CHAPTER 6

EXPERIMENTAL

6.1 PLANT MATERIALS

The rhizomes of *Kaempferia galanga* Linn. were collected at the Botanical Garden (Rimba Ilmu), University of Malaya, and identified in the herbarium in the Department of Botany, University of Malaya, with herbarium series number 038826. The botanical description of this species is as follows[7,124] :

General description :

Leaves two or three, almost horizontal and near the ground, to about 15 by 10 cm, apex rather broadly pointed, green with (often at least) a narrow reddish edge, much paler beneath; petiole and sheath about 3 cm long, broadly channelled. Inflorescence sessile, enclosed by the imbricating leaf sheaths without any sterile involucral bracts; flowers twelve or more. Bracts about 4 by 1 cm (outer ones) down to 2.5 cm long near the centre. Bracteoles two to each flower, narrow, facing the bract, to about 3.5 cm long. Calyx about 3 cm long. Corolla-tube 4.5-5 cm long; lobes 2.5 cm long, white, narrow, spreading. Staminodes spreading, obovate, about 2.2 by 1.4 cm, white. Lip about 2.3 cm long and 2.5 cm wide, divided 2 / 3 to the base, the lobes entire or somewhat lobed, the whole lip white with two longitudinal violet bands; in the basal half. Anther white, sessile with a white bilobed reflexed crest, the lobes rounded.

Distribution :

This species is said to be native in India. It is widely cultivated throughout China and south-eastern Asia, particularly Malaysia, Indonesia and Singapore.

Native name :

Cekur.

Diagram 6.1 *Kaempferia galanga* Linn.

Plate 6.1 *Kaempferia galanga* Linn.

6.2 PHYTOCHEMICAL ANALYSIS

In this study, *Kaempferia galanga* Linn. underwent standard phytochemical analysis, the general procedures and instrumentations of which are described as follows.

6.2.1 Instrumentation

All solvents, except those used for bulk extractions (distilled), are AR grade. Aluminium supported silica gel 60 F_{254} plates were used for thin layer chromatography (tlc). The plates were defatted with hexane and activated at 75°C for one hour and stored in a dessicator until required. TLC spots were visualized under ultra-violet light (254 nm and 365 nm). The plates were then put into a container containing iodine to detect the presence of unsaturated components, or exposed to ammonia vapour or sprayed with vanillin-sulphuric acid reagent to detect the presence of phenolics and terpenes. Silica gel 60 F_{254} (230 - 400 Mesh ASTM*) and DC-Fertigplatten SIL G-25UV_{254} were used in preparative thin layer chromatography on 20 cm x 20 cm plates on layer thickness 0.25 mm up to 1 mm depending on the quantity of the sample worked upon. Silica gel 60 (70 - 230 Mesh ASTM*) and silica gel 60 (230 - 400 Mesh ASTM*) were used for column and flash chromatography, respectively.

* 230 - 400 Mesh ASTM is equivalent to silica gel of size 0.04 - 0.063 mm
70 - 230 Mesh ASTM is equivalent to silica gel of size 0.063 - 0.200 mm

Melting points were measured on Fargo MP-1D (220V) melting point apparatus and were uncorrected. The ultraviolet absorption spectra were recorded on UV-Visible recording spectrophotometer (model Shimadzu UV-160A) with methanol as solvent. The infrared spectra were obtained on a Perkin Elmer 1600 Double-Beam recording spectrophotometer with chloroform as solvent. NMR analysis were carried out on a JEOL JNM-LA400 FT NMR System with deuterated chloroform ($CDCl_3$) as solvents and tetramethylsilane (TMS) as internal reference; chemical shifts were reported in ppm on δ scales and coupling constants were given in Hz. The mass spectra were obtained on Shimadzu GC-MS (GC-17A, MS QP-5000).

6.2.1.1 *Column chromatography*

Silica gel 60 (70 - 230 Mesh ASTM) and silica gel 60 (230 - 400 Mesh ASTM) were used for column and flash chromatography, respectively. The ratio of silica gel to sample is approximately 30:1. The gel was made into a slurry with a suitable solvent before packing into the glass column. The glass columns used were of various dimensions; the 3 cm x 80 cm dimension column was used for fractionation of crude extract, the 3 cm x 65 cm, 2 cm x 50 cm and 2cm x 25 cm dimensions were for separation of compounds from partially purified fractions, and the 1 cm x 30 cm dimension column was for purification of samples or small quantity of samples.

6.2.1.2 *Preparative thin layer chromatography*

Plates of size 20 cm x 20 cm were cleaned using soap and rinsed with water, then with acetone and were dried in the oven. The slurry was prepared by adding 60g of silica gel 60 F_{254} (230 - 400 Mesh ASTM) or DC-Fertigplatten SIL G-25UV_{254} to 120 ml distilled water in a closed container, and was shaken vigorously to obtain a smooth mixture. The slurry was then spread onto the clean and dry plates using the Shandon spreading jig with thickness ranging from 0.25 mm to 1 mm, depending on the amount of sample to be worked upon. Then, the plates were activated in the oven for over an hour at 110°C before use.

6.2.1.3 *Visualising reagents*

(a) Iodine vapour

The TLC plate was placed in a container or tank containing iodine / iodine vapour. Brown spots on yellow background indicated the presence of unsaturated compounds.

(b) Ammonia vapour

The plate was placed above a tank containing a solution of ammonia. Fluorescence under UV before and after exposure to the ammonia vapour was marked.

Fluorescence under long wave only might indicate the presence of flavonoids while fluorescence under short and long wave might indicate the presence of phenylpropanoids.

(c) Vanillin-sulphuric acid reagent

0.5 g vanillin in 2 ml concenctrated H_2SO_4 was added with cooling to 8 ml ethanol before spraying onto the TLC plate. The plate was then heated at 100 - 105°C until full development of colours had occurred.

The occurrence of pink, red, brown, purple, grey, dark green and black colours indicated the presence of phenylpropenes and simple terpenes.

6.2.1.4 *Recrystallization of pure compounds*

The compounds which crystallized out of the crude extracts under cold condition were purified by recrystallization. A solvent (in which these compounds dissolved only when the temperature was raised until boiling point) was heated to boil and added to the partially pure compound until all of it just dissolved. The solution was then filtered into a conical flask using a Whatman PS filter paper. The flask was covered with a glass watch and the solution was left to cool naturally to room temperature.

6.2.2 Extraction of plant material

Plant extractions were carried out by cold percolation or exhaustive extraction using the soxhlet extractor, following the general procedure described below.

Dried, ground rhizomes (5 kg) of the plant were extracted with pet. ether (40 - 60°C) and then air-dried overnight under the fume cupboard. They were then

reextracted with CH_2Cl_2 . Both the pet. ether extract and CH_2Cl_2 extract were concentrated under reduced pressure at room temperature to yield crude extracts each weighing 116.2 g and 142 g, respectively (table 6.1).

6.2.2.1 *Bioassay-guided fractionation of the crude CH_2Cl_2 extract*

The crude CH_2Cl_2 extract was tested with the brine shrimp lethality bioassay (refer chapter 6.3.1) and the analysis of the data obtained showed that the extract exhibited potent bioactivity with an ED_{50} value of 7.92 $\mu g\ ml^{-1}$ (refer chapter 4.11). The extract was then screened for antihypertensive activity on anaesthetized rats (refer chapter 6.3.2) and results indicated that the extract lowered the mean arterial pressure (MAP) of the rats in a dose dependent manner (refer chapter 4.1.2).

The extract was further fractionated using techniques as described in chapter 4.1.3 to isolate the bioactive compound. Scheme 6.1 outlines the bioassay-guided fractionation and isolation of the bioactive compound of *Kaempferia galanga* Linn. Table 6.2 shows the pure compounds obtained from this plant.

6.2.2.2 *Study of the crude pet. ether extract*

The crude pet. ether extract consisted of a dark brown oil of low viscosity. A small amount of the extract was dissolved in $CHCl_3$ and injected into the GC-MS to analyse the volatile components present in it. The column used was Shimadzu CBP-

TABLE 6.1 PERCENTAGE YIELDS OF THE CRUDE PET. ETHER AND CH_2Cl_2 EXTRACTS OF THE RHIZOMES OF *KAEMPFERIA GALANGA* LINN.

Crude extracts	Weight	% Yield
Pet. ether	116.2 g	2.39
CH_2Cl_2	142.0 g	2.92

SCHEME 6.1 FLOWCHART FOR THE BIOASSAY-GUIDED ISOLATION OF THE BIOACTIVE COMPOUND OF *KAEMPFERIA GALANGA* LINN.

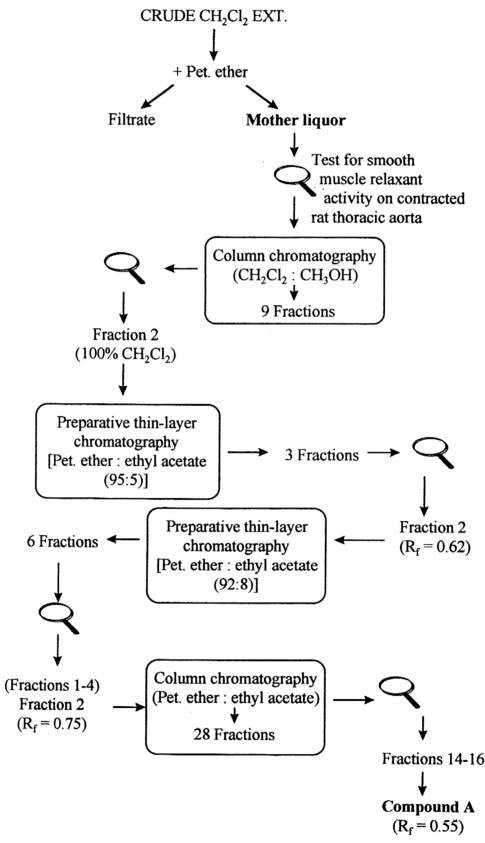


TABLE 6.2 PHENOLICS ISOLATED FROM THE RHIZOMES OF *KAEMPFERIA GALANGA* LINN.

Crude extract	Compound	Name of compound	% Yield of compounds from crude extract
CH_2Cl_2	A	Ethyl cinnamate	9.75
	B	*p*-Methoxycinnamic acid	0.28
Pet. ether	C	Ethyl *p*-methoxy-cinnamate	50.21

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 minutes and an increment of 10°C per minute until final temperature of 320°C was reached. The interphase temperature was 280°C. The injection was carried out under split mode with split ratio of 10. The compounds obtained were analysed and confirmed against standard compounds and by comparison with Standard NIST library[29].

Besides the study of volatile components, pure ethyl *p*-methoxycinnamate was also isolated from this extract (refer table 6.2).

6.3 BIOLOGICAL ASSAY

As mentioned in chapter one, the main purpose of this study was to isolate the compound that contributed to smooth muscle relaxant activity from *Kaempferia galanga* Linn. Below are the procedures of the bioassays carried out by the author.

6.3.1 Brine shrimp lethality bioassay

Since most active plant principles are toxic at elevated levels, a possible approach to developing an effective general bioassay is to screen for compounds that are toxic to zoologic organisms. The brine shrimp lethality bioassay provided a method for general screening for bioactive compounds in plants. This assay involved simple in-house procedures and was designed as a simple assay to monitor the bioactive plant extracts during fractionation and during preliminary screening.

The brine shrimp lethality bioassay was carried out according to McLaughlin *et. al.*[113,114]. The eggs of brine shrimp (*Artemia salina* Leach) were readily obtained from local aquarium and the artificial saline was prepared by dissolving sea salt (3.8 g) in 1 liter of water and then filtered. A small tank with 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 were 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 hours to hatch, after which the shrimps swam to the lighted side of the tank.

Small glass vials were used for incubations. For the crude CH_2Cl_2 extract, initially, three sets of tests were conducted at concentrations of 1000, 100 and 10 μg ml^{-1}, and each set was done in triplicate. A control was done by excluding the test sample.

Sample preparation was done by dissolving 20 mg of sample in 2 ml of solvent. 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 hours and the percentage of deaths at each dose were recorded. The experiment was repeated with sample concentrations of 0.5, 5, 6, 7, 8 and 9 μg ml^{-1}.

The data were analysed with Finney computer program obtained from Professor Jerry McLaughlin, Purdue University, USA, for the determination of LC_{50} values with 95% confidence intervals. Pure compounds with LC_{50} values > 200 ppm were considered inactive.

6.3.2 Antihypertensive activity on anaesthetized rats

Male Wistar rats weighing 200 - 250 g (supplied by the University of Malaya Animal Unit) with mean arterial pressure (MAP) of 130 ± 5 mm Hg 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 pressute 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 minute-stabilization period, the basal value of the MAP was recorded.

The crude CH_2Cl_2 extract 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.

6.3.3 Vasorelaxant activity on smooth muscles of rat aorta

The rat aorta were removed from 8-10 weeks old male normotensive Wistar rats (200-250g) supplied by the University of Malaya Animal Unit. The vessels were

cut into rings of about 3 - 4 mm in length (0.03 - 0.05 g wet weight) and mounted in 2.5 ml organ baths containing Krebs solution of the following composition (in mM); NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; $NaHCO_3$, 23.8; ethylenediaminetetraacetic acid (EDTA), 0.01; glucose, 5.5. The high K^+ solution was prepared by substituting NaCl with KCl (80mM) 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; the pH of the solution was adjusted to 7.2 and gassed with oxygen containing 5% CO_2. The solution in the baths were changed every 20 - 30 minutes. The aortic rings were equilibrated for 20 minutes before stretching to approximately 1g, and allowed to equilibrate further for at least 60 minutes. In some experiments, the endothelium was removed by gently rubbing the intimal surface of the tissue with a blunt forcep, otherwise, all other experiments were performed with the endothelium intact.

For Ca^{2+}-free Kreb solution, Ca^{2+} was omitted and 50 μM EGTA was added to the solution. Aortic rings were washed 4 - 5 times and allowed to be incubated in the solution for not more than 5 minutes. Such protocol ensured total elimination of extracellular Ca^{2+} without deleterious effects on intracellular Ca^{2+}.

Drugs involved in these experiments included phenylephrine (PE), indomethacin (Research Biochemical Incorporated, USA) and methylene blue (Sigma Chemical Co., USA). All the drugs were prepared in distilled water, except for indomethacin which was dissolved in dimethylsulphoxide (DMSO) and then

diluted with distilled water to make a solution. The final concentration of DMSO did not exceed 0.1 % v/v.

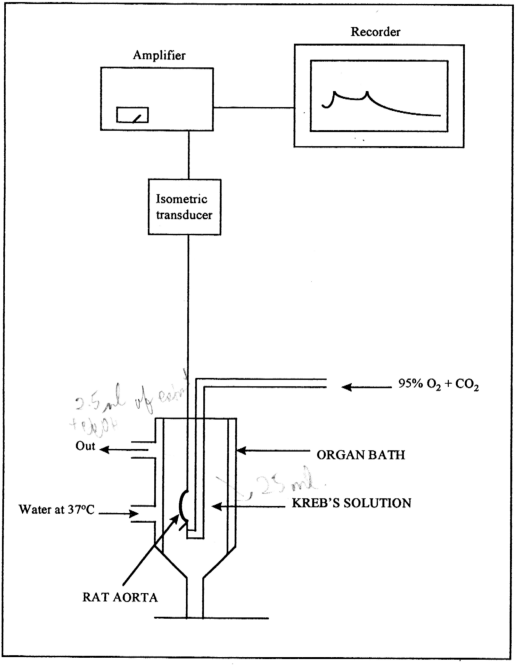
For data analysis, the mechanical responses were calculated as the relative percentage of the control. All data were expressed as the mean ± S.E.M. Significant differences between responses were analysed by analysis of variance followed by the Student's t-test, and P values of less than 0.05 were considered significant. Figure 6.1 shows a diagrammatic sketch of the organ bath and instrumentation for the recording of muscle contractions.

In this study, the effects of compound A and related compounds on the contractions of the isolated rat aorta were studied, and the mechanisms involved were investigated to a certain extent (refer chapter 4.3).

6.3.3.1 *Effects of compound A and related compounds on the contractions induced by high K^+ and PE in endothelium intact rat aorta*

In this experiment, the rat aorta were precontracted with 80 mM K^+ solution or 0.1 μM PE. When the contractions reached maximum, the compounds (A, D, E, F, G, H and I; refer chapter 4.3) were added to the baths every 8 minutes, respectively, at cummulative end concentrations (1×10^{-4} M, 2×10^{-4} M, 4×10^{-4} M, 8×10^{-4} M and 16×10^{-3} M). From this experiment, the IC_{50} values of the active compounds were also determined (refer table 4.4).

FIGURE 6.1 THE ORGAN BATH AND INSTRUMENTATION FOR RECORDING MUSCLE CONTRACTION



6.3.3.2 *Time course of the inhibitory effects of compound A and related compounds on contractions induced by high K^+ and PE in endothelium intact rat aorta*

The rat aorta were again precontracted with 80 mM K^+ solution or 0.1 μM PE. When the contractions to either spasmogens reached maximum, a single concentration of the compounds (based on the IC_{50} values) were added to the bath. Relaxation was allowed to take place for a period of time, and a graph was plotted to reveal the time profile of the activities of the compounds.

6.3.3.3 *Effects of compound A and related compounds on the contractions induced by PE in endothelium intact and denuded preparations of the rat aorta*

In the endothelium denuded preparation, the endothelium was removed from the internal surface of the aorta by gently rubbing the internal wall of the muscle with a blunt forcep. The aorta were precontracted with 0.1 μM PE and when the contractions reached maximum, a single concentration of the compounds (based on the IC_{50} values) were added to the bath.

6.3.3.4 *Effects of methylene blue and indomethacin on the relaxant actions of compound A and related compounds on the contractions induced by PE in the endothelium intact rat aorta*

In this experiment, the rat aorta were pretreated with methylene blue (10 μM) or indomethacin (20 μM) for 20 minutes before contracting the muscles with

0.1 μM PE. When the contractions reached maximum, a single concentration of the compounds (based on the IC_{50} values) were added to the bath.

6.3.3.5 *Effects of compound A and related compounds on the PE-induced transient contractions of the rat aorta*

In this experiment, Ca^{2+}-free Kreb solution was involved where Ca^{2+} was omitted and 50 μM EGTA was added to the solution instead. Aortic rings were washed 4 - 5 times and allowed to be incubated in the solution for not more than 5 minutes. This was to ensure total elimination of extracellular Ca^{2+} without deleterious effects on intracellular Ca^{2+}. The rat aorta were then preincubated with the compounds for 10 minutes prior to the addition of PE to the Ca^{2+}-free solution. The effects of the compounds on the PE-induced transient contractions of the aorta were then studied.

6.3.4 Cytotoxicity assay on KB cells

For the cell culture, the KB cells were maintained in Medium 199 with Earle's salt, supplemented with 10% heat-activated foetal calf serum (GIBCO, UK); L-glutamine (2 mM), penicillin (100 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and kanamycin (50 UI ml^{-1}). The culture medium was buffered with 20 mM Hepes. Once confluent, the cells were trypsinised and spun at1000 rpm for 5 minutes. The pellet was then resuspended in culture medium and split two-fold into new culture vessels.

For the cytotoxicity assay, 20 000 - 30 000 cells ml^{-1} were transferred into 24-well tissue culture plates. Compounds A and C were dissolved in DMSO (Sigma Chemical Co., USA) to a stock concentration of 20 mg ml^{-1}. The range of concentration investigated were 5, 10, 50 and 100 μg ml^{-1} and were done in quadruplicates. After the addition of the compounds, the KB cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air for 72 hours. Neutral red (1%) was added at the end of the incubation period and the cells were further incubated for 2 - 3 hours. The cells were then lysed with sodium dodecyl sulphate, SDS (1% in distilled water) to release the dye taken by healthy cells. After one hour of contact, the cells were removed and the optical density was read at 540 nm using a spectrophotometer (Hitachi 2000). The effective doses for 50% inhibition of cell growth (ED_{50}) of compounds A and C were calculated from the graphs plotted.

6.4 PHYSICAL AND SPECTRAL DATA OF ISOLATED COMPOUNDS

Compound A : Ethyl cinnamate (83)

$C_{11}H_{12}O_2$.

Isolated as colourless oil.

UV : $^{MeOH}\lambda_{max}$ (log ε) nm : 217.3 (4.12), 276.6 (4.39).

IR : ν_{max} ($CHCl_3$) cm^{-1} : 3060, 2992, 1957, 1889, 1705, 1638, 1458, 1377, 1304, 1159, 1011.

MS : m/z (relative abundance): 176 (28.8%), 148 (11.2%), 131 (100%), 103 (49.6%), 77 (40%).

^{1}HNMR : ($CDCl_3$) ppm : 1.28 (3H, t, CH_3)

4.21 (2H, q, CH_2)

6.38 (1H, d, J_2 = 16.1 Hz, H-2)

7.63 (1H, d, J_3 = 16.1 Hz, H-3)

7.31 (3H, m, H-6, 7, 8)

7.46 (2H, m, H-5, 9).

^{13}CNMR : ($CDCl_3$) ppm : 14.24 (CH_3), 60.39 (CH_2), 118.23 (C-2), 127.95 (C-5, 9), 128.78 (C-6,8), 130.11 (C-7), 134.41 (C-4), 144.48 (C-3), 166.88 (C-1).

Compound B : *p*-Methoxycinnamic acid (84)

$C_{10}H_{10}O_3$.

Isolated as white needles with melting point (m.p.) 176 - 177°C.

UV : $^{MeOH}\lambda_{max}$ (log ε) nm : 218.3 (4.04), 290.2 (4.21), 309.6 (4.15).

IR : ν_{max} ($CHCl_3$) cm^{-1} : 3049 - 2594, 3000, 2964, 2841, 1687, 1603, 1513, 1428, 1255.

MS : m/z (relative abundance) : 178 (100%), 161 (21.6%), 133 (14.4%), 118 (23.6%), 90 (18.8%), 77 (41.6%), 64 (16.4%), 63 (36.4%), 51 (25.2%).

^{1}HNMR : ($CDCl_3$) ppm : 3.82 (3H, s, OCH_3)

6.30 (1H, d, J_2 = 15.9 Hz, H-2)

6.90 (2H, d, $J_6 = J_8$ = 8.8 Hz, H-6, 8)

7.49 (2H, d, $J_5 = J_9$ = 8.8 Hz, H-5, 9)

7.73 (1H, d, J_3 = 15.9 Hz, H-3).

^{13}CNMR : ($CDCl_3$) ppm : 55.40 (OCH_3), 114.43 (C-6, 8), 114.64 (C-2),

126.85 (C-4), 130.11 (C-5, 9), 146.74 (C-3),

161.79 (C-7), 172.30 (C-1).

Compound C : Ethyl *p*-methoxycinnamate (85)

$C_{12}H_{14}O_3$.

Isolated as colourless prisms with m.p. of 48-49°C.

UV : $^{MeOH}\lambda_{max}$ (log ε) nm : 211.4 (4.08), 227.4 (4.11), 298 (4.35), 308 (4.38).

IR : ν_{max} ($CHCl_3$) cm^{-1} : 3033, 3000 - 2908, 2840, 1700, 1634 - 1463,

1307 - 1255, 1172, 1035.

MS : m/z (relative abundance) : 206 (62.8%), 178 (14%), 161 (100%), 133 (32%),

118 (114.4%), 90 (12.8%), 77 (14%), 64 (6.4%),

63 (12%), 51 (25.2%).

^{1}HNMR : ($CDCl_3$) ppm : 1.32 (3H, t, CH_3)

3.81 (3H, s, OCH_3)

4.25 (2H, q, CH_2)

6.31 (1H, d, J_2 = 15.9 Hz, H-2)

6.89 (2H, d, $J_6 = J_8$ = 8.8 Hz, H-6, 8)

7.46 (2H, d, $J_5 = J_9$ = 9.0 Hz, H-5, 9)

7.63 (1H, d, J_3 = 16.1 Hz, H-3).

^{13}CNMR : ($CDCl_3$) ppm : 14.25 (CH_3), 55.22 (OCH_3), 60.18 (CH_2),

114.22 (C-6, 8), 115.68 (C-2), 127.13 (C-4),

129.57 (C-5, 9), 144.12 (C-3), 161.25 (C-7),

167.18 (C-1).