Bioassay-guided isolation of a vasorelaxant active compound from *Kaempferia galanga* L.

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**Abstract**

Bioassay-guided fractionation was performed on a crude dichloromethane extract of *Kaempferia galanga* L. using chromatography techniques. Screening of the extract for biological activity started with the brine shrimp lethality bioassay, followed by the study of its antihypertensive activity on anaesthetized rats, which involved monitoring of the extract’s effect on mean arterial blood pressure. The components of the fractions obtained from the separation procedures were analyzed using gas chromatography (GC). The yield of the CH$_2$Cl$_2$ extract was 0.29% of the crude plant extract. Analysis of the data for brine shrimp lethality test using the Finney computer program showed that this extract exhibited potent bioactivity with an ED$_{50}$ value of 7.92 ± 0.13 μg ml$^{-1}$. Intravenous administration of the extract induced a dose-related reduction of basal mean arterial pressure (MAP) (130 ± 5 mmHg) in the anaesthetized rat, with maximal effects seen after 5–10 min of injection. The gas chromatogram showed that the common compound in the active fractions obtained from the bioassay-guided fractionation of the CH$_2$Cl$_2$ extract was ethyl cinnamate. This vasorelaxant active compound, ethyl cinnamate, was isolated as a colorless oil. Ethyl p-methoxycinnamic acid was also isolated as white needles but did not exhibit any relaxant effect on the precontracted thoracic rat aorta.

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**Keywords:** *Kaempferia galanga* L.; Ethyl cinnamate; Bioassay-guided isolation; Gas chromatography analysis.

**Introduction**

*Kaempferia galanga* L. is a common Malaysian Zingiberaceae species, known locally as cekur (‘kencur’ by the Indonesians and ‘shan-nai’ by the Chinese) (Perry and Metzger, 1980). It is used widely as a spice, a food flavoring, and as an ingredient in the preparation of ‘Jamu’, a local tonic consumed by the Malays. It is commonly prescribed as traditional treatment against hypertension, rheumatism and asthma (Zakaria and Mustafa, 1994). Sadikun (1987) reported that the plant exhibited anti-inflammatory actions and Hidir and Ibrahim (1991) reported its effects as a smooth muscle relaxant.

Mustafa et al. (1996) reported a vasorelaxant effect of the chloroform extract of *K. galanga* on the
precontracted rat thoracic aorta. Further to the study by Mustafa et al. (1996), the aim of the present study was to determine the chemical components present in the active fractions of *K. galanga*, followed by the isolation and elucidation of the structure of the compound(s) causing the vasorelaxant effect.

**Materials and methods**

**Separation and isolation of compounds**

Silica gel 60 F\(_{254}\) (230–400 Mesh ASTM) was used in thin layer chromatography (TLC). Kiesel gel (70–230 Mesh ASTM) was used in column chromatography (CC). UV light (254 and 365 nm) was used to examine TLC spots or bands. \(I_2\) vapor, ammonia vapor or vanillin–sulphuric acid reagent were used as staining reagents to detect the presence of phenolics and terpenes.

**Analytical methods**

**Gas chromatography method**

Fractions obtained from the experiment were analyzed on a Shimadzu gas chromatography (GC)–MS (GC-17A, MSQP-5000). The column used was Shimadzu CBP-1, 30 mm in length and 0.22 mm in diameter. The injector port was at 280 °C and temperature programming was set at an initial oven temperature of 60 °C for 2 min and an increment of 10 °C min\(^{-1}\) until the final temperature of 320 °C was reached. The interphase temperature was 280 °C. Injection volume of the fractions was 0.2 \(\mu\)l and injections were carried out under split mode with split ratio of 10. Analysis of compounds in the different fractions was confirmed by comparison with standard compounds and Standard NIST library.

**Identification of compounds**

Melting points of pure compounds were measured on a Fargo MP-ID (220 V) melting point apparatus and were uncorrected. Spectral data on pure compounds were obtained as follows: UV on a Shimadzu UV-160A, IR on a Perkin Elmer 1600 series double-beam recording spectrometer, MS on a Shimadzu GC-MS (GC-17A, MSQP-5000), NMR on a JEOL JNM-LA400 FT NMR system with deuterated chloroform (CDCl\(_3\)) as solvent and tetramethylsilane (TMS) as internal reference standard.

**Bioassay-guided fractionation process**

*K. galanga* was obtained from the botanical garden of the University of Malaya and identified by one of the author (H. Ibrahim). The dried rhizomes (5 kg) of this plant were extracted using Soxhlet with petroleum ether and dichloromethane (CH\(_2\)Cl\(_2\)), consecutively. The dried crude petroleum ether extract and CH\(_2\)Cl\(_2\) extract (CEKCL) represented a yield of 2.4% and 2.9%, respectively, of the dried plant powder.

CEKCL was tested using the brine shrimp lethality bioassay, followed by evaluation of antihypertensive activity on anaesthetized rats. The extract was subsequently fractionated to isolate the pure compound(s) responsible for the antihypertensive activity (Fig. 1). CEKCL was mixed with petroleum ether, the precipitate filtered and the mother liquor collected and dried to obtain a mixture of oil (KDNP). KDNP was passed through column chromatography (petroleum ether : ethyl acetate - 95 : 5) then subjected to TLC (petroleum ether : ethyl acetate - 92 : 8) and developed until it reached R\(_f\) 0.62. The active fraction was subjected to column chromatography (petroleum ether : ethyl acetate - 75 : 25) and developed until it reached R\(_f\) 0.75. The maximum relaxation activity was obtained from fraction 4 - 6. These fractions were subjected to column chromatography (petroleum ether : ethyl acetate) and developed until it reached R\(_f\) 0.58. The active fractions were subjected to column chromatography (petroleum ether : ethyl acetate) and were developed until it reached R\(_f\) 0.55. The ethyl cinnamate was obtained from fraction 14 - 16.
through CC (3 × 65 cm) on silica gel (220 g); CH$_2$Cl$_2$:CH$_3$OH (100:0; 99:1; 98:2; 95:5; 90:10; 80:20; 90:10), to yield nine fractions. Fraction 2 (100% CH$_2$Cl$_2$) exhibited vasorelaxant activity on the contracted smooth muscle of isolated rat aorta, and was subsequently separated using TLC on silica gel; petroleum ether:EtOAc (95:5); of which the fraction with $R_f = 0.62$ was active. The active fraction was separated using TLC, petroleum ether:EtOAc (92:8), yielding six fractions (fractions 1*-6*), four of which were active, with the third being the most active (fraction 1*, $R_f = 0.89$; 2*, 0.75; 3*, 0.58; 4*, 0.36; 5*, 0.13; 6*, 0.00). The four active fractions were analyzed using GC (Table 2). Fraction 2*, which contained the highest amount of sample, was subjected to CC (2000, 100 and 10 mm). The experiment was repeated with sample concentrations of 0.5, 5, 6, 7, 8 and 9 µg ml$^{-1}$.

The data obtained were analyzed with a Finney computer program provided by Professor Jerry McLaughlin, Purdue University, Indiana, USA, for the determination of LC$_{50}$ values with 95% confidence intervals. Pure compounds with LC$_{50}$ values > 200 ppm were considered inactive. The results were calculated statistically against controls using the Student t-test.

**Antihypertensive activity on anaesthetized rats**

Male Wistar rats weighing 200–250 g (University of Malaya Animal Unit) with mean arterial pressure (MAP) of 130 ± 5 mmHg were used in this study. The rats were anaesthetized with sodium pentobarbitone (40 mg kg$^{-1}$, intraperitoneal, i.p.). The left carotid artery and the femoral vein were cannulated for blood pressure measurement and intravenous (i.v.) injections, respectively. The arterial cannula was connected via a Statham pressure transducer to a Grass model 7 polygraph for arterial pressure measurement. After a 30-min-stabilization period, the basal value of the MAP was recorded. CEKCL was dissolved in 4% Tween-80 (in saline solution). After the end of the stabilization period, the control (4% Tween-80) or the crude extract (10, 33, 100 mg ml$^{-1}$) was administered as i.v. bolus injections and the MAP was monitored.

**Biological assay**

**Brine shrimp lethality bioassay**

The brine shrimp lethality bioassay was carried out according to McLaughlin et al. (1990), McLaughlin (1991) and Meyer et al. (1982). The eggs of brine shrimp (*Artemia salina* Leach) were readily obtained from a local aquarium shop and the artificial saline was prepared by dissolving sea salt (3.8 g) in 11 of water and then filtering. A small tank with a perforated dividing dam was made from a plastic soap container. About 10 ml of salt water was placed in the tank and a pinch of the eggs was placed on one side of the tank. An opaque cover was placed on top of this side of the tank. The other side of the tank was lighted by normal lamp. The eggs were left for 48 h to hatch, after which the shrimps swam to the lighted side of the tank. Small glass vials were used for incubation. For CEKCL, initially, three sets of tests were conducted at concentrations of 1000, 100 and 10 µg ml$^{-1}$, and each set was performed in triplicate. Saline was used as the control (excluding the test sample and solvent).

The sample was prepared by dissolving 20 mg of sample in 2 ml CH$_2$Cl$_2$. Volumes of 500, 50 and 5 µl were transferred from this solution into the glass vials, and these corresponded to 1000, 100 and 10 µg ml$^{-1}$, respectively. The solvent was dried by blowing with nitrogen. To each vial, 5 ml of brine were added, followed by 10 shrimps. Survivors were counted after 24 h and the percentage of deaths at each dose was recorded. The experiment was repeated with sample concentrations of 0.5, 5, 6, 7, 8 and 9 µg ml$^{-1}$.

The dried rhizomes of *K. galanga* L. were extracted with CH$_2$Cl$_2$ using a Soxhlet extractor and yielding 2.9% of the crude extract, which was then subjected to the brine shrimp lethality assay and screened for vasorelaxant activity.

**Results**

**Bioassay-guided study of the CH$_2$Cl$_2$ extract**

The dried rhizomes of *K. galanga* L. were extracted with CH$_2$Cl$_2$ using a Soxhlet extractor and yielding 2.9% of the crude extract, which was then subjected to the brine shrimp lethality assay and screened for vasorelaxant activity.

**Brine shrimp lethality bioassay on crude CH$_2$Cl$_2$ extract**

Table 1 shows the results of the preliminary screening of the CH$_2$Cl$_2$ extract from *K. galanga* L. at different concentrations using the brine shrimp lethality bioassay. Analysis of the data showed that this extract exhibited potent bioactivity, with an ED$_{50}$ value of 7.92 ± 0.13 µg ml$^{-1}$.

**Screening of the crude CH$_2$Cl$_2$ extract for antihypertensive activity**

Initial experiments were carried out to screen the crude CH$_2$Cl$_2$ extract for antihypertensive activity in anaesthetized rats. The concentrations of the crude extract used in this experiment were 100, 33 and
As shown in Fig. 2, administration of the crude extract induced a dose-related reduction of the basal MAP, (130 ± 75 mmHg), with maximal effects seen after 5–10 min injection; i.e., increasing concentration of the crude extract further decreased the blood pressure of the rats. The result of this experiment demonstrated that this crude extract showed a hypotensive property since it effectively lowered the MAP in anaesthetized rats.

Bioassay-guided fractionation of the CH₂Cl₂ extract

Since both of the above tests showed positive findings, a bioassay-guided fractionation was performed on the CH₂Cl₂ extract in order to isolate the bioactive compound(s) contributing to the lowering of MAP in anaesthetized rats (as shown in the flow chart in Fig. 1). After every fractionation process, the fractions were tested for vasorelaxant activities against contractions induced by high K⁺ and phenylephrine in the smooth muscles of the isolated rat aorta (Othman, 2002).

The chemical contents of the four active fractions in the gas chromatogram are shown in Table 2. All four GC-fractions exhibited vasorelaxant activity on smooth muscle of isolated rat thoracic aorta; fraction 3* showed the highest vasorelaxant activity among the four fractions. Analysis of the chromatograms showed that the common and the highest peak height found in all four fractions belonged to ethyl cinnamate. In addition, fraction 3*, which was the most active, consisted of 59.59% ethyl cinnamate, suggesting that the active component contributing to the vasorelaxant activity could be ethyl cinnamate. MS m/z (relative abundance): 176 [M]+ (28.8%), 148 (11.2%), 131 (100%), 103 (49.6%), 77 (40%).

Ultimately, ethyl cinnamate was isolated as colorless oil (yield was 9.75% of CEKCL). This compound had an Rf value of 0.55 on TLC with solvent system petroleum ether:ethyl acetate (90:10). Comparison of the spectral data obtained with literature values confirmed the identity of ethyl cinnamate (Kiuchi et al., 1988; Pandji et al., 1993).

Another compound was also isolated from CEKCL. This compound was recrystallized from CH₂Cl₂ and purified as white needles with melting point 176–177°C (yield was 0.28% of the extract). Analysis of the spectral data gathered and comparison with the literature values led to the conclusion that the compound was p-methoxycinnamic acid (Kiuchi et al., 1988).

Discussions

Chemical constituents of K. galanga have been reported previously (Kiuchi et al., 1988; Kosuge et al., 1985); however, no report on the vasorelaxant activity-guided isolation of ethyl cinnamate has been published. In this study, it was shown that the CH₂Cl₂ extract exhibited hypotensive activity by lowering the basal MAP in anaesthetized rats. Bioassay-guided fractionation and isolation led to the identification of the active component, ethyl cinnamate. Another compound
isolated and identified as \( p \)-methoxycinnamic acid did not, however, exhibit vasorelaxant activity towards the contracted smooth muscle of the rat aorta (Rozana et al., 1996). This study demonstrated the use of CC for isolation of bioactive fractions in a bioassay-guided study. TLC and GCMS were also used as tools to monitor the different components found in the various fractions. Further pharmacological study of ethyl cinnamate has been reported by Othman et al. (2002). The vasorelaxant effect of ethyl cinnamate, the main component of the active extract, supports the traditional use of the parent plant in alleviating hypertension. Other therapeutic uses of smooth muscle relaxants include the treatment of angina, asthma and other forms of generalized muscle spasms. The rhizomes of *K. galanga* have been used to treat many conditions by the people of the various regions where it is found. The most common indications, besides hypertension, include rheumatism, asthma, headaches, cough, toothaches and use as a poultice for the application on bruises and wounds (Perry and Metzger, 1980). In Malaysia and Indonesia, this plant is used to make a gargle, the leaves and rhizomes are chewed to treat coughs, or pounded and used in poultices or lotions applied to relieve many ailments; the juice of the rhizome is expectorant and carminative, and is a part of children’s medicine and tonics. The rhizome is also used to treat abdominal pain, and as an embrocation or sudorific to treat swelling and muscular rheumatism. In China, this plant is used as a remedy for toothache, as a stimulant, and as a carminative to treat cholera, contusions, chest pains, headache and constipation (Ibrahim and Rahman, 1988; Mustafa et al., 1995). The findings from this study may provide the rationale for the various uses of this plant in traditional medicine.

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References


