

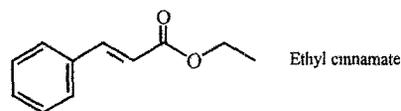
# Vasorelaxant Effects of Ethyl Cinnamate Isolated from *Kaempferia galanga* on Smooth Muscles of the Rat Aorta

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## Abstract

From the rhizomes of *Kaempferia galanga*, ethyl cinnamate (EC) was isolated and its vasorelaxant effect was examined on the rat aorta. EC inhibited the tonic contractions induced by high K<sup>+</sup> and phenylephrine (PE) in a concentration-dependent manner, with respective IC<sub>50</sub> values of 0.30 ± 0.05 mM and 0.38 ± 0.04 mM. The relaxant effect against PE-induced contractions was greater in the presence of endothelium. Pre-treatment of the aorta with methylene blue and indomethacin significantly reduced the relaxant effect. These results suggest that the inhibitory effects of EC may involve inhibition of Ca<sup>2+</sup> influx into vascular cells and release of nitric oxide (NO) and prostacyclin from the endothelial cells. Thus, the vasorelaxant effect of EC mediated through multiple pathways may explain the traditional use of the parent plant in treating hypertension.

*Kaempferia galanga* L. (Zingiberaceae) grows wild or is cultivated in India, China, South-east Asia, particularly Malaysia, Indonesia and Singapore [1]. It is widely used as flavouring in food and as an important element in the preparation of 'jamu' (a local health tonic). It is also known to treat ailments such as hypertension, rheumatism and asthma. The rhizomes of this plant are used to treat abdominal pain, boiled with other roots for treating women after childbirth, also to treat swelling and muscular rheumatism [2], [3]. The results of our previous study [4] showed that the smooth muscle relaxant activity of the crude extract was mainly due to the inhibition of Ca<sup>2+</sup> influx through the voltage- and receptor-operated channels, and Ca<sup>2+</sup> sensitivity of contractile elements. In the present study, EC (1) was isolated, purified from



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the rhizome of the plant and identified as the major compound contributing to the vasorelaxant activity.

In rat aorta, high  $K^+$  (80 mM) caused a tonic contraction while PE (0.1  $\mu$ M) caused an initial phasic contraction followed by a tonic contraction [5]. The cumulative applications of EC inhibited the sustained contractions induced by high  $K^+$  and PE with  $IC_{50}$  values of  $0.30 \pm 0.05$  mM and  $0.38 \pm 0.04$  mM, respectively (Fig. 1). Contractions induced by high  $K^+$  (80 mM) are due to membrane depolarization, which activates L-type voltage dependent channels (VDC) and thus permits  $Ca^{2+}$  entry. In addition to VDC, receptor agonists, such as PE, activate receptor-operated  $Ca^{2+}$  channels (ROC) to induce the sustained contraction [6]. In a preliminary experiment, we showed that verapamil at 10  $\mu$ M, markedly reduced both high  $K^+$  ( $95 \pm 2\%$ ;  $n = 5$ )- and PE ( $82 \pm 5\%$ ;  $n = 5$ )-induced contractions of the rat aorta. The present findings suggest that EC shares a similar relaxant action to verapamil, a calcium channel blocker [7].

It had been shown that most of the  $Ca^{2+}$  channel blockers have additional intracellular sites of action [8]. The relaxant action of EC against PE-induced contractions was compared between endothelium intact and denuded preparations. Following the removal of the endothelium, inhibitory action of the compound against the contractions of the aorta was markedly attenuated (Fig. 2) suggesting the involvement of NO and prostacyclin. NO activates soluble guanylate cyclase of vascular smooth muscle, and the resulting increase in cyclic GMP levels produces relaxation [9]. Methylene blue is an inhibitor of guanylate cyclase, while indomethacin abolishes the generation of prostacyclins by inhibiting the enzyme cyclo-oxygenase, which is involved in the metabolism of arachidonic acid. In this experiment, the rat aorta was pre-treated with methylene blue (10  $\mu$ M) or indomethacin (20  $\mu$ M) for 20 minutes before contracting the muscle with 0.1  $\mu$ M phenylephrine. The relaxant effect of ethyl cinnamate was strongly inhibited by pre-treatment with methylene blue (36%) and indomethacin (71%), thus confirming the additional involvement of NO and prostacyclins in mediating the vasorelaxant action of the compound (Fig. 3). These findings suggested that EC acts upon various sites causing the relaxation of vascular smooth muscles and presence of this compound in *Kaempferia galanga* may explain the traditional use of the plant in treating hypertension. Interestingly, EC is contained in all red wines as flavour, which might be responsible for the known vasorelaxant effect of red wine [10].

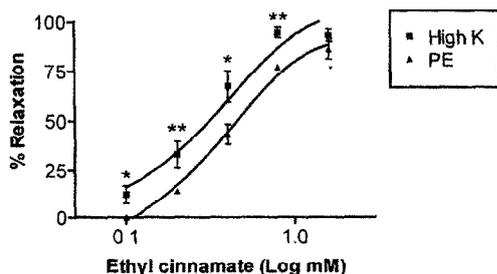


Fig. 1 Effect of ethyl cinnamate on high  $K^+$  and phenylephrine (PE)-induced contractions in endothelium intact rat aorta. Ethyl cinnamate was added cumulatively every 10 minutes to the rat aorta pre-contracted either with high  $K^+$  (80 mM) or PE (0.1  $\mu$ M). Each point represents the mean  $\pm$  S.E. of 4 experiments. \*,  $P < 0.05$ , compared with the contractions induced by PE alone.

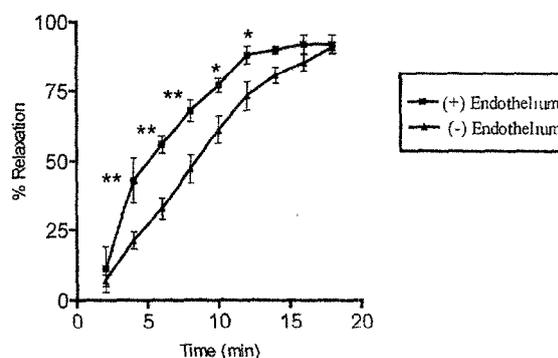


Fig. 2 Effect of ethyl cinnamate on the contractions induced by phenylephrine (PE) in endothelium-intact and denuded preparations of the rat aorta. The aorta were pre-contracted with PE (0.1  $\mu$ M) and when the contractions reached maximum, 0.30 mM ethyl cinnamate was added to the bath. Each point represents the mean  $\pm$  S.E. of 5 experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , compared with the values obtained in the absence of endothelium.

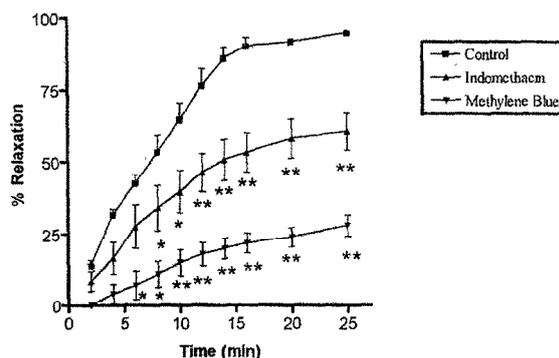


Fig. 3 Effects of methylene blue and indomethacin on the relaxant action of ethyl cinnamate on the contraction induced by phenylephrine in endothelium intact rat aorta. Either methylene blue (20  $\mu$ M) or indomethacin (20  $\mu$ M) was pre-incubated with the tissue 10 minutes before inducing a contraction with PE and subsequent addition of ethyl cinnamate. Each point represents the mean  $\pm$  S.E. of 4–7 experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the values obtained in the absence of methylene blue and indomethacin.

## Materials and Methods

Silica gel 60 F<sub>254</sub> (230–400 Mesh ASTM) was used in thin layer chromatography (TLC). Kieselgel (70–230 Mesh ASTM) was used in column chromatography (CC). UV light (254 and 365 nm) was used to examine TLC spots or bands.  $I_2$  vapour was used as staining reagent. Spectral data were obtained as follows: UV on a Shimadzu UV-160A, IR on a Perkin-Elmer 1600 series double-beam recording spectrometer, NMR on a JEOL JNM-LA400 FT NMR system and MS on a Shimadzu GC-MS (GC-17A, MSQP-1000).

*K. galanga* was obtained from the botanical garden of the University of Malaya and was identified by a botanist, Halijah Ibrahim. The dried rhizomes (5 kg) of this plant were extracted using Soxhlet with petroleum ether and dichloromethane ( $CH_2Cl_2$ ), consecutively. The dried crude  $CH_2Cl_2$  extract (CEKCL) represented a yield of 2.4% of the dried powder. CEKCL was mixed with petroleum ether, the precipitate was filtered and the mother li-

quor was collected and dried to get a mixture of oil (KDNP). KDNP was passed through CC (3 × 65 cm) on silica gel (220 g); CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH (100:0, 99:1, 98:2, 95:5, 90:10, 80:20, 90:10) to give nine fractions. Fraction 2 (100% CH<sub>2</sub>Cl<sub>2</sub>) exhibited vasorelaxant activity and was separated using TLC on silica gel; petroleum ether:EtOAc (95:5); of which the fraction with R<sub>f</sub> = 0.62 was active. This was separated using TLC, petroleum ether:EtOAc (92:8), yielding six fractions, four of which were active with the third fraction being most active (Fractions 1, R<sub>f</sub> = 0.89; 2, 0.75; 3, 0.58; 4, 0.36; 5, 0.13). The overlapped TLC spots suggested that fractions 1 to 3 may contain similar compounds and since fraction 2 yielded the longest amount of sample, this fraction was further fractionated by CC (2 × 50 cm) on silica gel; petroleum ether:EtOAc (100:0, 90:10, 80:20, 70:30). Fractions with a single spot on TLC (R<sub>f</sub> = 0.55); petroleum ether:EtOAc (90:10), were grouped together (compound **1**; colourless oil; 100% pure; 9.75% of the CH<sub>2</sub>Cl<sub>2</sub> extract). Based on spectral data and comparison with the literature values of known compounds [11], [12], **1** was identified as ethyl cinnamate (copies of the original spectra are obtainable from the author of correspondence). Compound **1** was reconstituted in absolute ethanol and diluted with freshly prepared physiological solution on the day of the experiment. The final concentration of the solvent was kept below 0.1%, which had no effect of its own. Drugs used for pharmacological experiments were PE hydrochloride, indomethacin (Research Biochemical Incorporated, USA) methylene blue and verapamil (Sigma Chemical Co., USA). All drugs were prepared in distilled water except indomethacin, which was dissolved in dimethyl sulphoxide (DMSO) and then diluted with distilled water to make a solution. The final concentration of DMSO did not exceed 0.1% v/v.

The thoracic aorta were removed from 8 to 10 week-old male Wistar rat (200–250 g) supplied by the University of Malaya Animal Unit and approved by the Animals Care and Use Committee (No. FAR-2691998). The vessels were cut into rings of about 3–4 mm in length and mounted in 5 ml organ baths containing Krebs solution of the following composition (in mM): NaCl, 136.9; KCl, 5.4; CaCl<sub>2</sub>, 1.5; MgCl<sub>2</sub>, 1.0; NaHCO<sub>3</sub>, 23.8; ethylenediaminetetraacetic acid, 0.01; glucose, 5.5. The high K<sup>+</sup> solutions were prepared by substituting NaCl with KCl (80 mM) in an equimolar amount. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a Grass FT03 transducer for isometric tension recording. The baths were warmed to 37 °C and pH of the solution adjusted to 7.2, and gassed with oxygen containing 5% CO<sub>2</sub>. The aortic rings were equilibrated for 20 min before stretching to approximately 1 g, and allowed to equilibrate further for at least 60 min. In some experiments, the endothelium was removed by gently rubbing the intimal surface with a blunt forceps; otherwise, all other experiments were performed with the endothelium intact. The contractile responses were calculated as the relative percentage of control. All data were expressed as the mean ± S.E. Significant difference between responses were analysed by the Student's t-test and P values of less than 0.05 were considered as significant.

#### Acknowledgements

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## CHEMICAL COMPOSITION OF COMPONENTS OF VI-28

### Radix Ginseng

- 2-3% Ginsenosides (triterpene saponins)
- 0.05% essential oil (limonene, terpineol, citrol, polyacetylenes)
- sugar
- starch

### Cornu Cervi Pantotrichum

- 34% Ash
- 12% moisture
- nitrogen
- fats
- collagen
- glycosaminoglycans (chondroitin sulfate, keratin sulfate, hyaluronic acid, dermatan sulfate, chondroitin sulfate proteoglycan, decorin)
- lipids (polysaccharides)
- growth hormone and prostaglandins (IGF-1, IGF-2)

### Semen Cuscutae

- quercetin 3-O-beta-D-galactoside-7-O-beta-D-glucoside (I)
- quercetin 3-O-beta-D-apiofuranosyl-(1-->2)-beta-D-galactoside (II)
- hyperoside (III)
- isorhamnetin (IV)
- kaempferol (V)
- quercetin (VI)
- d-sesamin (VII)
- 9(R)-hydroxy-d-sesamin (VIII)
- Vitamin A

### Fructus Cnidii

- osthol
- imperatorin
- xanthotoxin
- isopimpinellin
- bergapten

## Kaempferiae Rhizoma

- cineol
- borneol
- 3-carene
- camphene
- kaempferal
- kaempferide
- cinnamaldehyde
- p*-methoxycinnamic acid
- ethyl cinnamate
- ethyl *p*-methoxycinnamate

## Inactive Ingredient

- rice powder

## Capsule

- Gelatin Capsule

## METHOD OF MANUFACTURE

### Phase A

Radix ginseng; Cornu Cervi pantotrichum and Rhizome kaempseriae are ground to a fine powder under low temperature.

### Phase B

Fructus cnidii and Semen cuscutae are water decocted, filtered, the solution is oven dried, and reduced to a fine powder via grinding process.

Phase A and B are mixed in proper proportion to form the final mix. The final mix is heat treated at 80°C for 24 hours, and then filled into gelatin capsules.

## MICROBES AND PESTICIDE CONTROL

In the control of microbes, it is believed the method of manufacture, namely the use of heat treatment 80°C for 24 hours, is sufficient to address any microbes that may be present.

Regarding pesticide monitoring, the final mix is subject to pesticide monitoring in accordance with the Hong Kong Standards and Testing Centre (report of 2003-02-14 enclosed herewith).

## TEST ARTICLES UTILIZED IN STUDIES

The FDA has asserted in its reply to the original Notification that, at page 3, second full paragraph, there are discrepancies with the description of the test articles used in studies submitted with the original Notification. Specifically, the FDA states that one study reported was conducted with VI-28, and other studies were conducted with test substances similar to VI-28, however "the relationship of these test materials to the botanical preparations that are the subject of the notification was not stated".

With reference to the letter faxed to Dr. Walker on 21 January 2004 (attached herewith), it was previously stated that VI-28 to be marketed consists of the botanical preparations that were the subject of the original Notification (and subsequently this re-submitted Notification). Because the botanical preparations are not modified chemically in the preparation of VI-28 and the preparations are believed to be safe, it is believed that VI-28 to be marketed is safe. The test materials used in the studies are qualitatively and quantitatively akin to VI-28 to be marketed in the U.S., i.e., it consists of the same botanical preparations used in the same amounts as stated in this Notification.