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A STUDY ON INSECTICIDAL PRINCIPALS OF SEEDS OF ANNONA SPECIES FOUND IN INDONESIA

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MAY 31, 1989

PREFACE

All Praise to Allah,by His Grace

this report can be completed

The work described in this report,except otherwise stated,was carried out by the author in the faculty of Pharmacy Gadjah Mada University,Indonesia,between January 1,1987 to May 1,1989.

The author wishes to thank sincerely her collaborators,Professor D. StC. Black University of New South Wales,for advices in chemical aspects ,Professor H. Miles University of Central Florida,for advices in the biological aspects,Dr. Oetoyo and Dr. Wardiarto for the assistances in screening of insecticidal activities,Dr. Hardjono Sastrohamidjoyo for the instrumental analysis.

Thanks are also gratefully conveyed to Dr. Kenneth N. Randolph and Mr. Wilbur Scarborough PSTC Project Officers for continuous assistances and encouragement.
All staffmembers in Faculty of Pharmacy and Central Laboratory of Physical and Chemical analysis for their help and assistances.

The Rector of Gadjah Mada and the Dean of Faculty of Pharmacy for the opportunity given to the author to conduct

this project.

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May 31,1989

SUMMARY

The object of this work was to isolate and characterize insecticidal compound found in the seeds of *Annona squamosa* and to carry out phytochemical screening on the ether extract of seeds of *Annona reticulata*.

Isolation of insecticidal compound was carried out using diethylether.The insecticidal fraction was precipitated from the diethylether extract by adding 5 times volume of petroleumether.The petroleum ether non soluble fractions was fractionized in a column of silicagel H 60 using chloroform and methanol(9:1).Purification was carried out in column of silicagel H60 using ethylacetate and diethylether(2:1) and preparative thin layer chromatography using the same solvents.

Testing of insecticidal activities of the fractions and of purified isolated compounds were conducted on *Sitophylus oryzae* reared in the laboratory.

Active compound could not be separated from *A. reticulata*, because of difficulties in obtaining enough material.From seeds of *A. squamosa* a week active compound has been isolated.

A white waxy material,with a weak insecticidal activity was

isolated(M.P. 38°C,Molecular formule $C_{37}H_{68}O_7$).

It is soluble in aceton,ethylacetate,methanol,slightly soluble in chloroform not soluble in water and petroleum ether.

Color reactions are:negative to ninhydrin,brown with Dragendorff reagent,Ferric chloride negative ,phosphomolybdic acid blue,sulfuric acid 25% and heating brown.Liebermann-burchardt negative.$SbCl_3$ negative.

UV-Vis spectrum did not show any absorption above 222 nm.

IR spectrum (KBr disc) showed absorption at: 3440(s) ,2925(s) ,2852(s) ,1780(s) ,1660(w),1468(m),1424 (w) ,1322(m) ,1244(vw) ,1120(m) ,1060(s) ,1020(m), 960(w) , 880(w) ,740(vw) cm^{-1}.

^{1}H-nmr spectrum (δ ,$CDCl_3$)showed resonance at ppm 0.86 a triplet(3H) ; 1.24 singlet ,1.25 quartet ;1.37, quartet; 1.38 doublet, 1.4 quartet ; 1.52 triplet ; 1.58 quartet ; 1.8 multiplet ;1.94 multiplet ,(alltogether 54 H) ; 2.2 triplet,(2H); 3.3 multiplet (1H) ; 3.7 multiplet (3) ;4.9 quartet (1H) ; 6.9 singlet (1H); total number of hydrogen 68.D_2O exchange showed an exchange of 1 H.

The masspectra and ^{13}C-nmr have not been obtained, so, a proposed structure would be reported sometime elswhere.

C O N T E N T

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Chapter I.

I N T R O D U C T I O N

1.Background

Various insecticides are currently used to control insects.Inorganic and synthetic organic compounds,plants' products,pheromone,bacteries and fungi are used effectively for pest control.

Many inorganic and synthetic organic compounds,while they are very potent insecticides,can cause toxiccation to human and other animals,can cause hazardous pollution,and have long duration of residual toxicities.Plants' products are most often used as insecticides for domestic purposes,because of the milder toxcicities on human and other animals.Lately pheromones and microbes are developped to control pest because of the specificity of effect and the safety on other animals.

Novel insecticides with better characteristics such as safety,specificity,facile availability are still needed.It is most likely that insecticides with such characteristics can be found among the constituent of insecticidal plants.Once the structure of such insecticides can be found,development of better insecticides may be conducted by modifying the structure.

In this investigation,isolation and testing of the

insecticidal effects of seeds of *Annona reticulata* L and of *Annona squamosa* were undertaken and the insecticidal compounds were characterized.

2.The Annonaceae family.

Annonaceae is a large family,comprising *ca* 120 genera and more than 2,000 species.Economically the family is of appreciable importance as a source of edible fruits:the pawpaw(Azumia),cherimoya,sweetsop,soursop,custard apple and ilama.Oils from some seeds of some plants may be used for the production of edible oils and soaps.Woods of some Annonaceous plants have been employed for alcohol production.Fragrant flowers of ylang-ylang(Cananga odorata) are an important raw material for parfumery.Finally,many members of this family are used in folk medicine for various purposes.

Chemical studies,and to a lesser extent pharmacological studies,on Annonaceous plant have been intensified in the last decade,though pioneering work started long ago.It is striking,however that Annonaceae is perhaps one of the chemically least known families.Most investigations have centred upon alkaloids,but Annonaceae also produce a wide range of nonalkaloidal compounds belonging to various phytochemical groups.It is clear that the family now required thorough phytochemical investigations in search of medically important as well as chemically interesting compounds.

Distribution and origin of the Annonaceae

The Annonaceae is a large family of aromatic tree,shrubs,or climbers,which occur in tropical and subtropical regions.According to Takhtayan,51 genera and ca 950 species are confined to Asia and Australasia,whereas in Africa and Madagascar there are 40 genera with ca 450 species,and on the American continent 33 genera and 740 species(7.8),thus Asia together with Australasia is the basic centre of the distribution of the Annonaceae.

Diagnostic feature of the Annonaceae

On the basis of morphology and habitat,the Annonaceae is a very homogenous plant family.All but one species are trees or shurubs,sometimes climbing,usually evergreen,with resin canals and septate pith in the stem.The leaves are alternate,entire,exstipulate,they are often recognizable in the field by a glaucous or metallic sheen.

The fragrant flowers frequently open before all parts are fully developed;they are terminal,leaf opposed or axillary,solitary or crowded,hermaphrodite or rarely unisexual,regular,mostly trimerous;the perianth is usually in three whorls of three (sepals persistent or deciduous,petals generally six in two series series,rarely in two series of two or the inner series absent.The stamens are usually numerous,,hypogynous,spirally arranged;the carpels are generally numerous and free,very rarely united in a one celled ovary with parieted placentas.Fruiting carpels are sessile or stipitate,mostly indehiscent;the fruit is

usually an aggregate of berries,but in a few genera,specially Annona ,the berries coalesce with an edible fleshy receptacle and the fruits are worth eating.The seeds have a copious,ruminous endosperm and a minute embryo;some of the seeds develop an aril after fertilization.

Briefly Annonaceous plants are recognized in tropical and subtropical regions by the alternate,exstipulate leaves,mostly trimerous flowers,numerous and odften truncate free stamens,free carpels and seeds with ruminate endosperm..(M. Leboeuf ,1982).

3. Botanical feature of *Annona*

Flowers extra axillary,opposite the leaves,or on old wood,solitary or 2-10 together;female flowers:sepals 3,valvate,connate at base; petals 6 in whorls of 3,or by absence of the inner set 3,coriaceous;all petals or the outer set valvate;torus convex-conical;stamens very numerous;connective broad,concealing the cells,truncate or very obtuse;ovaries numerous,united from the beginning into a fleshy syncarp,each with 1 ovule;style either longish,much accrescent after anthesis,forming soft spines on the fruit,or absent;stigmas thick;fruit a fleshy syncarp,composed of the united carpels,or the ripe carpels finally separating apically,many celled,many seeded. Trees,shrubs or dwarf shrubs.

Annona squamosa

Peduncle 1-2 flowered;ovary with obvious knobby,separate bulges;mature fruit glaucous,5-10 cm diam.;single carpels with strongly convex apex(fruit therefore strikingly bullate)mature ones move or less separating in upper half;ripe seeds shining black;inner petals less than 1 mm or absent.Leaves strikingly glaucous beneath,oblong-lanceolate,with acute-obtuse-rounded base,tapering to the apex or rounded,at last glabrous,6-171/2 cm by 21/2 - 71/2 cm;lateral nerves at either side of midrib 10 - 15;axils of primary nerves not sunk beneath.2.00 - 7.00; VI - XII; from tropical America.

Annona reticulata

Peduncle 2-10-flowered;ovary smooth;mature fruit brown or reddish,5-12 cm diam:single carpels with flat apex(fruit therefore smooth),even at maturity cohering;ripe seeds black brown;inner petals always present, 1 - 21/2 mm long.Leaves green beneath,oblong lanceolate,with acute or obtuse base,mostly acuminate,rarely tapering to the apex,with an acute acumen,often with undulate margin,sparingly pubescent on the nerves, 9-30 cm by 31/2 - 7cm; lateral nerves at either side of midrib 8-23;axils of primary nerves often sunk beneath.3.00 - 7.00;from tropical America.

Annona species in Indonesia

Annona species found in Indonesia are *A. muricata* in Java often cultivated as fruit tree;*A. glabra* L,once found cultivated in a village near Bogor;*A. squamosa*

cultivated in Java as fruit tree,especially in East Java and Madura,less planted in West Java;*A. reticulata* often cultivated as fruit tree throughout Java.(Heyne,K.,1950,Backer,1972).

Annona squamosa L.

Indigenous names:kaneel appel,Attier,Pomme canelle,Zucker apfel,Custard apple,sugar apple,sweetsop.
Aceh:Delima bintang,Serba bintang.
Malay:Ata (Timor), Atis (Manado), Delima srikaya,Srikaya;
Minagkabau:Sarikaya;Lampung:seraikaya;Dayak:Sarikaya(ngadju);
Sunda:Sarikaya,Srikaya;Java:Serikaya,Surikaya,Srikaya,Srikawi
Madura:Sarkaya,Kerikaya,Surikaja;Bali:Srikaja;Bima:Garosakaj
Makassar:Sirikaya;Boegis:Atis;Maluku:Atis.

Annona squamosa L is an erect shrub,2 a 3 m high,came from Antillen,planted for the sweet smelling fruits in garden,especially in east Java and Madura.The roots are reported in Indische Vergift rapporten as poisonous.

The crushed leaves are highly appreciated as remedies to ripen abcesses;according to Filet people used them against scabies and in the Landbouwer extract of leaves as well as of crushed seeds are used to bath dogs to free them from lices.The only in rainy season available fruits are much sought.At the Antillen the juices are fermented to a soft fine liquer,similar with cider.

The seeds,with hard seedcoat contains 45% yellow not

drying oil and acoording to Dymock 1/3 resinous matter used to control head lices in India and Indonesia.

Annona reticulata L

Indigenous names: Couer de boeuf,Ochsenherapfel,Bullock's heart.
Aceh:Serba raksa;Malay:Buah nona,Jambu nona; Lampung:Nona;Sunda:Manowa,Nona;Madura:Binowa,Nona;Flores:Anona Sulawesi:Manunang,buwah nunang;Makasar:Sirikaya doke,S. susu;Bugis:Sirikaya lasetidong,Soesa,S.susu;Roti:Nona due lok;Timor:Ata kare';Maluku:Buwah nyonya,Java:kemulwo,manowa.

Annona reticulata is a fruit tree,3 to 6 m high,came from tropical America; at the time of Rumphius already spread extensively,so that he was not sure wether it was imported or indigenous.

The roots was reported deadly poisonous.According to Filet,the bark ,fine powdered can be used against cold caused by milt enlarging by putting the powder on the milt are.The leaves finely crushed are taken against worms.The leaves contain narcotic and poisonousnsap;they are often used to destroy lices of pets.Greshoff found a little alkaloid,a greenish fluorescent material.From the seeds a harsh extract is made to control diarrhoe and dysenteri(Heyne,K.,1950)

4. Phytochemistry Of *Annona squamosa* and *Annona reticulata*

Annona muricata L,*Annona squamosa* L and *Annona reticulata*

are found extensively in Indonesia wild or cultivated.Mostly grown in remote areas or lime mountainous dry area for the edible fruits.

Chemical studies and to a lesser extent pharmacological studies on Annonaceous plants have intensified in the last decade.Most investigations have centred on alkaloids,but *Annona species* produce also a wide range of non alkaloidal compounds belonging to various phytochemical group.

Non alkaloidal constituent of *Annona squamosa*

The non alkaloidal constituent of *Annona squamosa* L. considered here are carbohydrates,lipids,amino acids and proteins,polyphenols,essential olis,terpenes and aromatic compounds.Reducing sugar have obtained from the edible portion of the fruit of *Annona squamosa*,glucose and fructose(Ngifu,C.K.,1976).

Oils and fatty acids have been isolated from fruits and seeds of *Annona squamosa* L.Studies on the various fatty acids contained in the oil extracted from the seeds and or the fruits of *A. squamosa* L. show that they are semi drying oils,in which predominates in varying proportions,oleic and linoleic acids,saturated acids as normally found in fatty acids(myristic,palmitic,stearic,arachidic acids). Other unsaturated acids palmitoleic and linolenic acids exist only in small or trace amount.

Glycerides of one or more hydroxylated fatty acids of unusually high MW,possessing toxic and insecticidal

properties,have been reported from several *Annona*,especially *A.reticulata*.

Amino acids and proteins

From the fruit pulp of *Annona squamosa* 14 amino acids have been isolated,of which citrulline and γ-aminobutyric acids predominated(Ventura,M.M.,1961).

Asparagine,histidine,alanine,tyrosine and lysine have been also isolated from fruits,whereas in the seeds 16 amino acids were detected.(Rao,S.V.,1955).

Essential oils

A large number of Annonaceae are fragrant due to the presence of essential oils.The constituents of these oils are usually either well-known monoterpenes and sesquiterpenes or aromatic compounds.

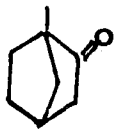
In *A. squamosa*,essential oils from seeds contains α-β-pinene and caryophyllene(Farnsworth,N.R.,1974) , terpenes in the fruit peel oil are and b pinenes,limonene, -farnesene,*trans*-ocimene,and in leaves pinene,caryophylene and a cadolinous sesquiterpen.

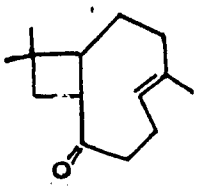
Terpenes

Monoterpenes:Camphor and borneol have been found in the roots and bark of *A. squamosa*,along with an oxygenated unidentified monoterpene,$C_{10}H_{14}O$.

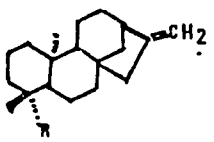
Sesquiterpenes:Bohlmann and Rao isolated from roots of *A.squamosa* caryophyllene accompanied by several kaurane type

diterpenes(Leboef,M. et al,1982).





camphor β-caryophyllene



R = CH_2OH

R = CH_2OAc

(-)kaur-16-en-19-ol (-)kaur-16-en-19-asetat

Triterpenes,sterol and saponins:Friedeline has been found in the leaves of *A. squamosa*, sitosterol in the seeds,root and bark of *A. squamosa*

Vitamins and carotenes

Vitamin C content was determined in the fruit pulp of *A. squamosa*. Carotene or carotenoids were isolated from the fruits.

Cyanogenetic glucosides

The occurence of cyanogenetic compounds was recorded in *A. squamosa*.

Non alkaloidal nitrogen heterocycles

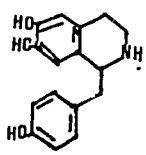
Squamolone was first reported in 1872,isolated from

A.squamosa as 4-oxoperhydro-1,3-diazepin-2-one but in 1980 it was found to be 1-carbamoyl-2-pyrolidinone instead and an unequivocal synthesis of squamolonene and of its isomer was carried out. A nitro compound was isolated from the leaves and stems of *A. squamosa*

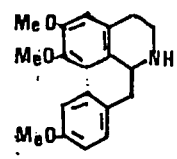
Alkaloids

Almost all alkaloids of Annonaceae possess an isoquinoline derived structure and they are considered in the following order:simple isoquinolines,benzyltetrahydro-isoquinolines,bisbenzylisoquinolines and bis benzyl tetrahydroisoquinoline;protoberberines and tetrahydroprotoberberines,aporhinoids *sensu stricto*;7-substituted aporphines,oxoaporphines,phenanthrene(open aporphines);miscellaneous isoquinoline type alkaloids. Only a few alkaloids devoid of an isoquinoline skeleton.

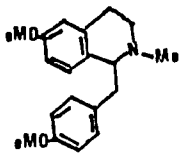
Benzyltetrahydroisoquinoline found in *Annona squamosa* are hegenamine, o-methylarpavine and reticuline.The aporphines are analobine ,annonaine,corydine, ;glaucine;isocorydine,norcorydine,norisocorydine,xylopine, noeushinsumine,lanuginosine,liriodenine.



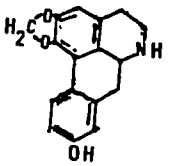
higenamine



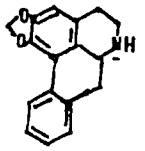
O-methylarpavine



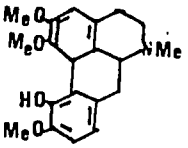
reticuline



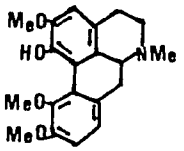
anolobine



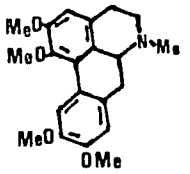
anonaine



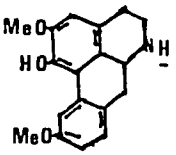
isocorydine



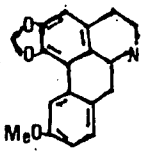
corydine



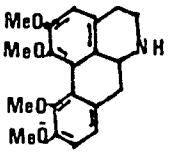
glaucine



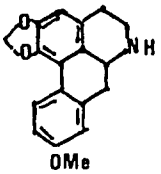
norcorydine



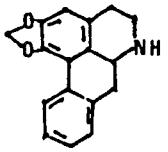
roemerine



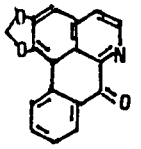
norisocorydine



xylopine



michelabine



liriodenine

5.Insecticidal activities of seeds of *Annona squamosa*

Insecticidal activities of extracts of seeds using different solvents have been investigated on several kinds of insects

Mc Indoo & Sievers(1924) mentioned five species,the seeds

of which are reputed to have insecticidal action.They are *A. cherimola*,*A. glabra*,*A. reticulata*,*A.squamosa* and *A.spinescens*.Insecticidal effect of alcoholic extracts of *A. squamosa* showed contact insecticidal properties to aphides *Aphis fabae* and *Macrosiphonella sanbornii*(6.2) It was found that the seed oil ,made by extracttion with ether,contained 1,2% petroleum ether insoluble resin.This resin obtained by treating etheric extract of seeds with five times of its volume with petroleum ether(b.p. 40 - 60 ^{o}C)and kept at 0°C or room temperature,separated as a browny cheesy mass and was recovered by decantation(1-2)(Ghanekar & Ayar,)Harper et al showed that this resinous concentrate so obtained(2.55% on the oil,0.8% on the seeds i.e a 120 fold concentration was very highly toxic to aphides approaching that of rotenon.As test insects for contact poison test*Aphis fabae*(black bean aphide) and *Macrosiphonella sanbornii* Gill(chrysanthemum aphis) were used.Test of tthe petroleum ether insoluble fraction of two *Annona species* in alkohol and saponin on *Macrosiphonella sanbornii* gave lower result to rotenon in the same medium. When a sulfonated medium lorol aceton medium was used,the toxicities of rotenon and petroleum insoluble fraction of *Annoa squamosa* were of the same order,though had neither the intensity of effect nor the range of insecticidal action of that compound.In both test apparatus and technique of Tattersfield & Morris(1924) and apparatus and technique of Potter(1941) were used.Examination of test insects was

carried out 40 - 48 hours after spraying because of the slowness of paralytic action of *Annona* extract.

Effect of insecticidal fraction of *Annona squamosa* on the urea,uric acid and creatinin contents of fecal matter and hemolymph of the cockcroach *Periplaneta americana*Linne was studied.They found that the LD50 of azadirachtin,insecticidal fraction of *A. squamosa* and oleoresin of *Chrysanthemum* for male adults of *P. americana* obtained by bloodstream injections were 1.500,300 and 0.5 microgram/gr body weight respectively.This result showed that to a certain insect insecticidal fraction of *A. squamosa* is more active than azadirachtin.

Chapter II

P R O C E D U R E

1.MATERIAL

Seeds of *Annona squamosa* used in this study were collected from Yogyakarta,the villages Bayat and Wonosari.Seeds of *Annona reticulata* were collected from Boyolali area.

Reagents and solvents used in this study,unless otherwise stated were analytical reagents from E. Merck.
Thin layer chromatography was carried out using prepared plate from E. Merck.Column chromatography was carried out using silicagel H 60 as stationary phase.

UV- Vis spectra were obtained from Hitachi double beam spectrophotometer.

Gas liquid chromatogram were obtained from gas chromatograph Hitachi 163.

IR spectra were obtained from Hitachi IR spectrophotometer.

NMR spectra were obtained from Jeol FX in shool of chemistry,The university of New South Wales.

Elemental analysis was carried out in shool of chemistry,The University of New South Wales.

As test insects *Sitophylus oryzae* reared in the laboratory was used.

2.EXPERIMENTAL

2.1 Preparation of seeds sample

Seeds were obtained from ripe fruits and dried in the sun,decorticated and grounded in a mill.

2.2 Isolation of insecticidal fraction

100 gr of fine grounded decorticated seeds was extracted in a Soxhlet apparate with diethylether for 6 hours.The extract was evaporated and petroleum ether(boiling point 60-80°C) 5 times of volume was added than left overnight.A brownish mass was precipitated and separated by filtration.

2.3 Fractionazation of petroleum ether insoluble fractions

10 gr of the petroleum ether insoluble fractions was frctionized in a column of 40 gr silicagel H 60 put in a glass column of 3 cm diameter and 40 cm high.Elution was done firstly with chloroform,than chloroform methanol(9:1), chloroform-methanol(1:1) and methanol.The fractionization was monitored by thin layer chromatography using sulfuric acid 25% as detection reagent.

2.4.Testing the insecticidal activities

To the petroleum ether insoluble fraction obtained, testing of insecticidal activities were carried out.As test insect *Sitophylus oryzae* was used to test the contact poison activity of the extract.

Testing of insecticidal activities on stored product beetle ,*Sitophylus oryzae* was done according to the modified method of FAO no. 15,FAO-Plant Protection buletin.

Test insectcs were reared in the laboratory in jars at room temperature,using rice grain as breeding material.Rice grain was disinfected by heating at 60°C for one hour.

For testing a room with normal illumination,range of temperature 28 - 30 °C and approximately 70%RH was used. Identification of species was as described in page 31.Before testing all progeny were removed and held at room temperature for a further two weeks.Most of them would be about three to five weeks old,representing a varied range of development.

Test solution:

A serial dilution using a factor of 1/2 were prepared by weighing the extract and dissolved in the prepared solvnet mixture(0.5 ml).Dosis of 500 mg,250 mg.125 mg,62.5 mg 31.25 mg/0.5 ml of petroleum insoluble fractions were tested.

To the fractionized extract eluted from column and isolated pure compound ,a sample of each of 100 mg were tested. The figure of dosis and mortality were referred in table 1,2 and 3 respectively.

Application to filter papers:

Papers were labelled with pencil before impregnation.The papers were supported on glass ring.The 0.5 ml of extract solution were spread on filter papers with 1 ml pipettes using using a progressively spiral to ensure distribution.They were allowed to dry for approximately one minute and than transferred to a sheet of glass plate.Fresh test papers were used for each test.

Esposure to the treated papers and record of responses.

The complete tests comprises three replicates of five concentration plus three replicates for control.Adult beetles were counted in 16 batches of 20 into small vials.The insects were held for one hour without food at room temperature and approximately 70% relative humidity.The 16 batches were then assigned at random to the test filter papers,to which they were confined by open plastic jars.After exposure of 24 hours ,than 48 hours,the insects were examined and the totals and number responding were recorded.The criterion of response was knockdown,defined as inability to move.A gentle push forward with forceps was usually sufficiant to determine response.

Interpretation and calculation

The knockdown figure were plotted on the dosage mortality by calculation.

Testing of insecticidal activities were also carried out to isolated single compound with the same procedure.

2.5.The found active compound were characterized by elemental analysis,phytochemical screening ,physicochemical and spectroscopic methods.

Chapter III

R E S U L T A N D D I S C U S S I O N

1.RESULT

1.1 Isolation of insecticidal principal of *A. squamosa*, *A. reticulata* and *A. muricata*.

1 Kg seeds of *A. squamosa* yielded 660 gr decorticated seeds,300 gr crude extract and 7.5 gr resinous brownish mass of the petroleum non soluble fraction.

1 Kg seeds of *A. reticulata* yielded 600 gr of decorticated seeds ,250 gr crude extract and 7.0 gr resinous brownish mass of petroleum non soluble fraction.

1 Kg seeds of *A. muricata* yielded 550 gr decorticated seeds,220 crude extract and 3 gr resinous mass.

Isolation were done in a Soxhlet extractor.

1.2 Table 1.Toxicity of resinous precipitate of *A. squamosa*.

Sample	Dosis	mortality % after 24 hrs	mortality % after 48 hrs
A.squa mosa	500 mg	100 %	100 %
	250 mg	68.2%	90.9 %
	125 mg	21.5%	41.6 %
	62.5ng	5.0%	20.0 %
	31.25mg	0.0%	10.5 %
Controll	15.62mg	0.0%	0.0 %

Table 2. Toxicity of resinous precipitate of petroleum non soluble fraction of *A. reticulata* and *A. muricata*

Sample	Dosis	mortality % after 24 hrs	mortality % after 48hrs
A. reticulata	500 mg	100 %	100 %
	250 mg	75 %	100 %
	125 mg	20 %	70 %
	62.5mg	0 %	20 %
	31.25mg	0 %	5 %
	15.6 mg	0 %	0 %
Control		0 %	0 %
A. muricata	500 mg	80 %	100 %
	250 mg	0 %	18 %
	125 mg	0 %	0 %
	62.5mg	0 %	0 %
	31.25mg	0 %	0 %
Controll		0 %	0 %

Table 3. Toxicity of isolated compounds and Rf value in chloroform-methanol(9:1) of isolates

Sample	Dosis	Mortality % after 24 hrs	Rf value in Chloroform: Methanol (9:1)
isolate 1	100 mg	3.5 %	0.74
compound 1	100 mg	2.0 %	0.53
compound 2	100 mg	45.5 %	0.51
compound 3	100 mg	0 %	0.36
controll		0 %	

Table 4. Thin layer chromatogram spectrum of isolated compound no. 2 in several solvents and solvents combination

Solvent/solvents combination	Rf value	Detection reagent
chloroform	0.04	sulfuric acid 25%
petroleum ether	0	
chloroform-methanol	0.51	
chloroform-isopropanol(197:3)	0.04	
isopropylether-acetic acid(24:1)	0.0	
petroleum ether-ether-acetic acid(90-10-1)	0.0	
aceton-benzene -water(90:30:8)	0.73	
Ethylacetae-ether(2:1)	0.33	

Table 5. Color reaction on thin layer chromatogram of compound no 2

Reagent	Result	
Licbermann Burchard	negativ	not a steroid or triterpene
$FeCl_3$ solution	negative	not a phenolic
phosphomolybdic acid	blue after heating	
vanillin sulfuric acid	yellow brown after heating	
$SbCl_3$-chloroform	negative	no terpenic compound
anthron	light yellow after heating, no sugar	
Molish	negative	

Table no 6 Fatty Acid Composition of isolate no 1 from the Gas liquid chromatogram calculated as methyl ester

palmitic acid	15.58	%
stearic acid	10.18	%
oleic acid	48.92	%
linoleic acid	24.96	%
linolenic acid	0.36	%

Table 7. Physical properties of compound no.2

Melting point	: 38 $_{o}C$
Solubility	: slightly soluble in Chloroform soluble in methanol,aceton,ethylacetate, ether. not soluble in water,petroleum ether.

Table no 8 Phytochemical screening of seeds of *Annona reticulata*

Sample	Reaction positive for
resinous mass(petroleum-non soluble fraction)	alcaloids steroids phenolic compound cyanogenic compound lipid

Gas liquid chromatogram of fatty acids methyl ester of isolate 1, was obtained using following condition:

column :15 % DEGS, 3 mt length,stailess steel

detector :Flame ionization detector

column temperature: 200 ^{o} C

injection temperature:240° C

gas carrier : N_2 50 ml/minute

H_2 0.8 kg /cm^2

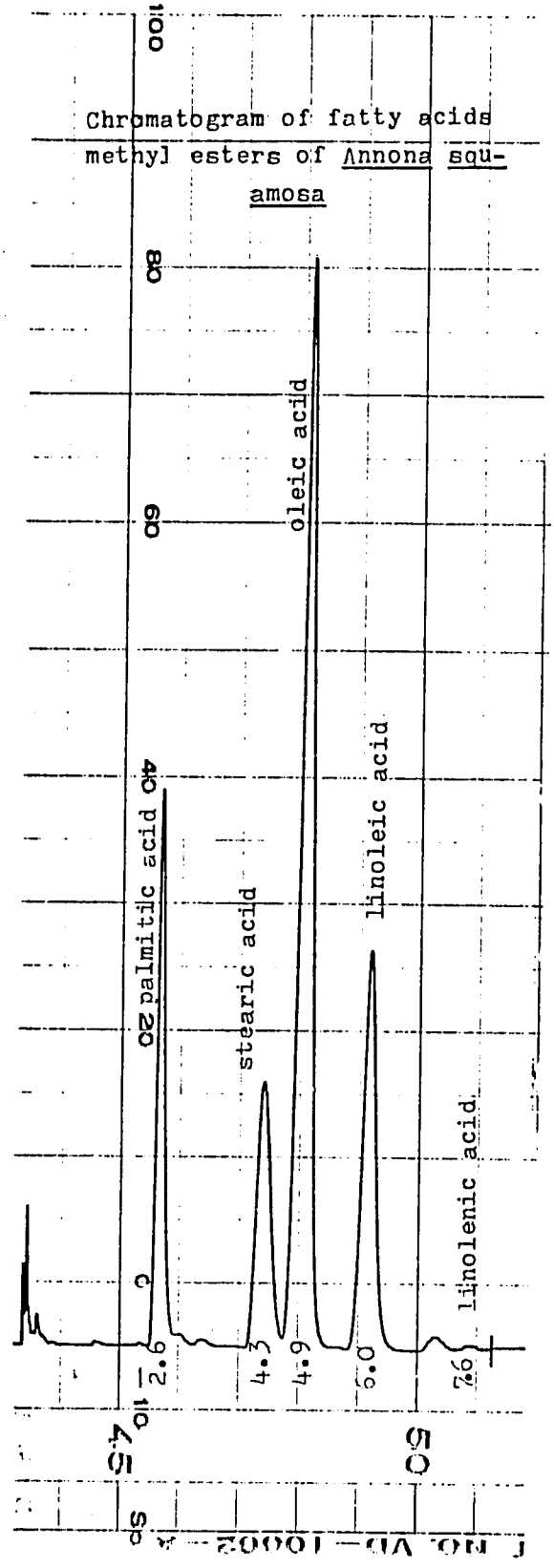
O_2 1.8 kg/cm^2

The chromatogram and the spectroscopical data are in the following pages.

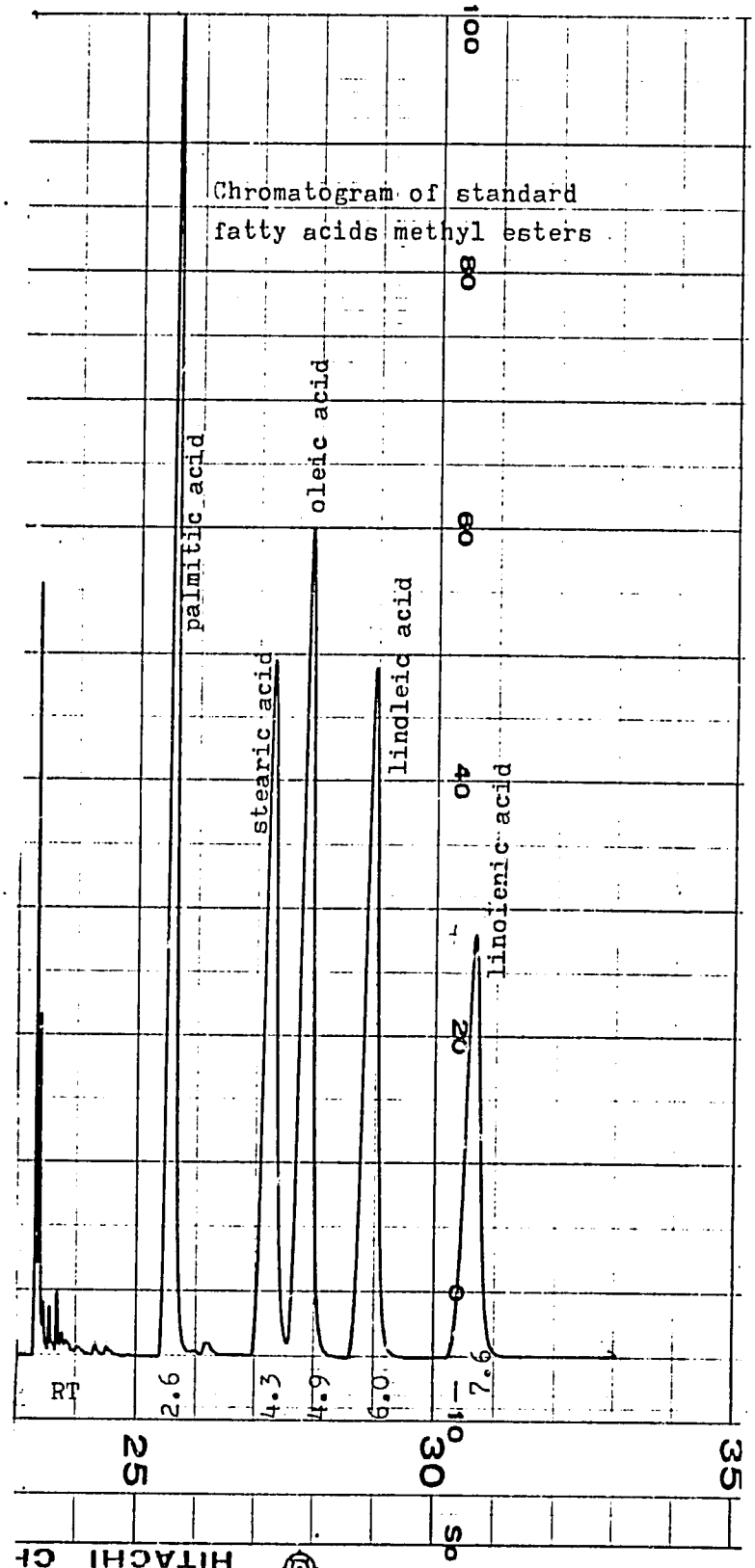
Identification of *Sitophylus oryzae*

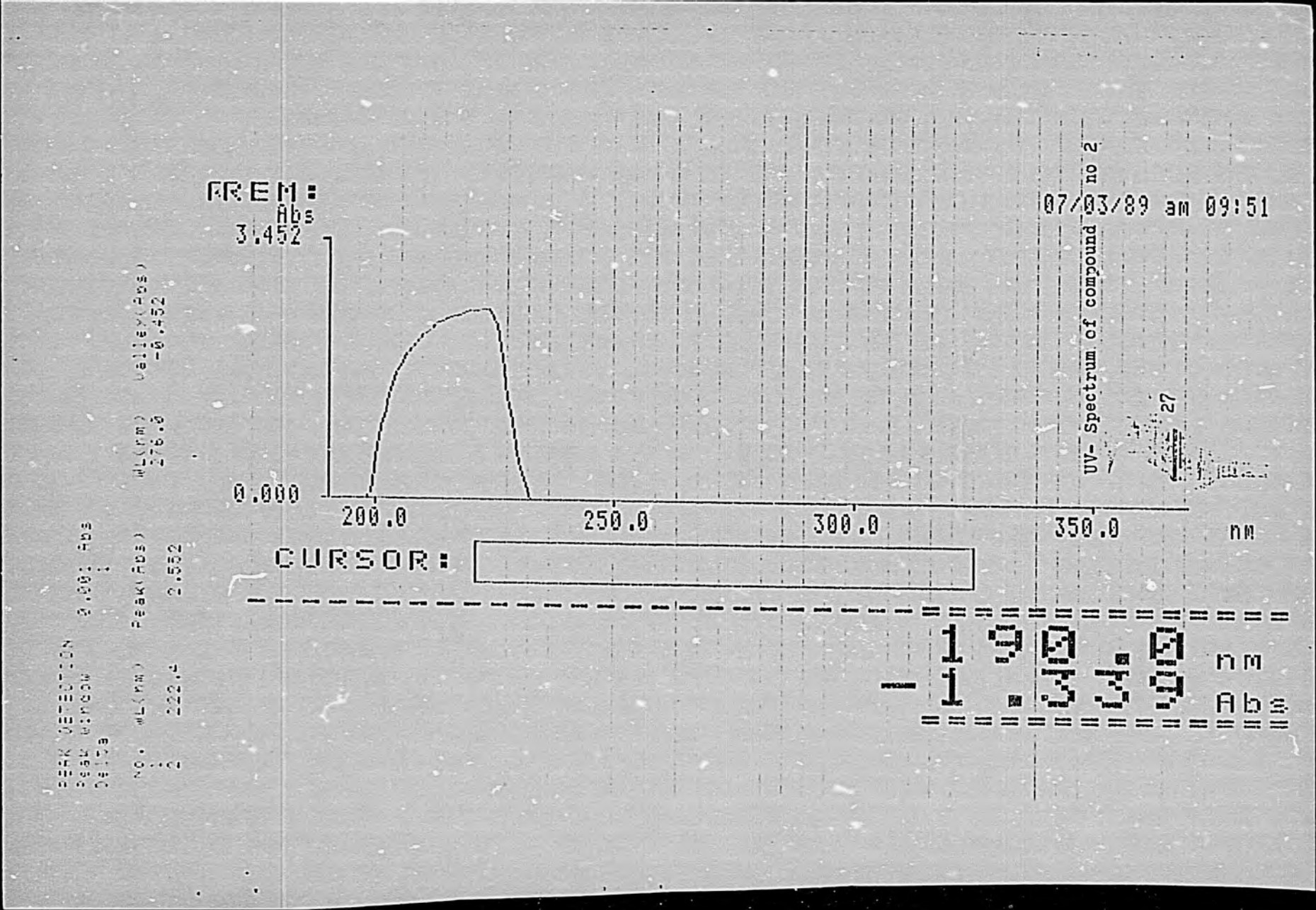
The *Sitophylus oryzae* are distinguihed from most other stored product beetle in that the head is elongated in front of the eyes to form a well defined rostrum or snout and the antennae are elbowed and clubbed.

Sitophylus oryzae is a dark brown species, 2.3 to 3.5 mm long with fully developped hindwings and two reddish-yellow markings on each wing cover.The prothorax is densely covered with round or irregularly shaped punctures.



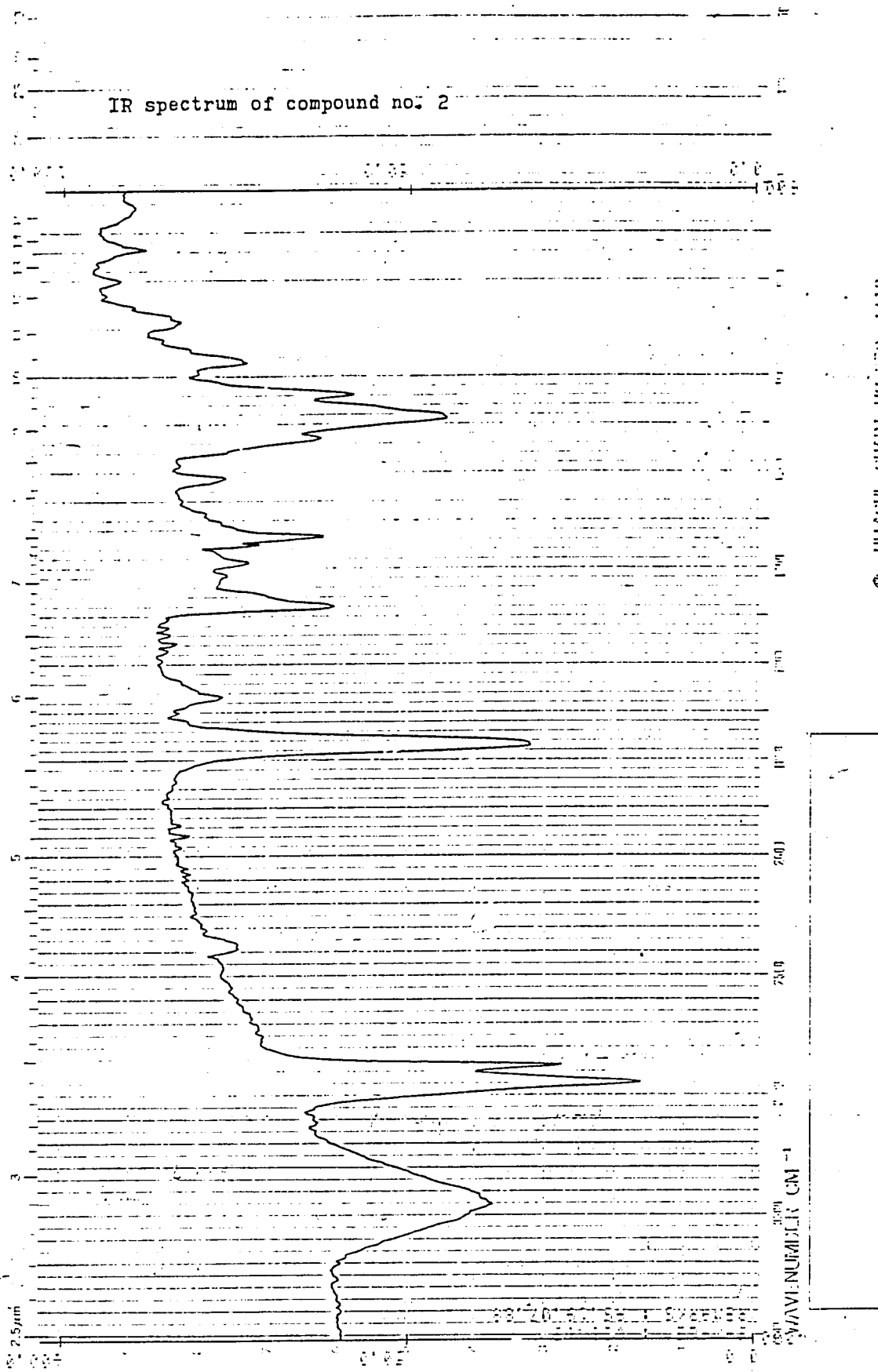
Chromatogram of fatty acids methyl esters of *Annona squamosa*


Chromatogram of standard
fatty acids methyl esters
palmitic acid
stearic acid
oleic acid
lindleic acid
linolenic acid
RT
2.6
4.3
4.9
6.0
7.6
100
80
60
40
20
0
25
30
35
HITACHI

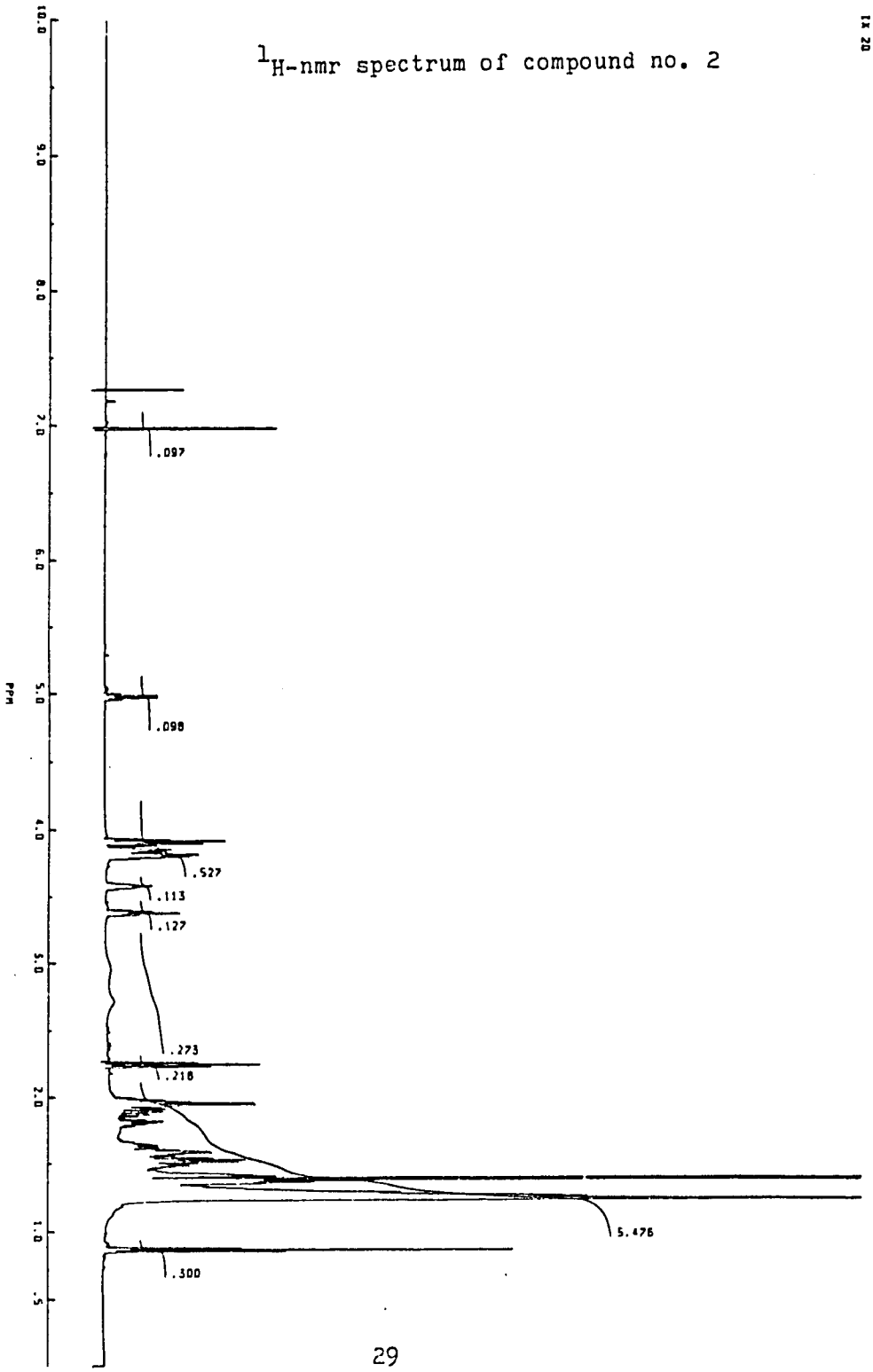


PEAK DETECTION
Peak Window 0.001 Abs
Delta 1
No. WL(nm) Peak(Abs) WL(nm) Valley(Abs)
1 276.0 -0.452
2 222.4 2.552
FREM:
Abs
3.452
0.000
200.0
250.0
300.0
350.0
nm
07/03/89 am 09:51
UV- Spectrum of compound no 2
27
CURSOR:
190.0 nm
-1.339 Abs

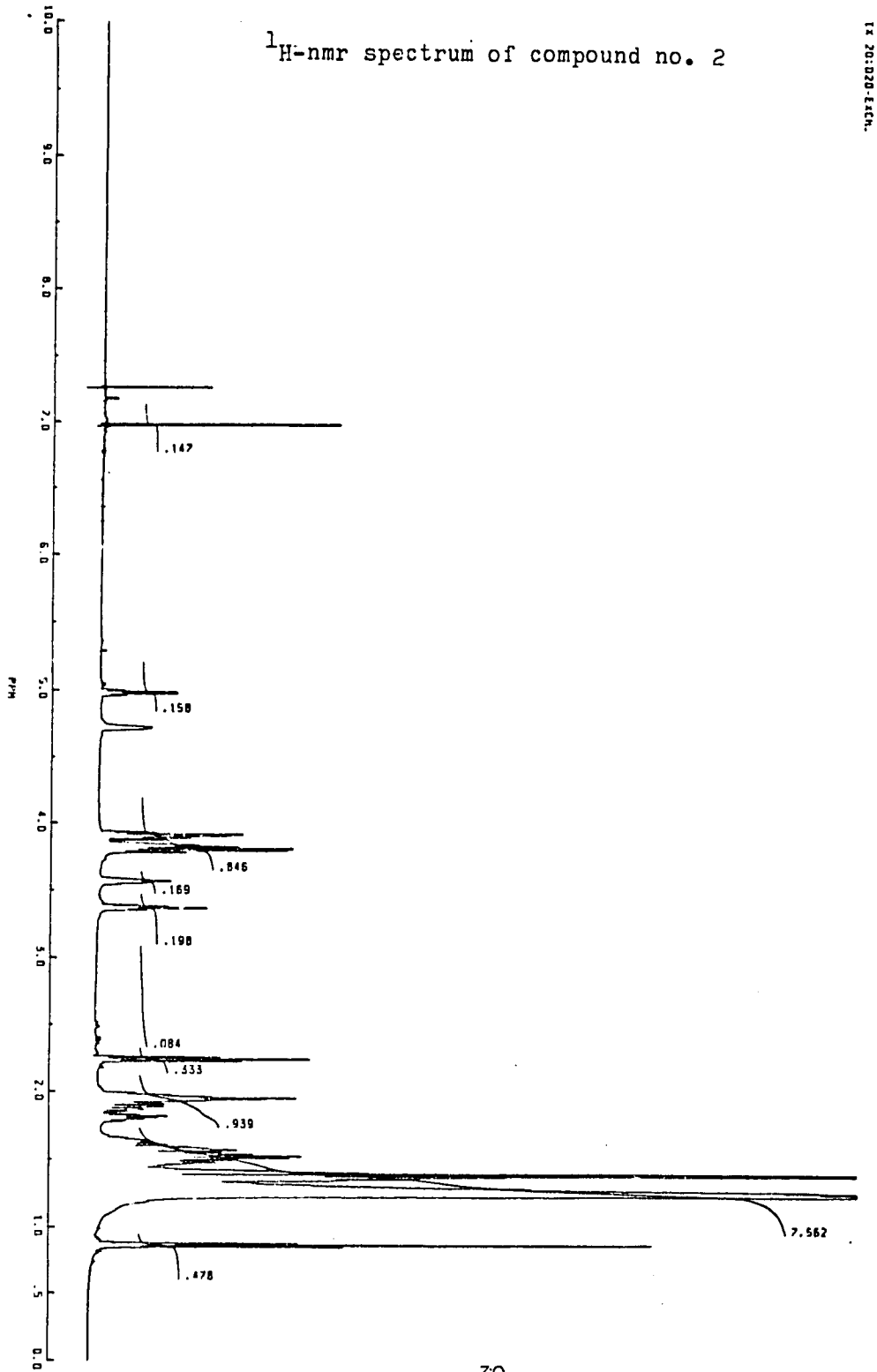
IR spectrum of compound no. 2



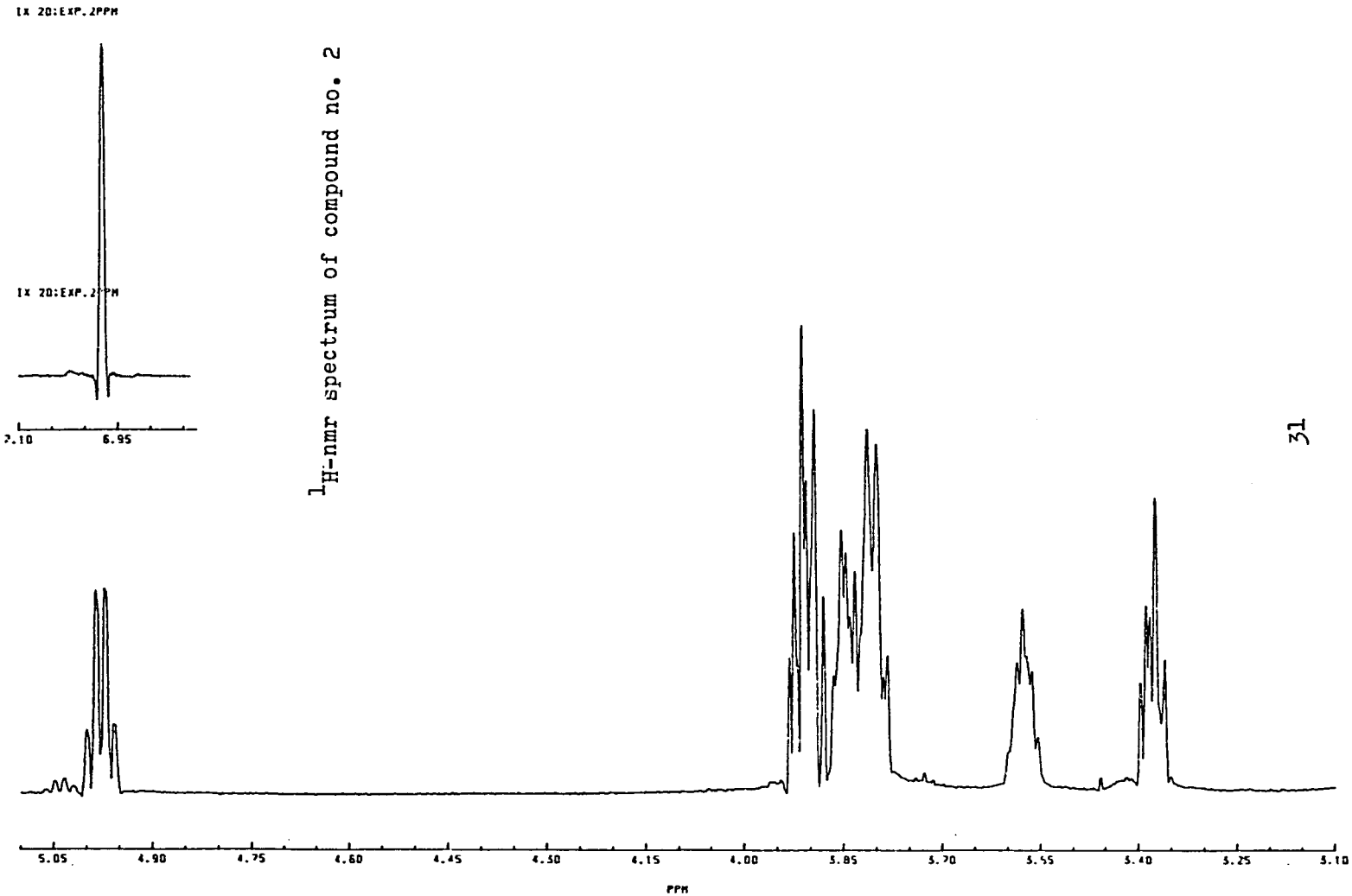
^{1}H-nmr spectrum of compound no. 2



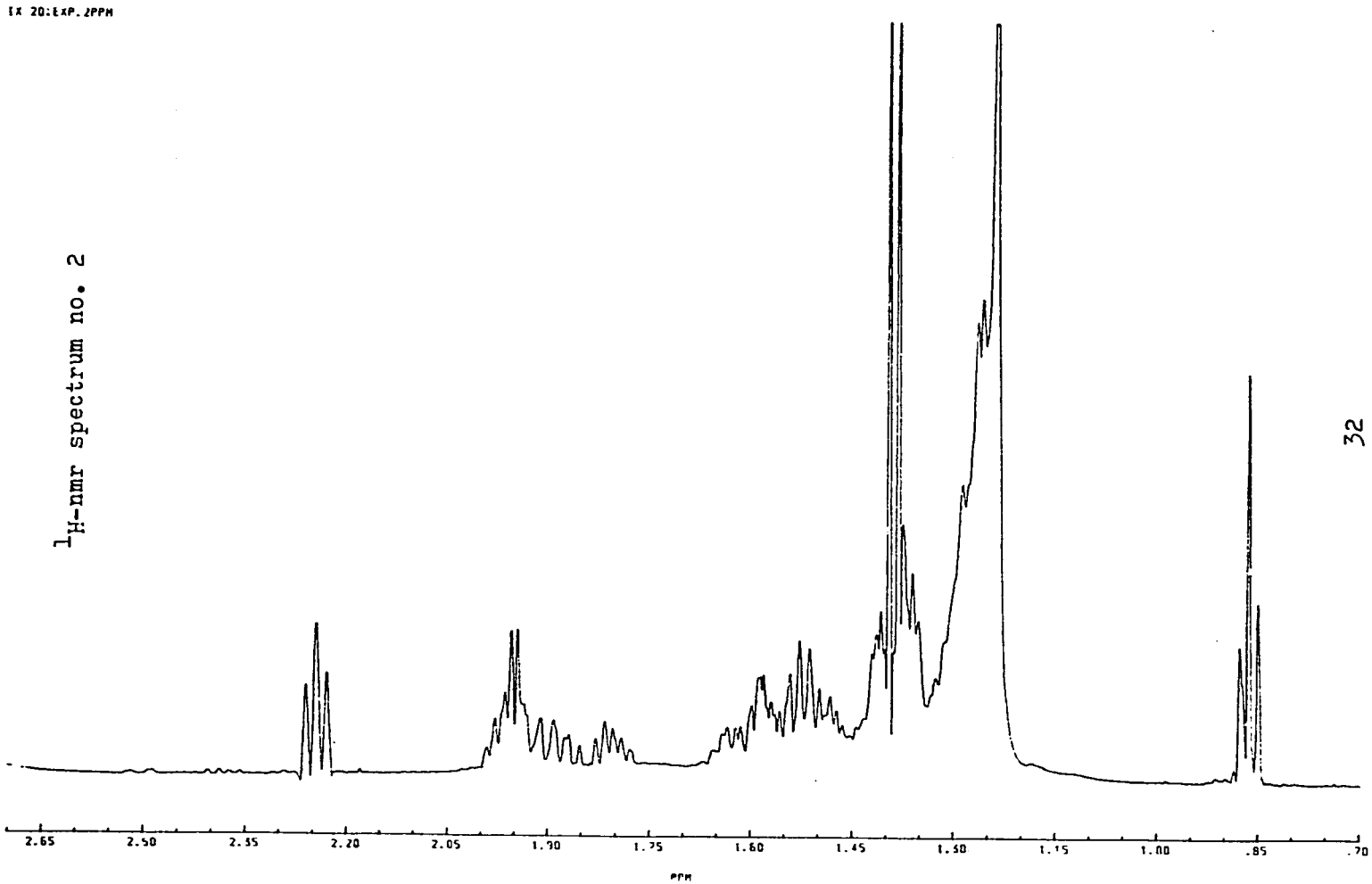
^{1}H-nmr spectrum of compound no. 2



^{1}H-nmr spectrum of compound no. 2



^{1}H-nmr spectrum no. 2



2.DISCUSSION

Several *Annona species* are found wildly or cultivated throughout Indonesia;mostly they are found in East Java,Madura and Central Java,less in West Java.

Sample for this study were collected from Central Java.*Annona species* found in this area are *Annona muricata*,*Annona reticulata* ,*Annona squamosa* and *Annona cherimolia*.*A. muricata* are grown in gardens for the edible fruits,used widely to make refreshing soft drink.They available every season in the market.The fruit flesh is wide and thick,the seeds are light brown in colour,less in number compared to other *Annona species*.

Annona squamosa are also found widely cultivated in gardens or wild.They are also found planted in lime mountainous area of Yogyakarta in the villages Bayat and Wonosari,from which most of the samples were collected.

Annona reticulata is much more rare compared to *A. squamosa* and *A.muricata*.The sample used for this study were collected from Boyolali area and in season around September.

Annona cherimolia is the most rare.It is found planted in the botanical garden of Gadjah Mada University.

In this study,seeds of *A. muricata*,*A. reticulata* and *A.squamosa* were tested on *Sitophylus oryzae*.From the preliminary study as shown in the table 2,the seeds of *A. muricata* had a very week activity,so a further study were only carried out on *A. squamosa* and *A. reticulata*.However,not enough seeds of *A. reticulata* were

obtained.A further chemistry study on the active compound was carried out only on *A. squamosa*.Preliminary study was done on the whole seeds,since the seed coat did not show any activity,a further extraction were carried out on the decorticated seeds.1 Kg of seeds consists of 600-660 seed kernels and 340-400 sead coat,and yielded 7.5 gr resinous precipitate.

Isolation of insecticidal compound was done firstly by extraction with ether in a Soxhlet apparate,after evaporation of solvent,5 times of volume of petroleum ether was added.A brownish yellow resinous matter was precipitated.Thin layer chromatography in chloroform-methanol(9:1),using sulfuric acid as detection reagent showed 9 spots.The resinous precipitate of whole seeds gave 11 spots.After testing the insecticidal activities on *Sitophylus oryzae* ,the resinous precipitate was fractionized in a column of silicagel H 6o.Fractionization was carried out firstly with chloroform. It seperated a yelllowish oily matter.Transesterification of the oil with methanol,benzene and sulfuric acid,than analysing it in GLC showed that the oil consist of palmitic acid 15.68 %,stearic aci 10.18 %,oleic acid 48.92%, linoleic acid 24.9%,and linoleic acid 0.36 %,caculated as methyl esters.

Elution was done than with chloroform-methanol(9:1),chloroform-methanol(1:1) than methanol.55 fractions were obtained.The first 20 fractions were the oily matter.Fractions with same spots were collected and

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Elution was done than with chloroform-methanol(9:1),chloroform-methanol(1:1) than methanol.55 fractions were obtained.The first 20 fractions were the oily matter.Fractions with same spots were collected and

fractionized again with suitable solvents combination.

3 Compounds were isolated using ethylaceate-ether(2:1).The 3 compounds as well as the first isolate(the oily matter) were tested on *Sitophylus oryzae*.The result was as shown in table 3.From the result it was assumed that compound 2 has the activity.

Characterization of compound 2 by thin layer chromatography gave 0 Rf in solvents combination for neutral lipids i.e. isopropyl ether-ether(24:1) than petroleum ether-ether-acetic acid(90:10:1) consecutively.Isolate no. 1 gave aspot at Rf 0.70, Rf 0.53 ,Rf 0.15.Developping lipid in this solvents combination,hydrocarbon will give approximately Rf of 0.90,triglycerides 0.70,diglyceride 0.50 and monoglycerides 0.15 ,while polar lipid 0 Rf(Harborne,198 . Using eluent of combination of aceton-benzene-water(90:30:8) the Rf was 0.75.It was in the range of Rf of diacyl digalactosyl glyceride,a polar glyceride.If this compound is a glyceride as suggested by the previous investigator(Harper et al),it must be a polar glyceride.In chloroform propanol the Rf was 0.04.After spraying with anthron reagent(for sugar) than heated at 70 ^{o} C for 20 minutes it gave a light yellow color:sugars should give green color.

Dragendorf reagent gave brown coloring ,$SbCl_3$ and Liebermann Burchardt reagent gave a negative result.vanillin sulfuric acid brown coloring,Ferricloride negative,phosphomolybdic acid blue coloring after heating.So it is no an alkaloid,nor

a terpenic or steroid.Sulfuric acid and heated gave pale brown color.This color indicated a lipid.

IR (film in chloroform) showed absorption at 3440(s),(OH absorption),2952(s) CH_2,1780 (s) carbonyl(lacton),1660(w) c=c ,1468,CH,1424 (w) c=c,1322,1380 CH_3 ,1120,1060,1020 (s) C-O-C, 960 cm^{-1}.

^1H -nmr(δ) in $CDCl_3$,ppm,showed a triplet at 0.86(3H) ; 1.24 ,singlet; 1.25,quartet ; 1.38 doublet ; 1.37 quartet ; 1.4 quartet ; 1.52 (triplet); 1.58 quartet/multiplet);1.9 (multiplet) (alltogether 54 H) ;2.2 triplet (2H) ; 3.3 (1 H); 3.5 multiplet (1H) ; 3.9 multiplet (5 H) ; 4.9 quartet ( ) ; 6.9 singlet (1H); total number of hydrogens were 68.

From the elemental analysis was found: C= 71.27 ; H= 10.86 and O= 17.87%.A molecul formule of $C_{37}H_{68}O_7$ fitted closely with the elemental analysis result;calculated C =71.15 ;H =10.89 ;O =17.94%.

D_2O exchange showed a decrease of 1 hydrogen.It suggested 1 OH.The presence of an olefinic hydrogen was supported by the IR spectrum so was the presence of an OH.The ^1H-nmr showed 3methyl groups at 0.86 ,1.24 and 1.3 ppm, an olefinic proton at 6.8 ppm probably $-\overset{|}{C}=\underset{H}{C}-$; it showed also another 3 methine protons.

The mass spectrum and the ^{13}C -nmr spectrum,which are very important to support characterization of compound by this time of writing have not arrived yet.So, a proposed structure and a further analysis of insecticidal activities

will be reported elswhere, perhaps in a journal or a seminar.

CONCLUSION AND SUGGESTION

1. An active compound has been isolated.
2. Characterization of compound need to be continued.
3. Continuation of study should also be carried out on the applied aspect on several pests in a relatively simple medium, though the activity on *Sitophylus oryzae* turned to be quite week.

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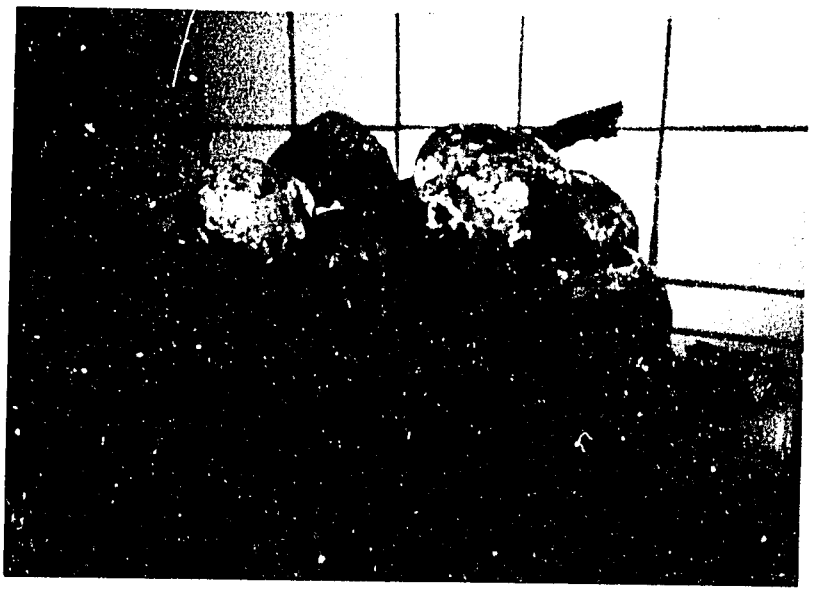
Fruits of *Annona squamosa*

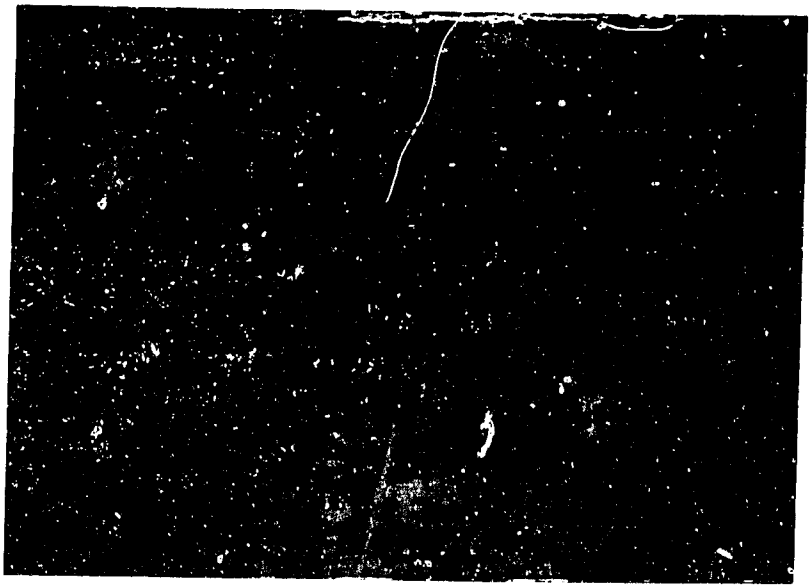
Fruits of *Annona muricata*

Fruits of Annona reticulata



Fruits of Annona reticulata

ripend with lime

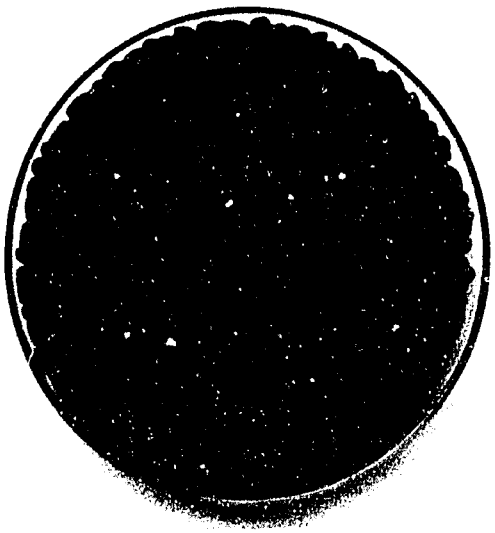


lime mountainous area planted

with *Annona squamosa* at Wonosari

lime mountainous area planted with

Annona squamosa at Bayat



Seeds of *Annona* squamosa

Seeds of *Annona muricata*