observed to have no significantly different effect from that of the control group with an $E_{max}$ value of 96.55 ± 1.16%, as demonstrated in Fig. 3.

### Effect of HHC on extracellular $Ca^{2+}$-induced contraction

To determine whether the vasorelaxant effect is mediated through $Ca^{2+}$ influx, a high concentration of KCl (80 mM) was used to depolarize the aortic rings. In the $Ca^{2+}$-free KCl (80 mM) solution, cumulative addition of $CaCl_2$ (10 μM to 10 mM) induced a progressively increased vascular tension (Fig. 4). Pre-treatment with HHC (1 μM, 10 μM, and 100 μM) significantly depressed the maximal contraction to 92.11 ± 3.27%, 83.5 ± 2.39%, and 63.08 ± 4.23%, respectively (vs. control group's

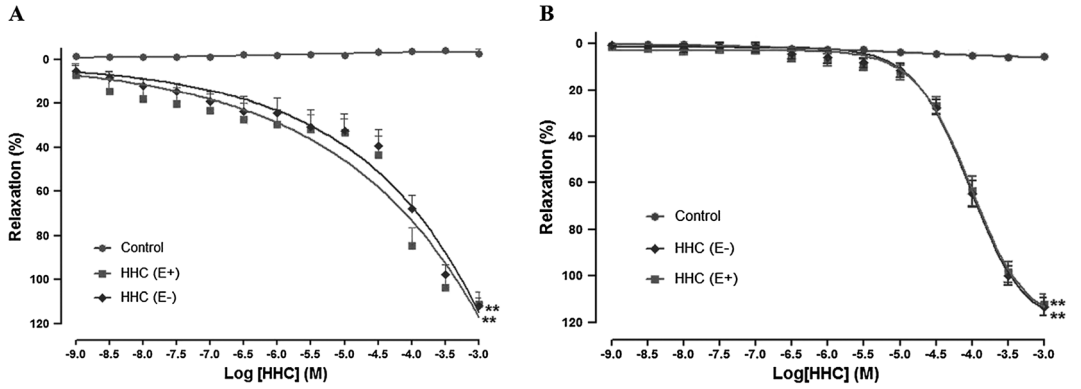


**Figure 2.** The cumulative concentration–response curve of the effect of HHC (1 nM to 1 mM) on isolated rat aortic rings pre-contracted with PE (10 μM) (A) and KCl (80 mM) (B), that is, in the endothelium-intact (E+) and endothelium-denuded (E−) rings. The data were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test, and expressed as mean ± S.E.M. of six rings. $^{**}p < 0.01$ vs. control group (0.1% DMSO).

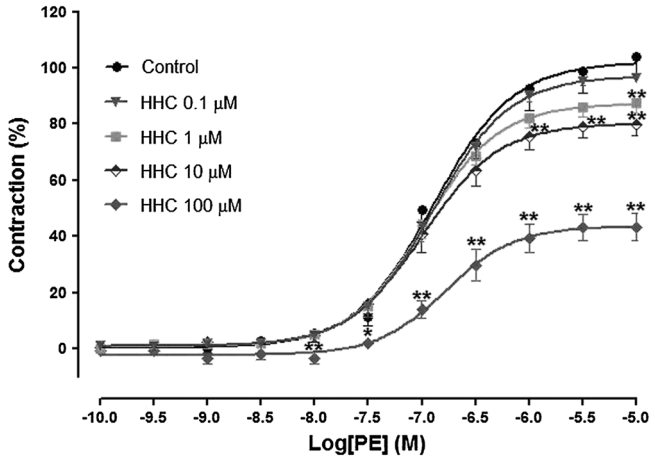


**Figure 3.** The inhibitory effects of HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) on the vascular contraction induced by cumulative 0.1 nM to 10 μM of PE in endothelium-intact rat aortic rings. The data were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test, and presented as mean ± S.E.M. of six rings. $*p < 0.05$ and $**p < 0.01$ vs. control group (0.1% DMSO).

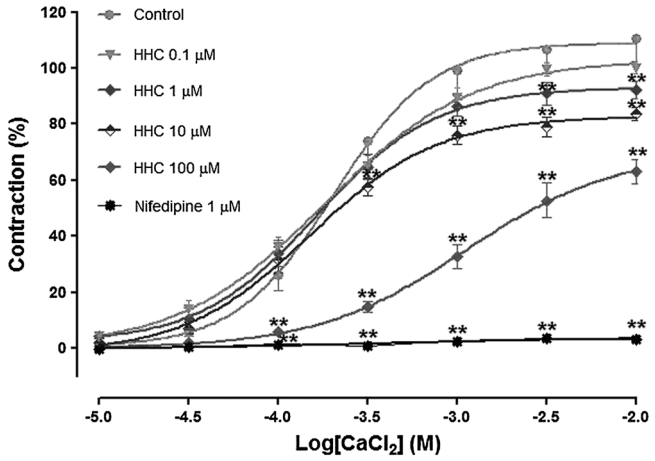


**Figure 4.** The effect of HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) and nifedipine (1 μM) on $CaCl_2$-induced contractile response in endothelium-denuded rings. The cumulative-concentration curves were determined in $Ca^{2+}$-free Krebs solution after intracellular and extracellular $Ca^{2+}$ depletion. The $CaCl_2$-contractile effect was dependent on the $Ca^{2+}$ influx via the VOCC, induced by KCl (80 mM). The data were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test, and expressed as mean ± S.E.M. of six rats. $**p < 0.01$ vs. control group (0.1% DMSO).

value of 110.43 ± 8.25%). However, pre-treatment with HHC at the concentration of 0.1 μM was observed to have no significantly different effect from that of the control group. Moreover, pre-treatment with nifedipine (1 μM), an L-type $Ca^{2+}$ blocker drug, was found to completely inhibit the contraction with an apparent $E_{max}$ value of 3.09 ± 1.14%.

## Effect of HHC on SR $Ca^{2+}$ release

In the $Ca^{2+}$-free Krebs solution, both PE (10 μM) and caffeine (20 mM) induced a transient contraction because of the release of intracellular $Ca^{2+}$ from the SR. As shown in Fig. 5A, pre-incubation with HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) for 30 min was observed to significantly attenuate PE-induced contraction which noticeably decreased the maximal contraction to 80.66 ± 1.96%, 70.68 ± 2.29%, 60.13 ± 2.23%, and 33.23 ± 3.74%, respectively (vs. control group's value of 88.65 ± 2.26%). HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) was also found to significantly reduce caffeine-evoked transient vasoconstriction to 77.19 ± 4.22%, 67.8 ± 4.35%, 64.58 ± 2.25%, and 49.95 ± 3.93%, respectively (vs. control group's value of 88.52 ± 3.26%) (Fig. 5B).

## Effect of HHC on $Ca^{2+}$ sensitivity of contractile proteins and on eliciting $Ca^{2+}$-independent vasoconstriction

In order to determine the possibility that HHC relaxed aortic rings through $Ca^{2+}$-independent mechanism, PMA (1 μM), the PKC activator, was applied to evoke a sustained contraction in the $Ca^{2}$-free Krebs solution. The results revealed that the cumulative application of HHC caused concentration-dependent relaxation with an $EC_{50}$ value of 93.36 ± 1.03 μM (Fig. 6). The contraction induced by PMA was found to have been significantly abolished by HHC, and it showed 117.6 ± 1.83% of relaxant action in the HHC treatment (vs. control group's value of −4.83 ± 3.08%).

## Role of $K^+$-channels in HHC-induced vasorelaxation

To test the involvement of $K^+$ channels in HHC induced relaxation in endothelium-denuded aorta rings, the aortic

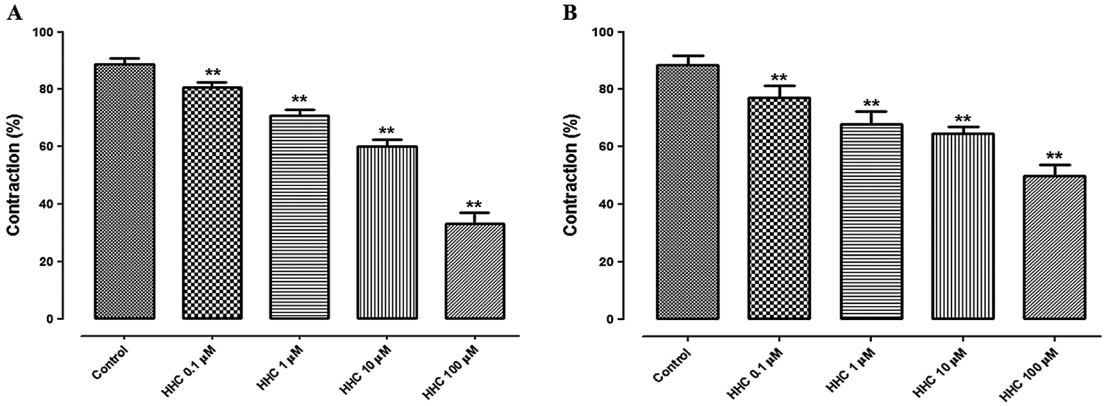


**Figure 5.** The inhibitory effect of HHC (0.1 μM, 1 μM, 10 μM, and 100 μM) on PE (10 μM) (A), and caffeine (20 mM) (B) induced-intracellular $Ca^{2+}$ release from the SR on endothelium-denuded aortic rings in the $Ca^{2+}$-free Krebs solution. The data were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test, and expressed as mean ± S.E.M. of six rats. $**p < 0.01$ vs. control group (0.1% DMSO).

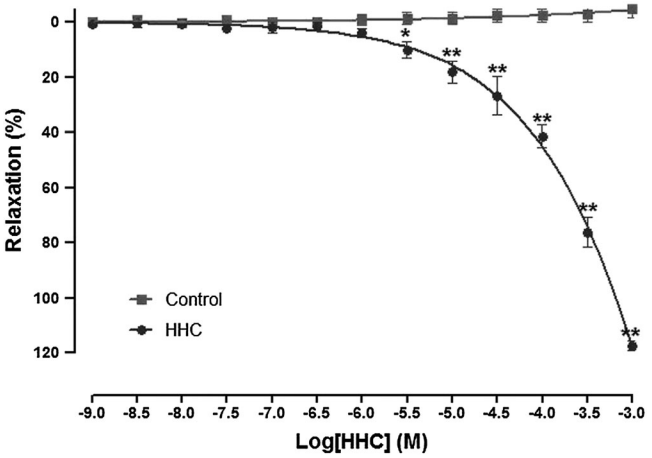


**Figure 6.** The inhibitory effect of HHC (1 nM to 1 mM) on PMA (1 μM) pre-contracted endothelium-denuded aortic rings in the $Ca^{2+}$-free Krebs solution. The data were analyzed with Student's *t*-test and expressed as mean ± S.E.M. of six rats. $*p < 0.05$ and $**p < 0.01$ vs. control group (0. 1% DMSO).

vessels were pre-treated with $K^+$ channel blockers, such as $BK_{Ca}$ blocker TEA (5 mM), $K_V$ blocker 4-AP (1 mM), $K_{IR}$ blocker $BaCl_2$ (1 mM), or $K_{ATP}$ blocker glibenclamide (10 μM) for 30 min before the contraction produced by PE (10 μM). As shown in Fig. 7, pre-treatment with $K^+$ channel blockers produced no effect on the HHC-induced vasorelaxation ($p > 0.05$). This result indicates that $K^+$ channel activation is not involved in HHC-induced relaxation.

### Involvement of β-adrenergic receptor in HHC-induced vascular relaxation

To test the involvement of β-adrenergic receptor in HHC-induced relaxation in endothelium-denuded aorta rings, we pre-treated aortic vessels with propranolol (1 μM), a non-selective β-adrenergic receptor blocker, for 30 min before contraction produced by PE (10 μM). The result found that propranolol significantly decreased the maximal relaxation to 107.56 ± 2.68% (vs. control group's value of

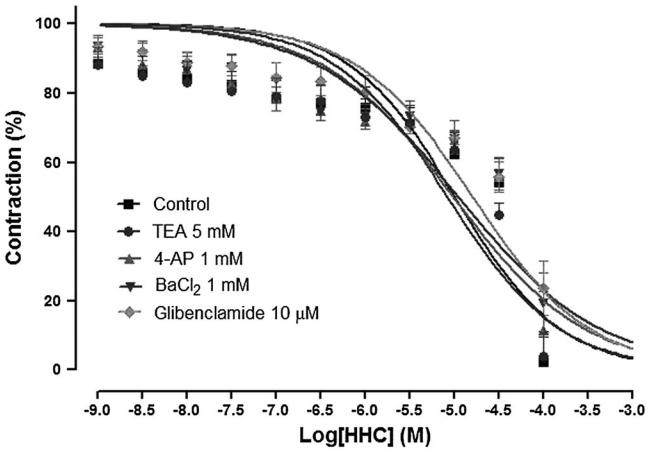


**Figure 7.** The effects of $K^+$ channel blockers on vasorelaxant response obtained by HHC (1 nM to 1 mM) in endothelium-denuded aortic rings pre-contracted with PE (10 μM) in the absence (control) or presence of 4-aminopyridine (4-AP, 1 mM), tetraethylammoniumchloride (TEA, 5 mM), glibenclamide (10 μM), and barium chloride ($BaCl_2$, 1 mM). The data were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test, and expressed as mean ± S.E.M. of $n = 6$ rats.

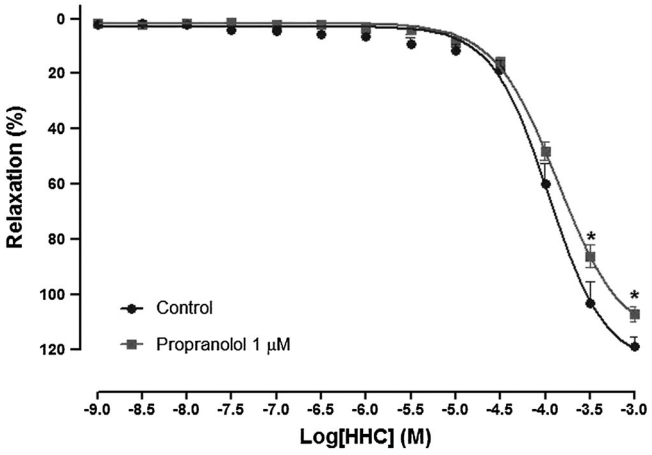


**Figure 8.** The vasorelaxant response induced by HHC (1 nM to 1 mM) in endothelium-denuded aortic rings pre-contracted with PE (10 μM) in the absence (control) or presence of propranolol (a nonselective β-receptor antagonist, 1 μM). The data were analyzed with Student's *t*-test and expressed as mean ± S.E.M. of six rats. $*p < 0.05$ vs. control group (0. 1% DMSO).

118.82 ± 3.62%) (Fig. 8). This result indicates that the β-adrenergic receptor is involved in HHC-induced relaxation.

## DISCUSSION

HHC, one of the major metabolites of curcumin, has been examined for its vasorelaxant effects and the underlying mechanisms of action involved in rat thoracic aorta. It is well known that one of the principal mechanisms of antihypertensive drugs is to lower the vascular resistance by dilating the blood vessels, thereby expressing their influence through vascular endothelium, or by acting directly on the vascular SMC, which are, respectively, called endothelium-dependent and endothelium-independent mechanisms. Our results demonstrate that HHC dose-dependently relaxed the endothelium-intact aortic rings pre-contracted with KCl and PE. However, mechanical removal of the endothelium was not found to significantly attenuate the relaxant effects of HHC in both PE and KCl pre-contracted rings. These findings suggest that the vasorelaxant effect induced by HHC occurs by endothelium-independent mechanisms, and that it directly acts through SMC.

Accumulation of intracellular $Ca^{2+}$ concentration through an influx of extracellular $Ca^{2+}$ via $Ca^{2+}$ channels as well as $Ca^{2+}$ release from intracellular stores is a critical factor in excitation–contraction coupling in smooth muscle contraction (Jackson, 2000). For this reason, we next focused on the vasorelaxant ability of HHC regarding whether it would be able to modulate the intracellular $Ca^{2+}$ ion level in the SMC. The influx of extracellular $Ca^{2+}$ occurs mainly through two types of transmembrane $Ca^{2+}$ channels, which are ROCC and VOCC (Berridge *et al.*, 2003). The contraction induced by PE is because of the activation of the $\alpha_1$-adrenergic receptor, which eventually results in an increase in the $Ca^{2+}$ influx pass through the ROCC whereas $K^+$-induced contraction is mediated by membrane depolarization and subsequent opening of the VOCC (Metzler *et al.*, 2013). Besides the activation of the ROCC, the binding of PE to $\alpha_1$-adrenergic receptors also activated the production of inositol-1,4,5-triphosphate ($IP_3$); $IP_3$ stimulates $IP_3$ receptors ($IP_3R$)

on the SR membrane and then mediates $Ca^{2+}$mobilization from SR, thus leading to transient contraction of vessels (Chen *et al*., 2009; Tao *et al*., 2013). Our results revealed that HHC was able to relax aortic rings precontracted with either PE or KCl, indicating that a vasodilator effect caused by the HHC might be acting as the blockade of both the ROCC and the VOCC. We next examined the inhibitory effect of HHC on $\alpha^1$-receptors: our results clearly demonstrated that pre-incubation with HHC (1 μM, 10 μM, and 100 μM) significantly reduced the vascular tension induced by PE cumulatively (0.1 nM to 10 μM) in a concentration-dependent manner. Thus, it seems likely that the ROCC-coupled $\alpha_1$-adrenergic receptors may be affected by the HHC compound. Moreover, the influence of HHC on the VOCC was also evaluated in $Ca^{2+}$-mediated contraction in high $K^+$-depolarizing solution. We found that HHC significantly inhibited the $Ca^{2+}$-induced contraction in high $K^+$-depolarized rings in a concentration-dependent manner, indicating that HHC can inhibit extracellular $Ca^{2+}$ movement through the VOCC. In addition, the contraction was observed to be completely abolished by nifedipine, a typical L-type VOCC blocker, confirming the involvement of L-type VOCC in the contractile response of HHC (Lee *et al*., 2005). Next, we further studied whether the vasorelaxant effect of HHC was because of the interfering of the $Ca^{2+}$ released from intracellular stores. As discussed above, PE induces $Ca^{2+}$ release from SR via $IP_3R$ activation, whereas caffeine induces $Ca^{2+}$ release from SR via $IP_3$-independent ryanodine receptors (RyR), intracellular $Ca^{2+}$channels on the SR membrane (Berridge *et al*., 2003). In our study, it was observed that HHC markedly decreased the transient contraction induced by both PE and caffeine in a dose-dependent manner. Therefore, it seems likely that the vasorelaxant actions of HHC involve a reduction in the $IP_3$-independent and $IP_3$-dependent mediated $Ca^{2+}$mobilizationfrom the internal stores. Aside from the activation of the ROCC and $IP_3R$, PE also generates a second messenger diacylglycerol (DAG) which further activates PKC activity which is involved in various physiological activities. PKC has been reported to promote vascular contraction by blockading the $K^+$ channels, followed by the opening of the VOCC and the ROCC (Lin *et al*., 2010). In addition, PKC also modulates vascular contraction by directly phosphorylating myosin light chain kinase (MLCK), which consequently phosphorylates the contractile proteins myosin light chain (MLC). Indeed, PKC induces increased $Ca^{2+}$sensitivity in the contractile proteins, and triggers a $Ca^{2+}$-independent vasoconstriction (Hilgers and Webb, 2005). In the present study, we used the PMA, a PKC activator, to evoke sustained contraction of the aortic vessels in the $Ca^{2+}$-free Krebs solution and we found that the HHC decreased the PMA-induced contraction in a concentration-dependent manner. Thus, it is likely that the PKC-mediated $Ca^{2+}$-independent vasoconstriction may be another target of HHC-induced relaxation.

It is well known that opening of $K^+$ channels leads to membrane hyperpolarization, and closure of VOCC, which inhibits $Ca^{2+}$ influx and subsequent vasodilation (Sun *et al*., 2011). Moreover, membrane hyperpolarization is reported to inhibit PKC activities (Yamamura *et al*., 2012), thereby promoting vasorelaxation. Our results revealed that the vasorelaxant effects of HHC were unaffected by pre-treatment with TEA (a blocker of $BK_{Ca}$), 4-AP (a $K_V$ blocker), $BaCl_2$ (a non-specific blocker of $K_{IR}$), and glibenclamide (a selective inhibitor of $K_{ATP}$), suggesting that the effect of HHC on smooth muscle relaxation did not seem to be because of the opening of the $K^+$ channels. Therefore, we excluded the ability of HHC to activate $K^+$ channel-mediated vasorelaxation.

β-adrenergic receptors, which are distributed on the plasma membrane of the vascular SMC in both rats and humans, are important regulators of the vascular tone (Li *et al*., 2010). β-Adrenoceptor-induced vasodilation is activated by an increase in the intracellular cyclic adenosine monophosphate (cAMP) concentration and by the activation of the $K^+$ channels (Huang and Kwok, 1998; Sun *et al*., 2011). To investigate the possible involvement of β-adrenoceptors in HHC-induced relaxation, we used propranolol, a β-adrenergic receptor blocker, in this protocol. The result showed that the propranolol partially inhibited the HHC-induced relaxant response when the volumes were 0.3 mM and 1 mM, indicating that the high concentrations of HHC-induced vasorelaxation might be attributed to β-adrenoceptors.

In conclusion, our results suggest that HHC induces relaxation in rat aortic rings through an endothelium-independent pathway. The possible mechanisms of vasorelaxation of HHC involved four mechanisms, including (1) activation through β-adrenoceptors, (2) inhibition of extracellular $Ca^{2+}$ influx via the ROCC and the VOCC, (3) inhibition of $Ca^{2+}$mobilization from intracellular stores as well as (4) inhibition of PKC mediated $Ca^{2+}$-independent contraction. It needs to be mentioned that additional studies are needed to test the other possible mechanisms underlying HHC-induced vasorelaxant effects, in addition to further *in vivo* study.

## Acknowledgements

This work was supported by the Faculty of Medicine Research Fund, Chiang Mai University, Chiang Mai, Thailand, and the Office of the Higher Education Commission. Supports from The Thailand Research Fund and the Center of Excellence for Innovation in Chemistry are gratefully acknowledged.

## Conflict of Interest

The authors declare no conflict of interest.

## REFERENCES

Adaramoye OA, Anjos RM, Almeida MM, *et al*. 2009. Hypotensive and endothelium-independent vasorelaxant effects of methanolic extract from *Curcuma longa* L. in rats. *J Ethnopharmacol* **124**: 457–462.

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. 2007. Bioavailability of curcumin: problems and promises. *Mol Pharm* **4**: 807–818.

Belviranli M, Okudan N, Atalik KE, Oz M. 2013. Curcumin improves spatial memory and decreases oxidative damage in aged female rats. *Biogerontology* **14**: 187–196.

Berridge MJ, Bootman MD, Roderick HL. 2003. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4**: 517–529.

Bhullar KS, Jha A, Youssef D, Rupasinghe HP. 2013. Curcumin and its carbocyclic analogs: structure–activity in relation to

antioxidant and selected biological properties. *Molecules* **18**; 5389–5404.
Brito TS, Lima FJ, Aragao KS, *et al*. 2013. The vasorelaxant effects of 1-nitro-2-phenylethane involve stimulation of the soluble guanylate cyclase–cGMP pathway. *Biochem Pharmacol* **85**: 780–788.
Changtam C, de Koning HP, Ibrahim H, Sajid MS, Gould MK, Suksamrarn A. 2010. Curcuminoid analogs with potent activity against Trypanosoma and Leishmania species. *Eur J Med Chem* **45**: 941–956.
Chen GP, Ye Y Li L, Yang Y, Qian AB, Hu SJ, 2009. Endothelium-independent vasorelaxant effect of sodium ferulate on rat thoracic aorta. *Life Sci* **84**: 81–88.
Dempe JS, Scheerle RK, Pfeiffer E, Metzler M. 2013. Metabolism and permeability of curcumin in cultured Caco-2 cells. *Mol Nutr Food Res* **57**: 1543–1549.
Deters M, Knochenwefel H, Lindhorst D, *et al*. 2008. Different curcuminoids inhibit T-lymphocyte proliferation independently of their radical scavenging activities. *Pharm Res* **25**: 1822–1827.
Dulbecco P, Savarino V. 2013. Therapeutic potential of curcumin in digestive diseases. *World J Gastroenterol* **19**: 9256–9270.
Hilgers RH, Webb RC. 2005. Molecular aspects of arterial smooth muscle contraction: focus on Rho. *Exp Biol Med* **230**: 829–835.
Hlavackova L, Janegova A, Ulicna O, Janega P, Cerna A, Babal P. 2011. Spice up the hypertension diet-curcumin and piperine prevent remodeling of aorta in experimental L-NAME induced hypertension. *Nutr Metab* **8**: 1743–7075.
Holder GM, Plummer JL, Ryan AJ. 1978. The metabolism and excretion of curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] in the rat. *Xenobiotica* **8**: 761–768.
Huang Y, Kwok KH. 1998. Beta-adrenoceptor-mediated relaxation inhibited by tetrapentylammonium ions in rat mesenteric artery. *Life Sci* **62**: L19–25.
Ireson C, Orr S, Jones DJ, *et al*. 2001. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. *Cancer Res* **61**: 1058–1064.
Ireson CR, Jones DJ, Orr S, *et al*. 2002. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. *Cancer Epidemiol Biomarkers Prev* **11**: 105–111.
Ishida J, Ohtsu H, Tachibana Y, *et al*. 2002. Antitumor agents. Part 214: synthesis and evaluation of curcumin analogues as cytotoxic agents. *Bioorg Med Chem* **10**: 3481–3487.
Jackson WF. 2000. Ion channels and vascular tone. *Hypertension* **35**: 173–178.
Lee SL, Huang WJ, Lin WW, Lee SS, Chen CH. 2005. Preparation and anti-inflammatory activities of diarylheptanoid and diarylheptylamine analogs. *Bioorg Med Chem* **13**: 6175–6181.
Li YJ, Bao JX, Xu JW, Murad F, Bian K.2010. Vascular dilation by paeonol—a mechanism study. *Vascul Pharmacol* **53**: 169–176.
Lim TG, Lee SY, Huang, Z, *et al*. 2014. Curcumin suppresses proliferation of colon cancer cells by targeting CDK2. *Cancer Prev Res* **7**: 466–474.
Lin YL, Dai ZK, Lin RJ, *et al*. 2010. Baicalin, a flavonoid from *Scutellaria baicalensis* Georgi, activates large-conductance $Ca^{2+}$-activated $K^+$ channels via cyclic nucleotide-dependent protein kinases in mesenteric artery. *Phytomedicine* **17**: 760–770.
Martins CV, da Silva DL, Neres AT, *et al*. 2009. Curcumin as a promising antifungal of clinical interest. *J Antimicrob Chemother* **63**: 337–339.
Matsuda H, Tewtrakul S, Morikawa T, Nakamura A, Yoshikawa M. 2004. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-alpha and IL-4 in RBL-2H3 cells. *Bioorg Med Chem* **12**: 5891–5898.
Metzler M, Pfeiffer E, Schulz SI, Dempe JS.2013. Curcumin uptake and metabolism. *Biofactors* **39**: 14–20.
Morsy MA, El-Moselhy MA. 2013. Mechanisms of the protective effects of curcumin against indomethacin-induced gastric ulcer in rats. *Pharmacology* **91**: 267–274.
Pan MH, Huang TM, Lin JK. 1999. Biotransformation of curcumin through reduction and glucuronidationin mice. *Drug Metabol Dispos* **27**: 486–494.
Somparn P, Phisalaphong C, Nakornchai S, UnchernS, Morales NP.2007. Comparative antioxidant activities of curcumin and its demethoxy and hydrogenated derivatives. *Biol Pharm Bull* **30**: 74–78.
Srimuangwong K, Tocharus C, Yoysungnoen Chintana P, Suksamrarn A, Tocharus J. 2012a. Hexahydrocurcumin enhances inhibitory effect of 5-fluorouracil on HT-29 human colon cancer cells. *World J Gastroenterol* **18**: 2383–2389.
Srimuangwong K, Tocharus C, Tocharus J, Suksamrarn A, Chintana PY. 2012b. Effects of hexahydrocurcumin in combination with 5-fluorouracil on dimethylhydrazine-induced colon cancer in rats. *World J Gastroenterol* **18**: 6951–6959.
Sun T, Liu R, Cao YX. 2011. Vasorelaxant and antihypertensive effects of formononetin through endothelium-dependent and -independent mechanisms. *Acta Pharmacol Sin* **32**: 1009–1018.
Tao L, Hu HS, Shen XC. 2013. Endothelium-dependent vasodilatation effects of the essential oil from Fructus Alpiniae Zerumbet (EOFAZ) on rat thoracic aortic rings in vitro. *Phytomedicine* **20**: 387–393.
Venkateswarlu S, Ramachandra MS, Subbaraju GV. 2005. Synthesis and biological evaluation of polyhydroxycurcuminoids. *Bioorg Med Chem* **13**: 6374–6380.
Xu PH, Long Y, Dai F, Liu ZL. 2007. The relaxant effect of curcumin on porcine coronary arterial ring segments. *Vascul Pharmacol* **47**: 25–30.
Yamamura H, Ohya S, Muraki K, Imaizumi Y. 2012. Involvement of inositol 1,4,5-trisphosphate formation in the voltage-dependent regulation of the $Ca^{(2+)}$concentration in porcine coronary arterial smooth muscle cells. *J Pharmacol Exp Ther* **342**: 486–496.
Zhang HS, Ruan Z, Sang WW. 2011. HDAC1/NFkappaB pathway is involved in curcumin inhibiting of Tat-mediated long terminal repeat transactivation. *J Cell Physiol* **226**: 3385–3391.