CHAPTER II

MATERIAL AND METHODS

2.1 Plant Materials

The air-dried leaves of *Aglaia odorata* Lous (1,000 g), *Anona squamosa* Linn. (346 g) and *Murraya paniculata* Jack (265 g) were collected from Bangkok, Thailand in April 1996. The air-dried stems and branches of *Azadirachta indica* var.*siamensis* Valeton (918 g and 855 g), the air-dried whole plants of *Cymbopogon nardus* Rendle (803 g), *Derris scandens* Benth (998 g), the dried heartwoods of *Mansonia gagei* Drumm. (1,004 g), the roots of *Trigonostemon reidiodes* (Kurz) Craib (858 g) and the dried rhizome of a local variety known as Phai, *Zingiber cassumunar* Roxb. (268 g) were bought from Vechapong Drug Store, Bangkok Thailand in 1996 (Fig.2.1).

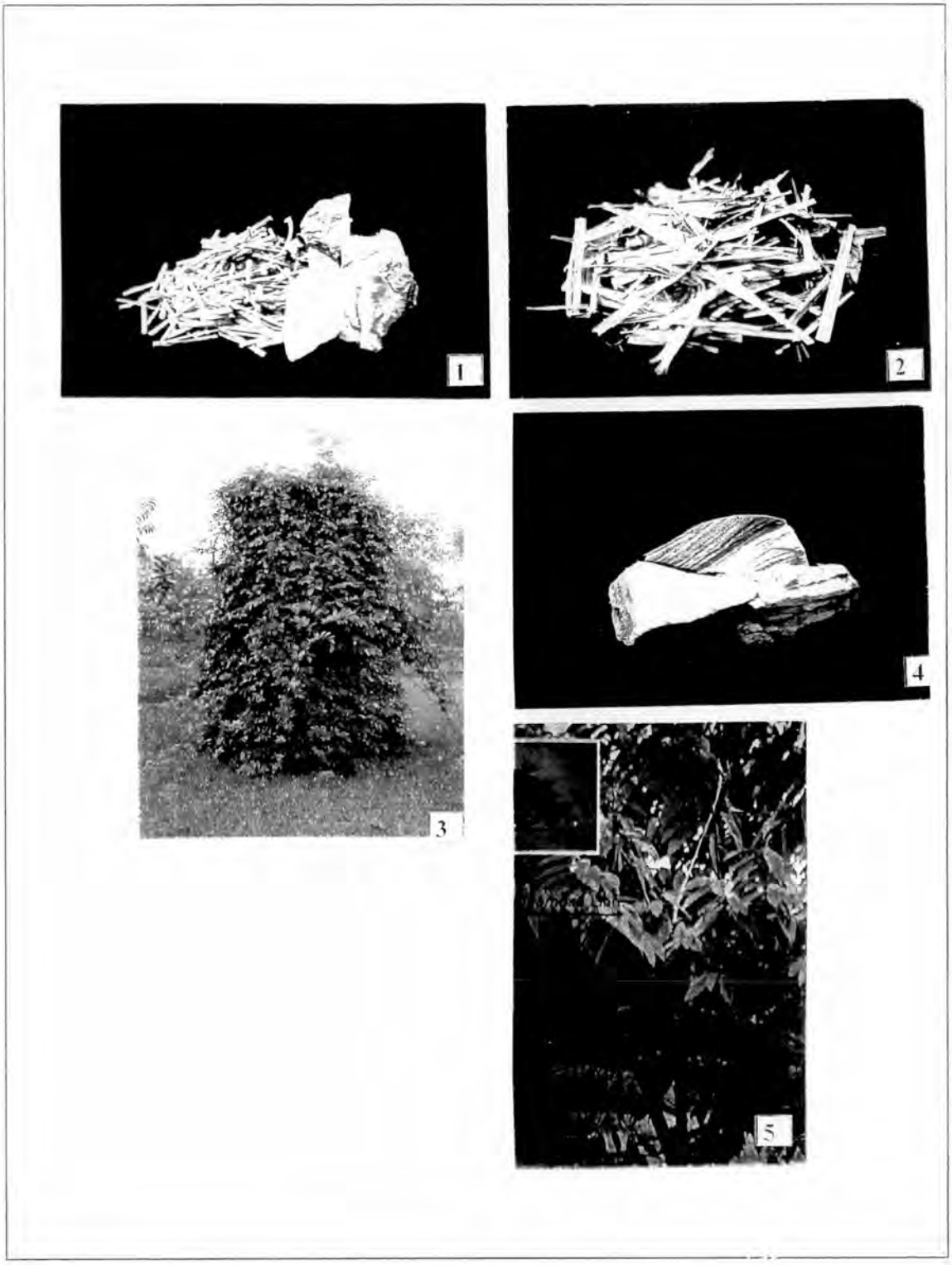


Fig. 2.1 Plant materials

1. *Azadirachta indica* var *siamensis* Voleton 2. *Cymbopogon nardus* Rendle

3. *Derris scandens* Benth 4. *Mansonia gagei* Drumm. 5. *Anona squamosa* Linn

Fig. 2.1 (cont.)

6. *Aglaia odorata* Lous 7 *Murraya paniculata* Jack

8 *Trigonostemon reidioides* (Kurz) Craib 9 *Zingiber cassumunar* Roxb

2.2 General Procedures

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Chromatotron equipment, Harrison Research Model 7924 T, was used for certain separation (Raksilp,1995). Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF_{254}) (Randerath, 1996). Column chromatography was performed on silica gel (Merck Kieselgel 60 G) (Fessenden, 1983).

The FT-IR spectra were recorded on a Fourier Transformed Infrared Spectrophotometer model Impact 410: solid samples were incorporated to potassium bromide to from a pellet. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained from a Bruker model AC-F 200 spectrometer and a Jeol, model JNMR-A500 which operated at 200.13 MHz for ^{1}H and 50.32 MHz for ^{13}C-nuclei. The GC-MS analysis was performed by a Fisson Gas-Liquid Chromatography Model GC 8000-Fisson Mass Spectrometer Model Trio 2000.

2.3 Chemicals

Solvent : All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grades.

Other substances : Merck' s silica gel 60 Art.7734 1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography; No.7731 for quick column chromatography and No. 7749 for chromatotron, respectively. Other chemicals, unless otherwise stated, were of the highest purity available purchasing from Fluka chemical company and used without further purification.

2.4 General Extraction for Preliminary Study

A plant sample was air-dried and milled. Each specimen was then soaked in 95% ethanol for 5 days at room temperature. The soaking procedure was repeated three times or until the color of extract was found to be pale. The ethanolic extract was evaporated by using rotatory vacuum evaporator to almost dryness.

2.5 Bioassay Experiments

2.5.1 Preparation of Greater Wax Moth *Galleria mellonella* Linn.

Larvae: Greater Wax Moth larvae were obtained from the damage combs of Bee Research Unit of Chulalongkorn University, Tambol Bangkhantak, Amphur Muang, Samutsongkhram province, Thailand. The wax moth adult larvae were reared on artificial diet and life stages were kept in environmental chambers 27 °C at Bee Research Unit, Department of Biology, Chulalongkorn University. The larvae food were modified according to those described by Dutky *et al.*, 1962. The mixtures were consisted of ceresol : bee pollen (3:1) and honey : distilled water : glycerol (1: 1.4 : 1.1).

2.5.2 Insect Antifeedant Bioassay Experiments

Preliminary test : Mixed larvae food (about 1) g was put in an aluminium foil bowl, size 3x3 cm^2. Then the proper solvent for each crude extract which provided good solubility, non-toxic or rapidly volatile such as acetone, methanol or dichloromethane was chosen. Two food bowls were compared. The first one is a control bowl ; mix 1 mL of solvent into food bowl and the second is a treatment bowl; mix crude extract solutions of various concentrations (percent wt by wt of food with 1 mL of the same solvent). After that each bowl was held at room temperature to allow the solvent evaporate and then weighed both bowls. Ten third-instar larvae were placed in the plastic box, after being starved for 3 hr. Placed them into an incubator at 34-36 °C for 48 hr. The larvae were weighed to determine the weight loss from treatment and 0control. Each crude extract was arranged with ten replicates.

The control of (consist of larvae food only) was mandatory to perform in order to compare loss moisture and to use for the calculation of % antifeedant. Antifeedant activity was expressed as % antifeedant and was calculated according to the following equation which was modified from that of Bentley *et al.* (1984).

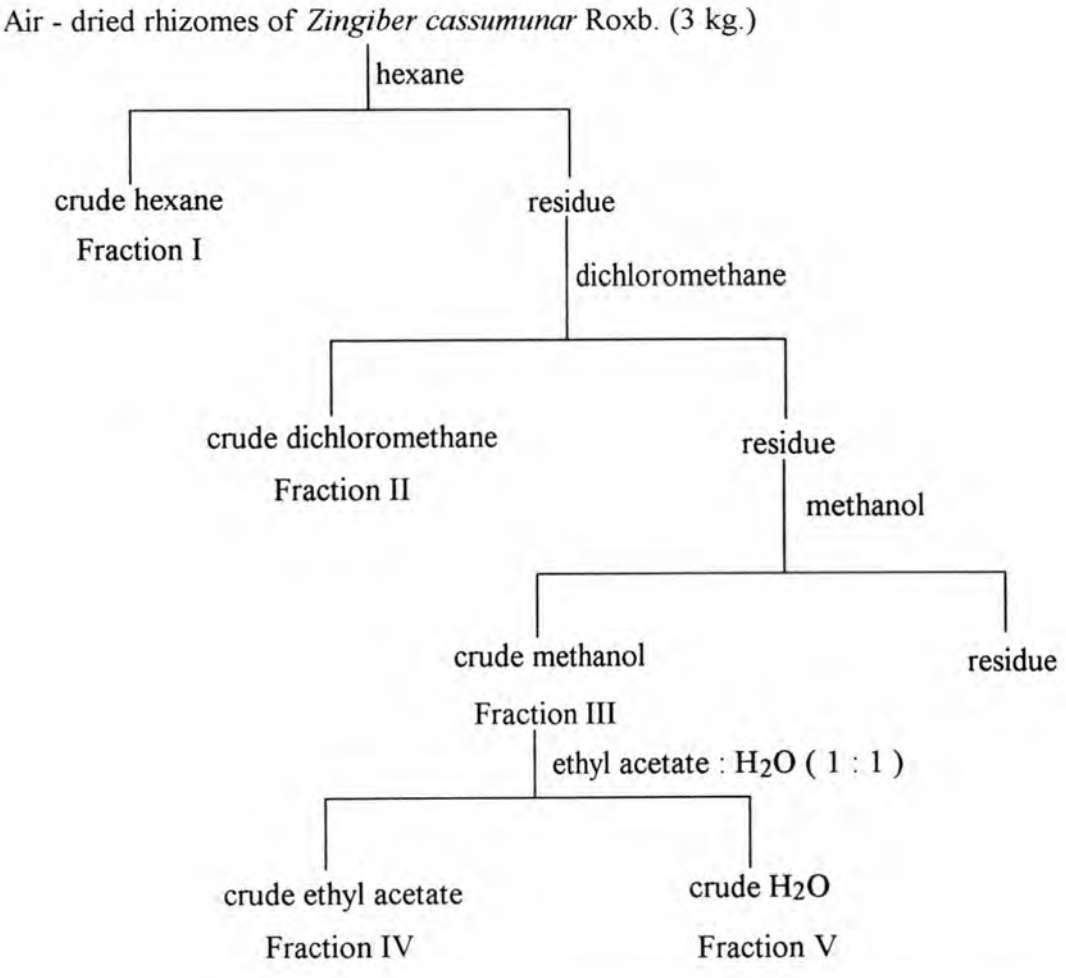
$$\% \text{ Antifeedant} = 1 - \{ (T / C) \} \times 100$$

where T is the weight loss of food in treatment bowl and C is the weight loss of food in control bowl .

2.5.3 Statistical Analysis : Data from each experiment was compared for differences between treatment and control by the two sample *t* tests. In each statistical test, the significant level for rejection of null hypothesis was $\alpha = 0.05$. Mean consumption values and mean % antifeedant were tested for significant difference using Fisher's least significant difference (LSD) procedure (Fisher, 1959).

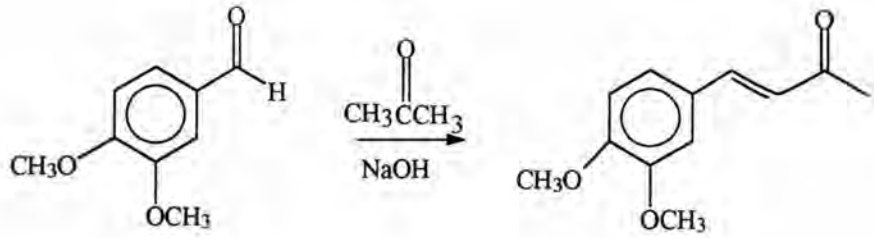
2.6 Extraction

The air dried rhizomes of *Z. cassumunar* Roxb.(3 kg) were minced to coarse powder. The plant initially extracted with *n*-hexane by soaking for 4-5 days at room temperature. The soaking procedure was repeated for three times. The solution was filtered and the solvent was evaporated yielding a hexane crude extract as viscous yellow liquid (Fraction I), 67 g (2.23% yield). The plant residue was then extracted with dichloromethane by the same fashion as that described for using hexane as a solvent, giving a dichloromethane extract as yellow liquid (Fraction II),120.26 g (4.01% yield). The residue left after dichloromethane extraction was further extracted with methanol to give dark yellow liquid of a methanol crude extract, 234.53 g (7.82% yield) (Fraction III). The methanolic crude extract was further partitioned between ethyl acetate and water in ratio 1:1 to yield an ethyl acetate fraction (Fraction IV) and water soluble fraction (Fraction V), 58.23 g (1.94% yield) and 100.19 g (3.34% yield), respectively. The extraction procedure of the rhizomes of *Z. cassumunar* is depicted as shown in Scheme 2.1.



Scheme 2.1 Extraction and fractionation of *Z. cassumunar* Roxb.

2.7 Synthesis of 3′,4′-Dimethoxyphenyl-(1 *E*)-butene-3-one



(1) **(2)**

To a solution of veratraldehyde **(1)** 2 g (12 mmol) in acetone 80 mL was added 10% NaOH 60 mL. After stirring for 30 min at room temperature, the mixture was acidified to pH 6 with 1 N HCl and extracted with CH_2Cl_2 250 mL twice. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and concentrated under reduce pressure. The residue was crystallized with EtOH to give the desired product **(2)** 1.28 g (64 % yield) as pale yellow plates m.p. 83.5-84.0 °C, R_f 0.32 (CH_2Cl_2).

The IR spectrum of Compound **2** (Fig. 2.2) revealed significant characteristic absorption peaks at 3030 and 1680 cm^{-1} for C-H stretching of alkenes and C=O stretching vibration of conjugated ketone. Other peaks were tentatively assigned as tabulated in Table 2.1.

Table 2.1 The IR absorption band assignments of Compound 2

wave number (cm^{-1})	intensity	tentative assignment
3030	weak	C-H stretching alkene
2970-2870	weak	C-H stretching of CH_3-
1680	strong	C=O stretching of enone
1600-1440	strong	C=C stretching

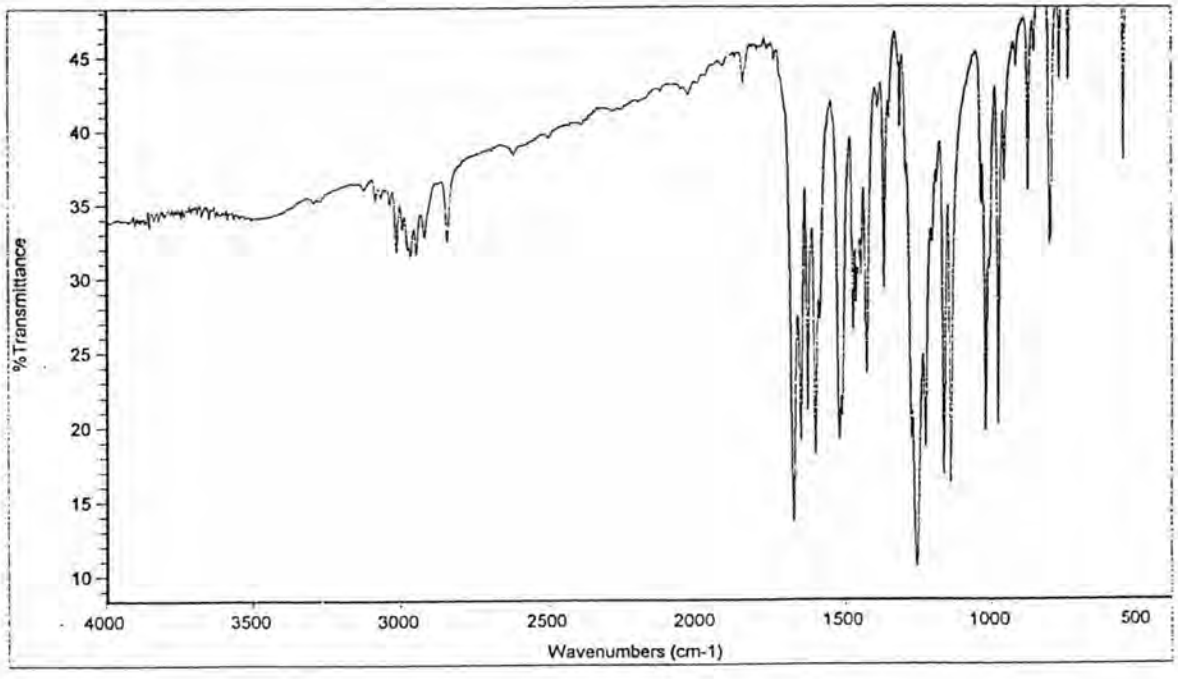


Fig. 2.2 The IR spectrum of 3′,4′-dimethoxyphenyl-(1*E*)-butene-3-one

The ^{1}H NMR spectrum of **(2)** (Fig. 2.3) showed signal at δ (ppm) : 2.33 (s,-CH_3), 3.86 (s, 2 -OCH_3), 6.57 (d, J = 16.18 Hz, =CH- Ar), 6.84 (d, J = 8.23 Hz, =CH-), 14.07 (d, J = 1.84 Hz, =CH- Ar), 7.09 (d, J = 8.21 Hz, =CH-) and 7.43 (d, J = 16.18 Hz, =CH- Ar).

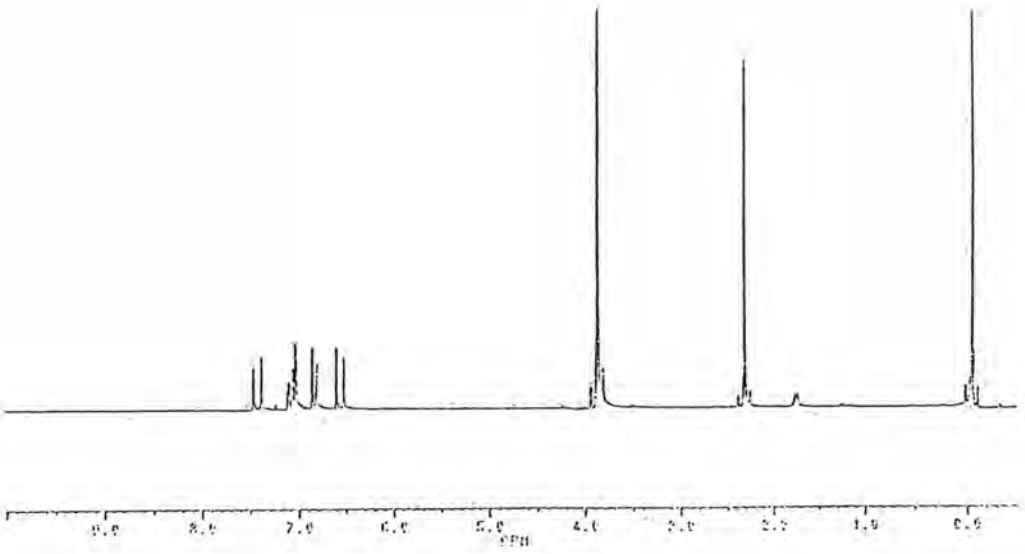


Fig. 2.3 The ^{1}H NMR spectrum of 3′,4′-dimethoxyphenyl-(1*E*)-butene-3-one

The ^{13}C NMR spectrum of **(2)** (Fig. 2.3) exhibited totally 11 signals at δ (ppm): 27.3 ($C(O)\underline{C}H_3$), 55.9 ($-OCH_3$), 56.0 ($-OCH_3$), 109.7 (=CH-), 111.1 (=CH-), 123.0 (=CH-), 125.2 (=CH-), 127.3 (=C-), 143.5 (=CH-), 149.3 (=C-OCH_3) and 151.4 (=C-OCH_3).

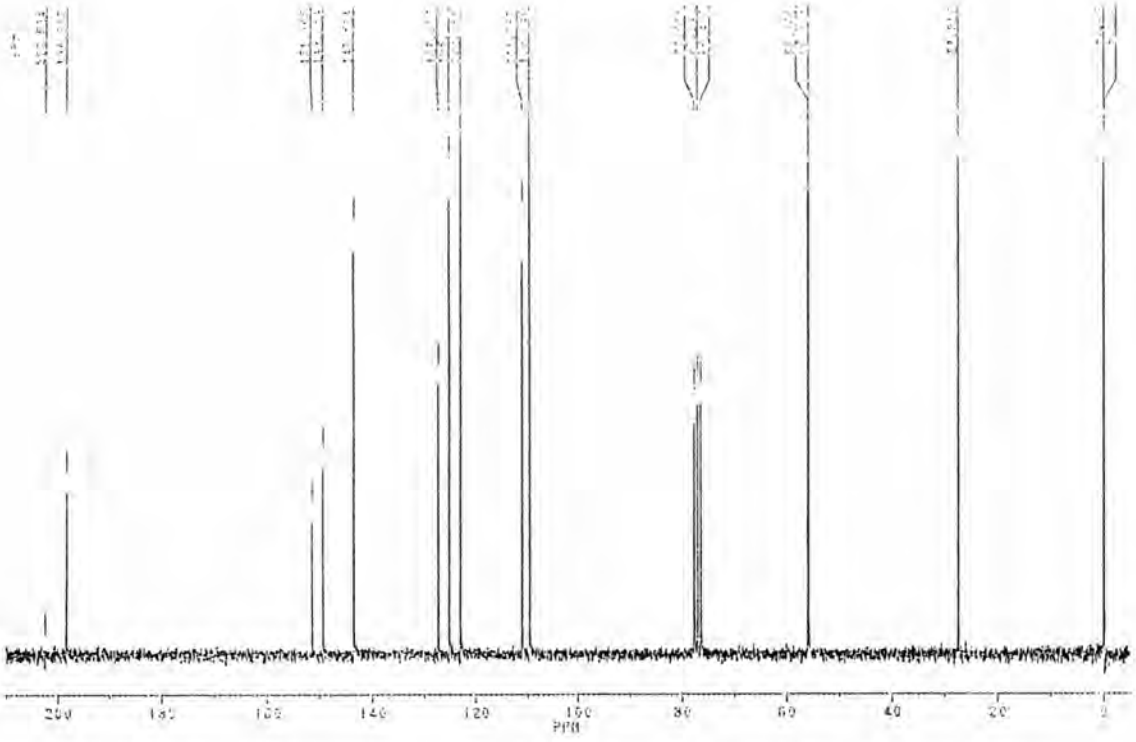


Fig. 2.4 The ^{13}C NMR spectrum of 3′,4′-dimethylphenyl-(1*E*)-butene-3-one