การทดสอบฤทธิ์ของไมตราจินีนและสารสกัดต่างๆ ที่ได้จากใบของต้นกระท่อม

ปริญญานิพนธ์

ของ

สรินทร เฉลิมชัยวินิจกุล

เสนอต่อมหาวิทยาลัยศรีนครินทรวิโรฒ ประสานมิตร เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาเคมีชีวภาพ
กันยายน 2535

คณะกรรมการควบคุมและคณะกรรมการสอบได้พิจารณาปริญญานิพนธ์ฉบับนี้แล้วเห็นสมควรรับเป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาเคมีชีวภาพ ของมหาวิทยาลัยศรีนครินทรวิโรฒได้

คณะกรรมการควบคุม

.....ประธาน
(ดร.วีระชัย สิงหนิยม)

.....กรรมการ
(ผศ.ดร.สุนันท์ ชัยนะกุล)

คณะกรรมการสอบ

.....ประธาน
(ดร.วีระชัย สิงหนิยม)

.....กรรมการ
(ผศ.ดร.สุนันท์ ชัยนะกุล)

.....กรรมการที่แต่งตั้งเพิ่มเติม
(ผศ.พงษ์ศักดิ์ กรรณล้วน)

บัณฑิตวิทยาลัยอนุมัติให้รับปริญญานิพนธ์ฉบับนี้ เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาเคมีชีวภาพ ของมหาวิทยาลัยศรีนครินทรวิโรฒ

.....คณบดีบัณฑิตวิทยาลัย
(ศ.ดร.สมพร บัวทอง)

วันที่ 25 เดือน กันยายน พ.ศ. 2535

EVALUATION OF EFFECTS OF MITRAGYNINE AND DIFFERENT FRACTIONS EXTRACTED FROM LEAVES OF MITRAGYNA SPECIOSA KORTH.

A THESIS

By

SARINTORN CHALERMCHAIVINIJKUL

Presented in partial fulfillment of the requirments for the
Master of Science degree in Biological Chemistry
at Srinakharinwirot University
September 1992

ACKNOWLEDGEMENTS

I wish to express my sincerest gratitude and appreciation to my advisor, Dr. Weerachai Singhaniyom, Head of the Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, for his kindness, valuable supervision, guidance and encouragement throughout the course of this study, and editing of this dissertation.

My deeply gratitude is hereby extended to my co-advisors, Asst.Prof.Dr.Sunan Chainakul, Department of Chemistry, Faculty of Science, Srinakharinwirot University, for her keen interest during the study and kindness in interpretation of the ultraviolet, infrared, nuclear magnetic resonance and mass spectra.

And I am deeply indebted and grateful to Assoc.Prof. Dr.Sunibhond Pummangura, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his valuable suggestions and discussion in interpretation of the ultraviolet, infrared, nuclear magnetic resonance and mass spectra .

I deeply in gratitude to Asst.Prof.Pongsak Kanluan, Head of the Department of Physiology, Faculty of Pharmaceutical Sciences, for his valuable helps, kindness, guidance and encouragement in laboratory work.

My deeply gratitude is extended to Dr.Saisanom Thammapituk, Department of Chemistry, Dr.Sumontha Phrombund, Department of biology, Faculty of Science, Srinakharinwirot University, for valuable supervision, guidance and encouragement during the course of study in this University.

Finally, I wish to express my appreciation and gratful to my parents for their love and understanding encourage me in every ways, I am so sincere thanks.

TABLE OF CONTENTS

LIST OF TABLES

PAGE

TABLE

LIST OF FIGURES

PAGE

FIGURE

PAGE

FIGURE

PAGE

FIGURE

PAGE

FIGURE

PAGE

FIGURE

PAGE

FIGURE

PAGE

FIGURE

ABBREVIATIONS

dec.	=	decompose
^{1}H NMR	=	proton nuclear magnetic resonance
^{13}C NMR	=	carbon nuclear magnetic resonance
cm.	=	centimeter
g.	=	gram
ip.	=	intraperitoneal
IR	=	infrared
J	=	coupling constant
KBr	=	potassium bromide
kg.	=	kilogram
L	=	liter
M^{+}	=	molecular ion
m/z	=	mass to charge ratio
mg.	=	milligram
MH_{z}	=	mega hertz
m.p.	=	melting point
ppm.	=	parts per million
S.E.M.	=	standard error of mean
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Mitragyna speciosa Korth is a large tropical tree growing in swampy teritory in the tropical and subtropical regions of Africa and South East Asia. The tree was named by Korthals, and has been classified in the genus Mitragyna which belongs to the subtribe Mitragyninae in the tribe Cinconeae of the family Rubiaceae(Holmes. 1895). However, the Mitragyna speciosa Korth has been found extensively South East Asia areas such as Thailand, India, Malasia, Indonesia and Philipine etc. In Thailand, it grows extensively in the central and southern parts of country and has several names depending on the growing areas, "Kratom" or "Kakuam" or "Ithang" in the central part, and "Thom" in the southern part(Suwanlert. 1975).

The Mitragyna speciosa Korth(Figure 1) usually grows to a height of 10-25 metres. The young stems bear 10-12 leaves arranged in opposite and decussate pairs, each pair accompanied by two interpetiolar stipules which initially appresses and protects the apical bud. The leaves(Figure 2) are simple elliptic, 17 cm. long and 10 cm. broad, the lower surface is slightly paler green than the upper surface. The upper surface shows distinct pinnate venation, but this is more marked on the lower surface where the midrib, lateral veins and secondary veins are prominent, the base is round or heart-shape. The petioles are 2-4 cm. long. The flowers are crowded in round, terminal inflorescences(heads) of 3-5 cm. long. The calyxtube is short and cup shaped, with rounded lobes. The fruit is oblongovoid and 5-7 mm. long, with 10 ridges(Shellard. 1965, 1974).

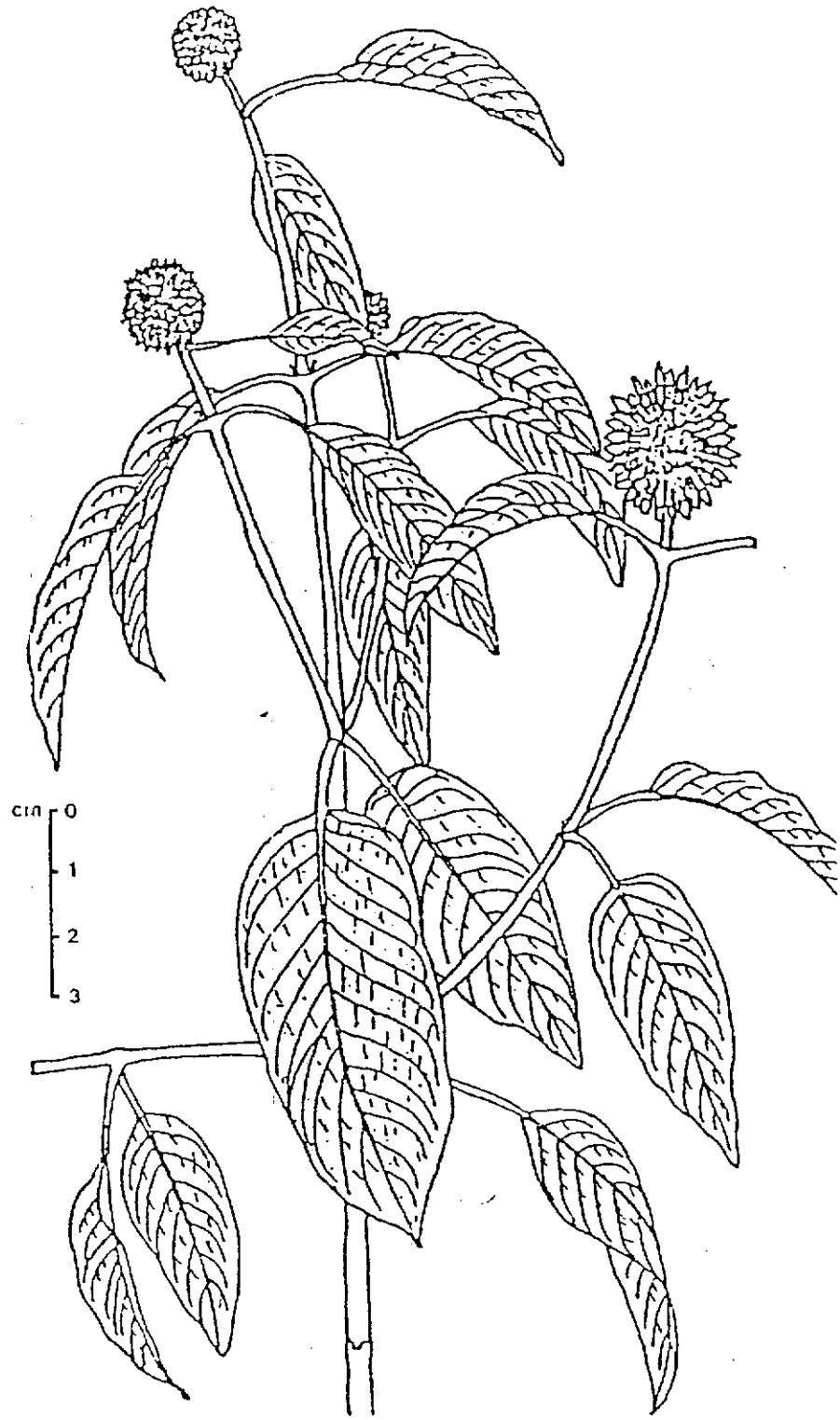


Figure 1 Mitragyna speciosa Korth.

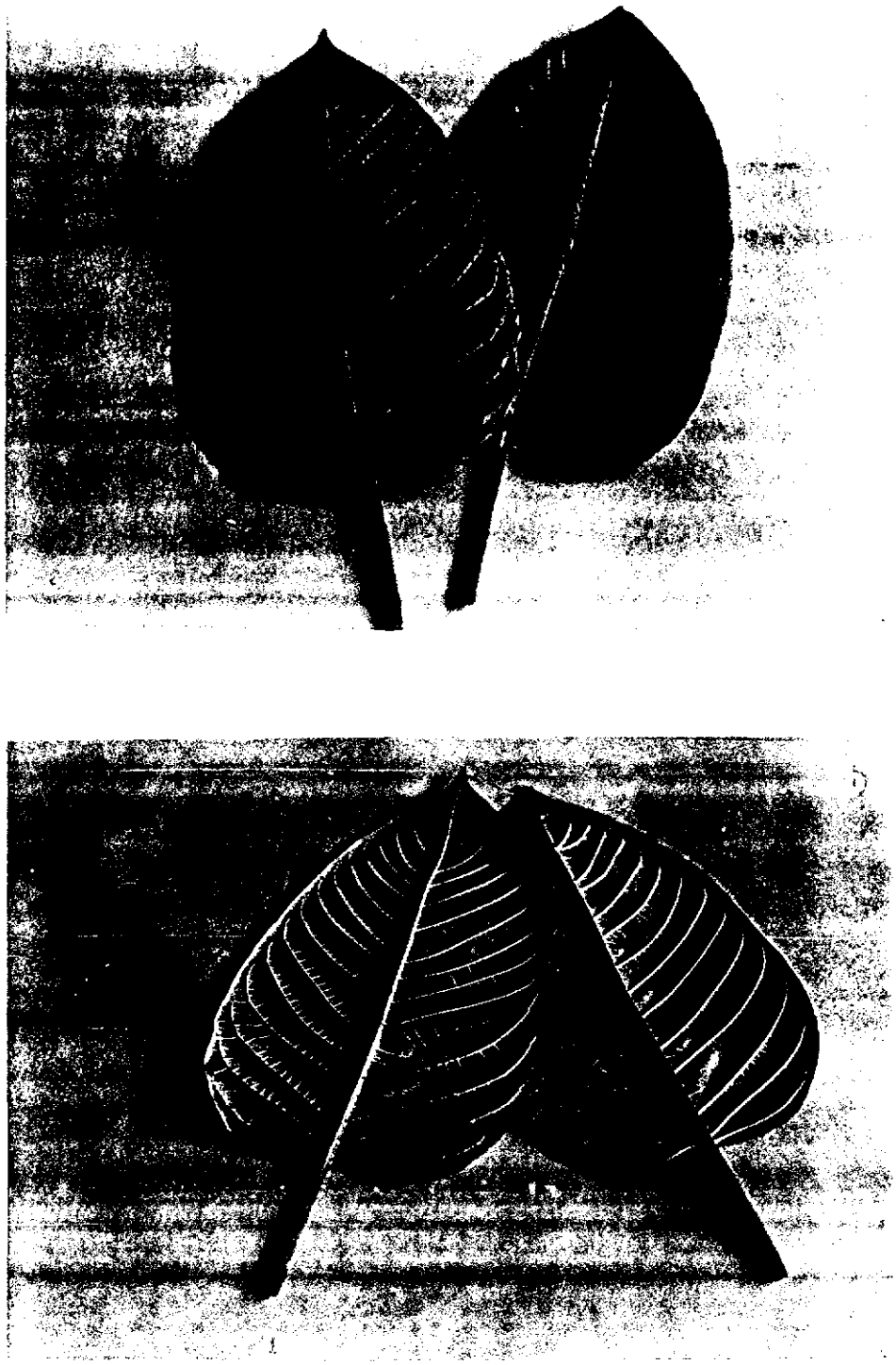


Figure 2 Photographs demonstrate leaves of Mitragyna speciosa Korth.

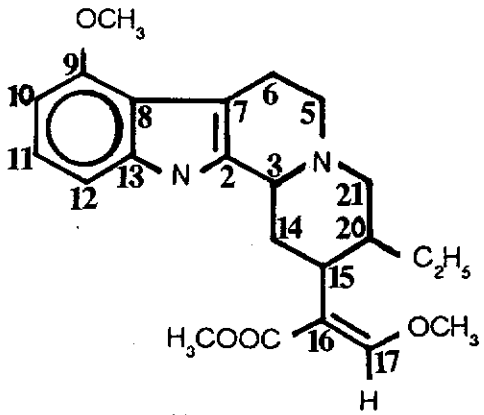
a. The upper surface

b. The lower surface

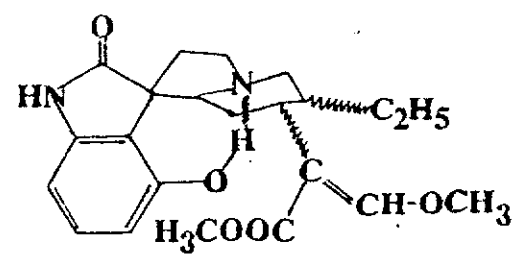
In the folklore medicine, the leaf of Mitragyna speciosa Korth has been used for the treatment of diarrhoea, wound poultice, cure for fever, colic, the expulsion of worms and suppressor of the opiate withdrawal syndrome. Some villagers use it as an ingredient for cooking and used as an opium substitute when opium itself was unavailable or unaffordable (Burkill. 1935). Generally, the leaves have been commonly used among gardeners, peasants and labourers to increase work efficiency, tolerance of hot sun(Amattayakul. 1960; Norakanphadung. 1966 and Suwanlert. 1975). Two kinds of leaves are popular among the kratom users, those with the red or white vein, the latter has stronger effect than the former. The general methods of consumption is by chewing the fresh leaf or by grinding the dried leaf or by smoking. For each preparation the vein in the leaf is extracted and in some cases salt is added to prevent constipation. Consumption is usually followed by a drink of warm water. From 10-36 leaves are usually taken from 3-10 times a day. Five to ten minutes after kratom consumption the user described himself as feeling happy, strong and active(Suwanlert. 1975 and wray. 1907). Kratom was weaker than morphine with shorter effects, less harmful than cocaine, it was said to stimulate like coca and have depressive effects like opium and cannabis, as if chewing caco leaves and smoking opium simultaneously (Norakanphadung. 1966). When an excess of the leaves is eaten, it caused vomiting and dizziness, numbness of body, twitching of the hands and feet, and an effect on the heart have also been reported(Marcan. 1929). The long-term users were claimed to be thin with distended stomachs, unhealthy complexions, dark lips, dry skin, anorexia, weight loss and insomnia(Suwanlert. 1975). The dosage is then increased in varying degree. Because of the harmful effects, the government of Thailand imposed the law "Kratom Act,B.E. 2486" which came into force on 4 August, 1943

and annouced that it's forbidden to plant the tree, and existing ones are to be cut down. Later, kratom is listed in the schedule V under narcotic Act,B.E. 2522.

In 1907, Hooper actually isolated an alkaloid from the leaves of Mitragyna speciosa Korth and was repleated in 1921 by Field who named the alkaloid "mitragynine" without identification of its chemical structure(Field. 1921). Until the 1906's, new analytical techniques were applied to these alkaloids, also in 1964 Zacharias et. al, showed by X-Ray crystallography that the C(17) -H is cis to the methyl ester at C(16). Many workers had previously been involved in the determination of general structure of mitragynine and it was shown by Joshi et. al (1963) to be an indole alkaloid having a methoxy group in the C(9) position and open E ring(E. seco). Later, Beckett(1965) isolated two alkaloids from the dried leaves of M. speciosa Korth, the first alkaloid is mitragynine (0.3 % yield) which its structure was elucidated by elemental analysis, UV and IR spectrometry showing a molecular formula of $C_{23}H_{30}N_2O_4$. Mass spectrometry confirmed the molecular weight of 398 that show chemical structure in the figure 3. It crystallises in absolute ethanol. The second alkaloid is an oxindole alkaloid, namely speciofoline(0.038 % yield; Figure 3), crystallised from a mixture of acetone and n-hexane(1:1).



mitragynine

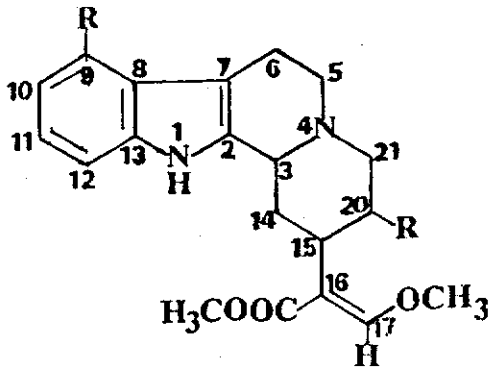


speciofoline

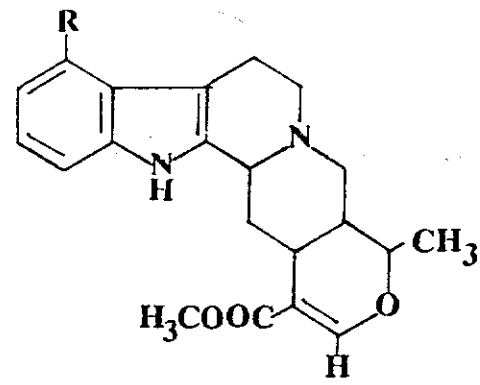
Figure 3 Chemical structures of mitragynine and speciofoline.

Beckett and Shellard(1966), isolated two types of alkaloids from the dried leaves, The first type is indoles and consist of 5 compounds with mitragynine(0.022 % yield) is the major alkaloid. The second type is oxindoles and consist of 3 compounds. They have general structure of closed or opened E ring with substitution occuring at C(9) position(Figure 4). All have asymmetric centers at C(3), C(15) and C(20). The closed E ring alkaloids have asymmetric centers at C(19).

General chemical structure of indole alkaloid.

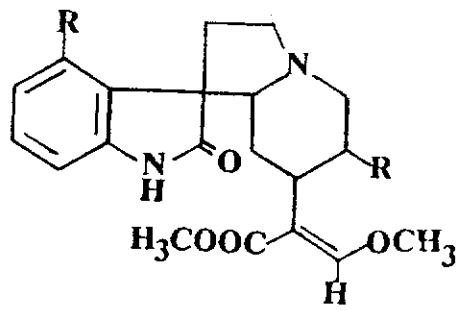


Open E ring

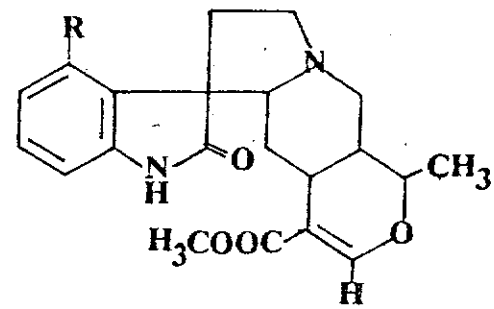


Close E ring

General chemical structure of oxindole alkaloid.



Open E ring



Close E ring

Figure 4 Chemical structure of different type of alkaloids in Mitragyna speciosa Korth.

Shellard(1974) isolated twenty-two alkaloids from the genus Mitragyna(Table 1), and found that their contents was varies according to locations and seasons. Mitragynine is a dominant alkaloid, which isolated only from Mitragyna speciosa Korth, The mitragynine had methoxyl group at position 4 of the indole ring, rendering it analogeous to the 4 substituted indoles psychedelics, e.g. psilocybin and lysergic acid amide. It was assumed to be the physiologically active constituent having morphine-like properties. According to laidlaw, mitragynine is a local anaesthetic(Field. 1921). Grewal(1932) performed a series of experiments on animal tissues and found mitragynine is a central nerve system stimulant rather than depressant. He indicated that mitragynine has a depressant effect on some isolated tissues, facilitates the passage of impulses in the autonomic nerve system and increases the excitability of the medulla and probably of the motor centers. He also found that mitragynine is a general protozoal poison, but is probably ineffective against bacteria, or pathogenic protozoa. Grewal(1932) subsequently administered mitragynine to five men and observed a cocaine-like effect. Moreover, 50 mg. of pure mitragynine acetate produced nousea and vomiting in some subjects

Mitragynine acetate was found to diminish the excitability of plain muscle, to anaesthetize the cornea, and to be toxic to animals in fairly low doses. In the psychological research, 6 human subjects submitted to various tests after taking doses of 50-100 mg. of mitragynine acetate and after doses of 650-1300 mg. of the powdered leaves. There were indications that both forms of drug produced the following effects; increase tolerance, increase steadiness and capacity for work; flushing of the skin, apparently due to dilation of superficial blood vessels(Marcan. 1934). Mitragynine hydrochloride or mitragynine ethanedisulfonate exhibited analgesic and

Table 1 Alkaloids in the genus mitragyna

Species of mitragyna	Indoles	Oxindoles
West African species		
- *Mitragyna stipulosa*		
Leaves	Hirsutine	Rotundifoline
		Isorotundifoline
		Rhynchophylline
		Isorhynchophylline
		Mitraphylline
		Traces of corynoxeine
		and isocorynoxeine
Stem-bark	None	as in leaves
Root-bark	None	as in leaves except
		for the corynoxeine
- *Mitragyna cilliata*		
Leaves	Mitraciliatine	Rotundifoline
		Isorotundifoline
		Rhynchophylline
		Isorhynchophylline
		Ciliaphylline
		Rhyncociline
Stem-bark	None	as in leaves
Root-bark	None	as in leaves
- *Mitragyna inermis*		
Leaves	Mitraciliatine	Rotundifoline
	Speciogynine	Isorotundifoline
		Rhynchophylline
		Isorhynchophylline
		Speciophylline
		Uncarine F

Table 1 (cont.)

Species of mitragyna	Indoles	Oxindoles
		syn-Rhynchophylline *N*-Oxides
		anti-Isorhyncho-phylline *N*-Oxide
Stem-bark	None	as in leaves but no *N*-Oxides
Root-bark	None	as in leaves but no *N*-Oxides
East African species		
-Mitragyna rubrostipulata		
Leaves	None	Rotundifoline
		Isorotundifoline
		Rhynchophylline
		Isorhynchophylline
		Mitraphylline
		Isomitraphylline
		anti-Rotundifoline *N*-Oxides
		syn-Rhynchophylline *N*-Oxides
Stem-bark	None	as in leaves
Root-bark	Hirsutine	as in leaves
	Hirsuteine	

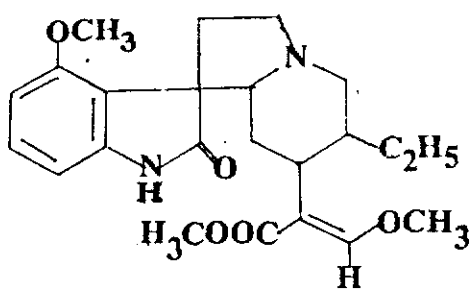
Table 1 (cont.)

Species of mitragyna	Indoles	Oxindoles
India and S.E. Asia		
- *Mitragyna hirtusa*		
Leaves		Mitraphylline
		Isomitraphylline
		Hirsutine
		Hirsuteine
		rhynchophylline
		Isorhynchophylline
		Mitrajavine
- *Mitragyna diversifolia*		
Leaves		Mitraphylline
		Isomitraphylline
		Ajmalicine
		Javaphylline
		Mitrajavine
		Angustine
- *Mitragyna parvifolia*		
Leaves	Hirsutine	Rotundifoline
		Isorotundifoline
		Rhynchophylline
		Isorynchophylline
		Akuammigine
		Akuammigine *N*-Oxides
		speciophylline
		speciophylline *N*-Oxides

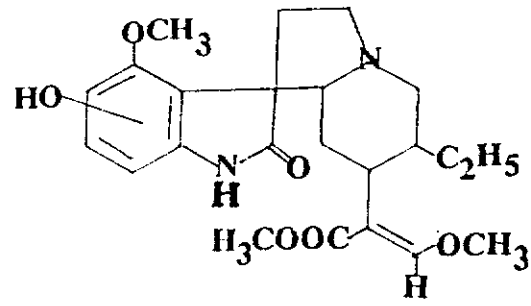
Table 1 (cont.)

Species of mitragyna	Indoles	Oxindoles
- *Mitragyna rotundifolia*		
Leaves		rotundifoline
		Isorotundifoline
		Isorhynchophylline
		Mitraphylline
		Isomitraphylline
		Ciliaphylline
		Corynoxeine
		Isocorynoxeine
		Specionoxeine
- *Mitragyna tubulosa*		
Leaves		Mitraciliatine
		Ciliaphylline
		Rhynchociline
		Rhynchophylline
		Isorhynchophyl-line
- *Mitragyna speciosa*	Mitragynine	Speciofoline
Leaves	Speciogynine	Isospeciofoline
	Speciociliatine	Ajmalicine
	Corynantheidine	corynoxine A
	Paynantheine	Corynoxine B
		Mitraphylline
		Isomitraphylline
		speciophylline
		Mitrafoline
		Isomitrafoline
		specionxeine
		Isospecionoxeine

antitussive properties in animal. Unlikes the narcotic analgesic, mitragynine had little effect on gastric mobility, failed to produced excitement in cats, was not antagonized by nalophine and had only weak respiratory depressant activity in anaesthetized animals at analgesic levels. In the mouse, no evidence of toxicity (tremors and convulsions) was observed after doses as high as 920 mg/kg.body weight. Mitragynine was found to be mush less active subcutaneously than orally, suggesting that the active analgesic moiety may be a metabolite(Macko. 1972). Thus, Zarembo(1974) prepared two active metabolites from microbial transformation of mitragynine by the fungus *Helminthosporum sp.* Their structures were identified by spectroscopic technique, as mitragynine pseudoindoxyl and hydroxy mitragynine pseudoindoxyl(Figure 5). The mitragynine pseudoindoxyl displayed analgesic activity in the D'Amour-Smith test almost 10 folds stronger than mitragynine when administered by both intraperitoneal and oral routes to animals. Hydroxy mitragynine pseudoindoxyl was 20-30 % more effective than mitragynine (Zarembo. 1974).



mitragynine
pseudoindoxyl



hydroxy mitragynine
pseudoindoxyl

Figure 5 chemical structure of mitragynine pseudoindoxyl and hydroxy mitragynine pseudoindoxyl.

Previous evidences seem to demonstrate contradict effects of mitragynine and kratom leaves on experimental animals or tissues as central nervous system depressant or stimulant. There is no direct evidence to prove this phenomenon. Thus, this study is aimed to investigate the effect of mitragynine, crude extract, crude alkaloids and non-alkaloids on locomotor activity of mice in order to gain direct evidence supporting whether they are CNS stimulant or depressant or both.

CHAPTER II

EXPERIMENTAL

General Procedures

Melting point were determined by means of Buchi melting point apparatus.

Infrared spectra were registered in KBr pallets with Jasco IR-700 spectrophotometer.

Ultraviolet spectra were determined in ethanol on a Shimadsu UV-160 spectrophotometer.

^{1}H-NMR spectra were recorded on a Cryomagnetic for NMR Spectroscopy BZH 200/52 (200 MH_z) using tetramethylsilane(TMS) as the internal standard.

^{13}C-NMR spectra were recorded on a Cryomagnetic for NMR Spectroscopy BZH 200/52 (50.283 MH_z) using tetramethylsilane (TMS) as the internal standard.

Mass spectra were measured with JEOL-DX 300 mass spectrometer.

Thin layer chromatography was performed on 250x750x0.25 mm. layers of silica gel G60 F254 (E.Merck), activated at 100 C for an hour, and protectively stored in a desiccator.

Column chromatography was carried out on silica gel (E.Merck, 70-230 mesh). All solvents were distilled before chromatography.

Removal of solvents was effected using a Buchi rotary evaporator connected to a water pump. This chapter consists of three parts namely, preparation of mitragynine, preparation of crude extract, crude alkaloids and non-alkaloids and verification of animal locomotor activity.

1. Preparation of mitragynine

1.1 Plant Materials.

The fresh leaves of Kratom (Mitragyna speciosa Korth) were collected during August to December, 1990 from a flowering tree growing in the campus of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The plant materials were identified by comparison with the herbarium specimens in the Botany section, Technical Division, the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

1.2 Solvents and chemicals

- Absolute ethanol
- Acetic acid,glacial.
- Alumina F254 type E.
- 25% Ammonia solution.
- Anhydrous sodium sulfate
- Chloroform
- Diethyl ether
- 95% Ethanol
- Ethyl acetate
- n-Hexane
- Silica gel G60 (E.Merck, 70-230 mesh.)
- Silica gel G60 F254 (E.Merck)

1.3 Procedure

Two kilograms of dried powder obtained from the leaves of Mitragyna speciosa Korth. were exhaustively extracted with 95 % ethanol(20 L) for 3-5 days. The alcohol was removed under reduced pressure to give the crude extract(199.83 g). The crude extract (149.8 g) was dissolved in glacial acetic acid(400 ml) then distilled water was added to give 5 % acetic acid solution(8 L), well shaken and left to stand overnight. The acedic filtrate was alkalinized to pH 9 with 25 % ammonia solution and extracted with chloroform, until testing for complete extraction was carried

out with Dragendorff's reagent. The combined chloroform extract was washed with distilled water and then dried over anhydrous sodium sulfate and evaporated under reduced pressure to 200 ml. Then 10 % oxalic acid was added followed by well shaking and left for an hour. The precipitated was filtered by paper-filter No. 1 and washed with 10 ml diethyl ether followed by 10 ml distilled water. Then, it was dissolved with 5 % acetic acid and alkalinized to pH 9 with 25 % ammonia solution and extracted with chloroform. Testing for complete extraction with Dragendorff's reagent. The combined chloroform extract was washed with distilled water, then dried over anhydrous sodium sulfate and evaporated under reduced pressure to dry crude alkaloidal extract (5.87 g, Figure 6)

Crude alkaloidal extract(3.91 g.) was dissolved in small volume of chloroform(15 ml) and gently p laced on top of silica gel packed-column (5 x 50 cm). The column was eluted with a mixture of n-hexane and ethyl acetate(2:3), then washed with methanol until no traces of alkaloids was detected. Fractions of 50 ml were collected and compared each fraction by TLC. Those fractions with similar alkaloidal were combined and evaporated under reduced pressure giving the following fractions:

Fraction 1 - 13 containing no alkaloid.

Fraction 14 - 25 containing three alkaloids assigned as Fraction-M(2.893 g)

Fraction 25 .. containing at least 5 alkaloids.

Fraction-M (2.893 g) was dissolved in absolute ethanol and left for an hour until the colouress needle crystals precipitated. The crystals were purified by several recrystallization in absolute ethanol. The final yield of presumed mitragynine was approximately 2.5 grams which was assigned as substance M(Figure 7).

EXTRACTION OF ALKALOIDS

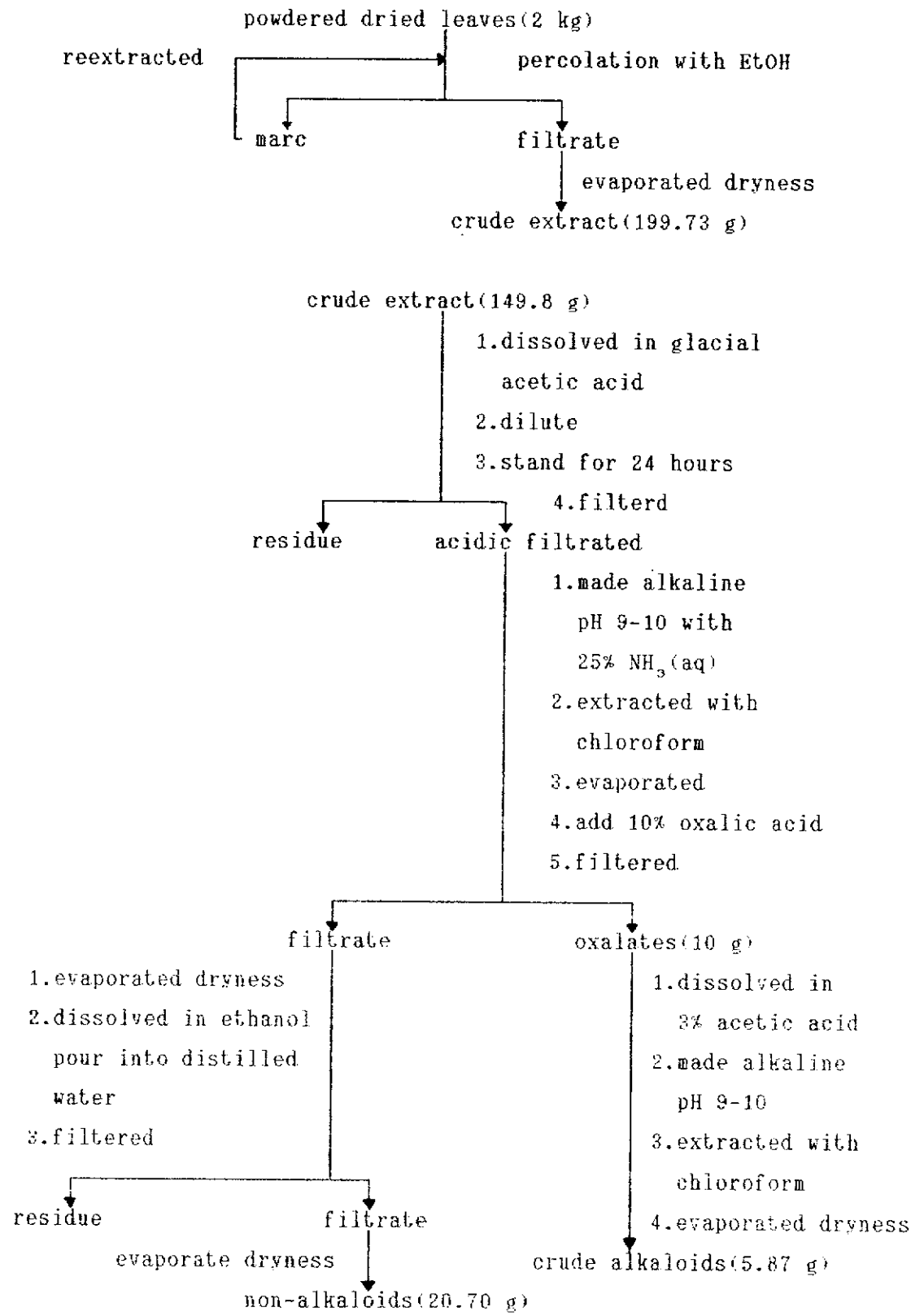


Figure 6 A step-wise procedure for the extraction of crude alkaloids and non-alkaloids from the dried leaves of Mitragyna speciosa Korth.

PURIFICATION

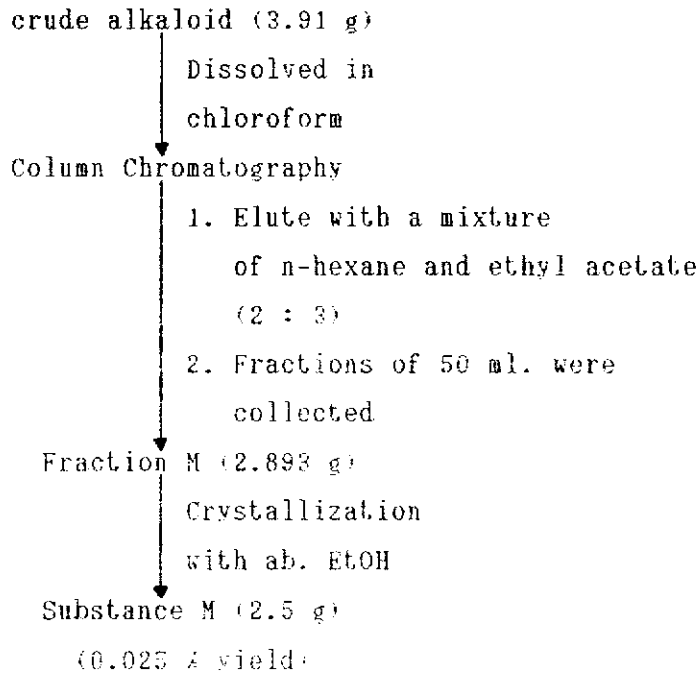


Figure 7 A step-wise procedure for the purification of mitragynine.

The substance M was dissolved in minimal amount of absolute ethanol. Then, the concentrated hydrochloric acid was added followed by diethyl ether. The solution was left until colourless needle crystalline was precipitated. The precipitated was purified by several recrystallization. The final yield of presumed mitragynine hydrochloride salt was approximately 1.875 grams which was assigned as substance M-HCl.

2. Preparation of crude extract, crude alkaloids and non-alkaloids.

Crude extract, crude alkaloids and non-alkaloids fraction were prepared in this study follwed as :

2.1 Crude extract

Crude extract(49.93 g.) obtained from the part I was dissolved in 95 % ethanol, then diluted to 1 % ethanol by distilled water. The solution was evaporated under reduced pressure to 1000 ml. (1 ml of the solution = 0.0499 g. of crude extract)

2.2 Crude alkaloids

Crude alkaloids(1.956 g.) obtained from the part I was dissolved in absolute ethanol (10 ml) then 3-5 drop of concentrated hydrochloric acid was added. The solution was evaporated under reduced pressure giving pale yellow amorphous solid. The process was repeated several times in order to get rid of hydrochloric acid which was checked by pH paper.

2.3 Non-alkaloids

The filtrate obtain from alkaloids precipitation was evaporated under reduced pressure until dry, giving dark brown residue. The residue was dissolved in 95 % ethanol followed by distilled water, then discarded the precipitation. The supernatant was evaporated under reduced pressure to give a brown residue(20.70 g.).

3. Verification of animal locomotor activity of substance M, crude extract, crude alkaloids and non-alkaloids.

3.1 Animals

Male swiss mices at 4-8 weeks of age and weight ranging between 30-40 grams were used in experiments. Each group of experiment employed 8 mices.

3.2 Instrument and chemical used

Polyethylene tube No.90, Syring(size 1 ml) with needle (size 26G), normal saline solution and 1 % ethanol.

3.3 Apparatus

Animal Locomotor Activity Monitoring system, consist of test chamber surrounded by sensors. The test chamber is square inshape (each side = 40 cm) and 20 cm in hight. The sensors consists of two single row of eight photoelectric cells and these cells will receive the light signals from the infrared cells(8 cells in a single row) from the opposite side of the chamber(Figure 8). The movement of animals in the chamber will be detected by the infrared light and this will be recorded by photoelectric cells at 5 minutes interval. The data was displayed by the casio printing calculator

3.4 Procedure

Substance M-HCl, crude extract, crude alkaloids and non-alkaloids fraction were administered into experimental mices through two routes, the oral and intraperitoneal. There are eight mices in each treatment, which performed within two-days period as followed:

3.4.1 Intraperitoneal

The day 1 : Mice was placed into the test chamber for 30 minutes, to get acquainted with the new environmental condition. Then, the mice was injected(Figure 9) with 0.3 ml normal saline solution and left for 90 minutes for normal locomotor activity recording. The number of activity counts were plotted against

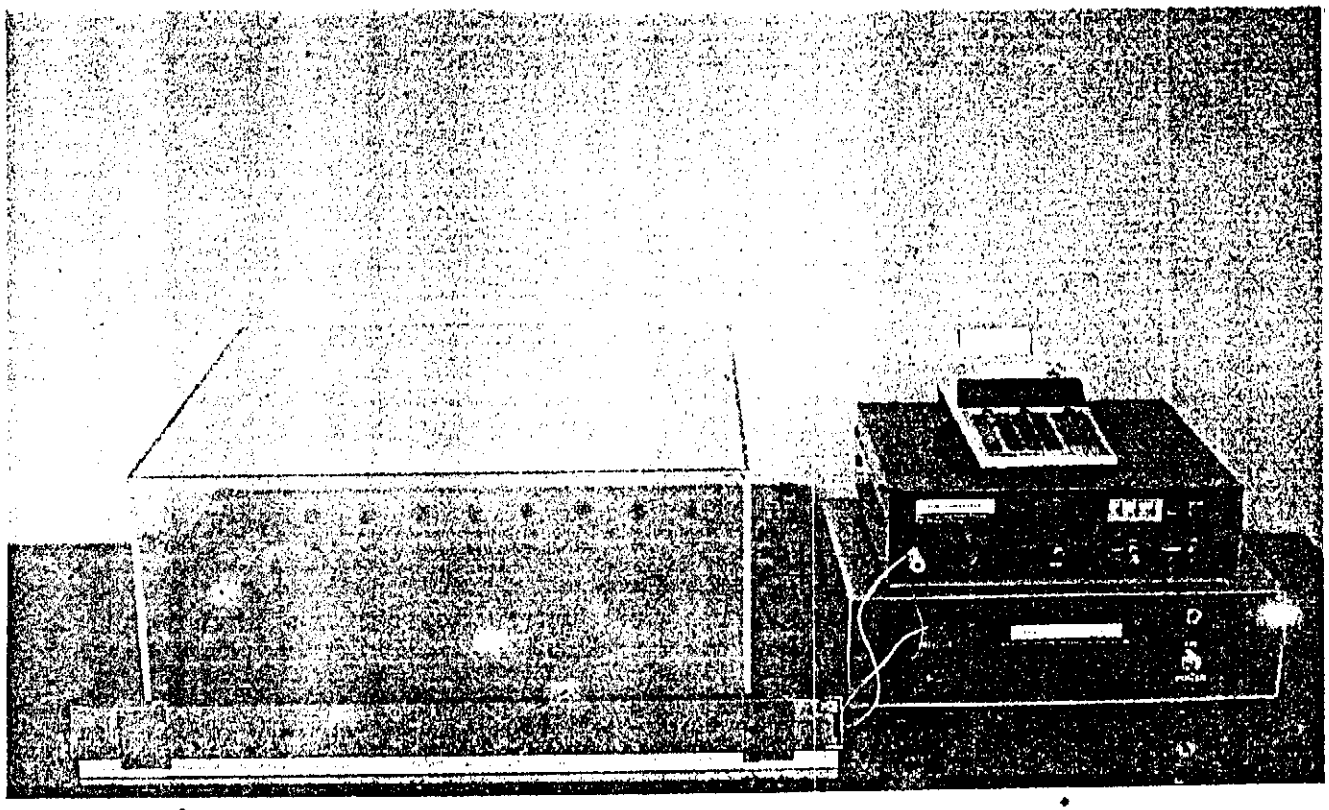


Figure 8 Photograph shows the animal locomotor activity monitoring system.

Figure 5 Photograph demonstrates the intraperitoneal injection of substance into experimental mice.

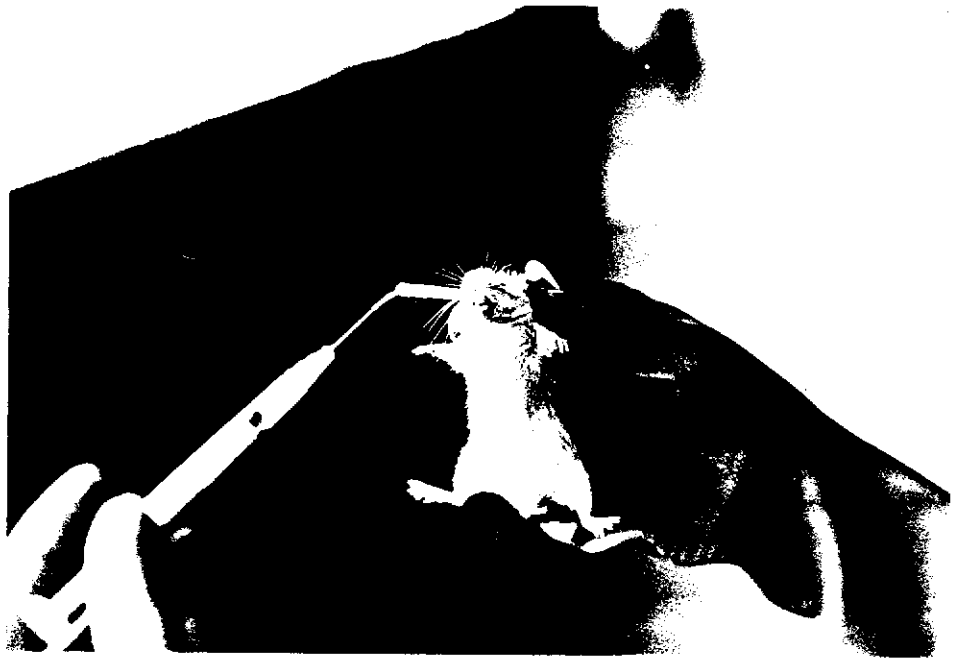


Figure 6 Photograph demonstrates the oral administration of substance into experimental mice.

time of 5 minutes intervals.

The day 2 : Mice was injected with 0.3 ml of substance M-HCl or crude extract or crude alkaloids or non-alkaloids at doses(20 or 50 or 100 or 150 or 200 mg/kg.body weight) instead of normal saline solution, and left for 90 minutes. The number of activity counts were plotted against time at 5 minutes intervals.

The crude extract used 1 % ethanol instead of normal saline solution. Experiments were performed during 10.00 am. to 4.00 pm. of each day. The experimental design was shown in table 2.

3.4.2 Oral Administration

The day 1 : Mice was place into the test chamber for 30 minutes, to get acquainted with the new environmental condition. Then inserted polyethylene tube into mouth until its tip reached the stomach(Figure 10). The injection was carried on first with 0.3 ml normal saline solution for recording normal locomotor activity. The number of activity counts were plotted against time at 5 minutes intervals.

The day 2 : Mice was injected with 0.3 ml of substance M-HCl or crude extract or crude alkaloids or non-alkaloids at doses 50 or 100 or 150 or 200 or 250 or 300 mg/kg.body weight instead of 0.3 ml normal saline solution, and left for 90 minutes. The number of activity counts were plotted against time at 5 minutes intervals.

For crude extract used 1 % ethanol instead of normal saline solution. The experimental design was shown in table 3.

Table 2 Table showing 8 mice in each group receiving different treatments at five doses through intraperitoneal route.

Treatments	Doses(mg/kg.body weight)				
	20	50	100	150	200
substance M-HCl	8	8	8	8	8
crude extract	8	8	8	8	8
crude alkaloids	8	8	8	8	8
non-alkaloids	8	8	8	8	8

Table 3 Table showing 8 mices in each group receiving different treatments at five doses through oral administration.

Treatment	Doses(mg/kg.body weight)					
	50	100	150	200	250	300
substance M-HCl	8	8	8	8	8	8
crude extract	8	8	8	8	8	8
crude alkaloids	8	8	8	8	8	8
non-alkaloids	8	8	8	8	8	8

3.5 Statistic analysis

Comparison between control and treated groups were calculated by the Wilcoxon match pair test. Comparisons among doses of each substance were calculated by the Kruskal wallis test and comparisons among high dose of each the substance were calculated by the Kruskal wallis test. And comparison between intraperitoneal and oral administration were calculated by Mann whitney U' test. All of the methods to were analyzed at a P value of 0.05 (two-side) which was considered as singnificant difference.

CHAPTER III

RESULT

I. Preparation of mitragynine, crude extract, crude alkaloids and non-alkaloids.

Two kilograms of Mitragyna speciosa Korth. dried leaves were first extracted with 95 % ethanol, crude extract was acidified with glacial acetic acid followed by alkalanization with 25 % ammonia solution and extracted with chloroform giving crude alkaloids and non-alkaloids. The crude alkaloids was processed by column chromatographic technique giving the substance M, presumably mitragynine.

The final yields of substance M, crude extract, crude alkaloids and non-alkaloids of dried sample are 2.5 g, 199.83 g, 5.87 g and 20.70 g respectively.

The substance M was further reacted with concentrated hydrochloric acid giving 75 % yield of substance M-HCl which is the colouress needle crystals.

The physical and spectroscopic characteristics of substance M evaluated as followed:

The substance M has melting point (m.p.) at 95-98 °C. The Rf value showed at 0.72, 0.65 and 0.82 when using a mixture of n-hexane and ethyl acetate (2:3), chloroform, and a mixture of chloroform and ethanol (9:1) as solvent systems respectively and using silica gel G60 F254 as adsorbent. However the Rf showed at 0.65 and 0.73 when using a mixture of n-hexane and ethyl acetate (1:1), and diethyl ether as solvent system, respectively and using alumina F254 as adsorbent. UV λ_{max} (EtOH) : 224($\log \varepsilon$ = 4.70) and 291.5($\log \varepsilon$ = 3.97) nm. (Figure 11); IR ν_{max} (KBr) : 3300(N-H),

3000-2900(C-H), 1700(C=O,ester) and 1625(C=C) cm^{-1} (Figure 12); ^{1}H NMR ($CDCl_3$) δ value (ppm) : 0.8(3H, t, H-18, J = 7.0 H_z), 1.2 (1H, t, H-20, J = 7.0 H_z), 1.75(3H, m, H-14, H-19), 2.5(3H, m, H-6, H-14, H-15), 3.0(6H, m, H-3, H-5, H-6, H-21), 3.7(6H, s, 9-OCH_3, 17-OCH_3), 3.9(3H, s, 22-OCH_3), 6.5(1H, d, H-10, J = 7.26 H_z), 6.9(1H, d, H-12, J = 7.44 H_z), 7.0(1H, t, H-11, J = 7.72 H_z), 7.4(1H, s, H-17) and 7.9(1H, s, -NH) ppm. (Figure 13); ^{13}C NMR ($CDCl_3$) δ value (ppm) : 12(C-18), 18(C-19), 23(C-6), 29(C-14), 39(C-15), 40(C-20), 50(C-23; -OCH_3), 52(C-5), 54(C-9; -OCH_3), 57(C-21), 60(C-3), 61(C-17; -OCH_3), 98(C-10), 103(C-12), 107 (C-8), 111(C-16), 117(C-7), 120(C-11), 132(C-2), 136(C-13), 153 (C-9), 160(C-17) and 169(C-22) ppm. (Figure 14); Mass spectra : m/z (rel) M^{+} 398(28.87), 397(24.07), 269(9.63), 200(7.72), 199 (7.19), 186(8.30), 129(6.88) and 75(10.68)(Figure 15).

The physical and spectroscopic characteristics of substance M-HCl has melting point at 238-243 °C and the Rf values showed at 0.65 when using a mixture of n-hexane and ethyl acetate (2:3) as a solvent system and using silica gel G60 F254 as adsorbent. However, the Rf values showed at 0.7 when using a mixture of chloroform and ethanol (9:1) as solvent system and using alumina F254 as adsorbent. UV λ_{max} (EtOH) : 223(log ε = 4.71) and 291.2(log ε = 3.96) nm.(Figure 16); IR ν_{max} (KBr) : 3400(N-H,broad), 3200-2900(C-H), 1700(C=O) and 1625(C=C) cm^{-1} (Figure 17); ^{1}H NMR (D_2O) δ value (ppm) : 0.7(3H, q, H-18, J = 6.98 H_z), 1.3(1H, m, H-20, H-14), 1.9(2H, m, H-19), 2.3(3H, m, H-14, H-15, H-6), 3.0(6H, m, H-3, H-5, H-6, H-21), 3.5(3H, s, 9-OCH_3), 3.6(3H, s, 17-OCH_3), 3.7(3H, s, 22-OCH_3), 6.4(1H, d, H-10, J = 7.3 H_z), 6.9(2H, m, H-11, H-12), 7.4(1H, s, H-17) (Figure 18); ^{13}C NMR (D_2O) δ value (ppm) : 11(C-18), 19(C-19), 23(C-6), 26(C-14), 36(C-15), 38(C-20), 51(C-23; -OCH_3), 52.5(C-5), 53(C-21), 55(C-9; -OCH_3), 62(C-3), 62.5(C-17; -OCH_3), 103(C-10), 106(C-12), 114(C-7), 123(C-8), 126(C-16), 127(C-11), 137(C-13),

153(C-9), 162(C-17), 169(C-2), 197.5(C-22; C=O)(Figure 19).

The chemical structure of substance M and substance M-HCl were identified by pattern of spectroscopy spectra and comparing to those of previous works.(Beckett,1965 and Keawpradub,1990)

Table 4 Spectroscopic and physical characteristics of substance M, substance M-HCl and mitragynine obtained from the previous work (Beckett,1965 and Keawpradub, 1990)

type of spectrum	substance M	substance M-HCl	mitragynine (Beckett,1965)	mitragynine (Keawpradub, 1990)
Melting point (°C)	95-98	240-244	97-98	93-95
UV λ_{max} (nm.)	in EtOH 224 (log = 4.70) 291.5 (log = 3.97)	in EtOH 223 (log = 4.71) 291.2 (log = 3.96)	in EtOH 226.8 (log = 4.69) 291 (log = 3.96)	in EtOH 224.2,291.5
IR ν_{max} (cm^{-1})	in KBr 3300(NH) 3000-2900(CH) 1700(C=O) 1625(C=C)	in KBr 3400(NH) 3200-2900(CH) 1700(C=O) 1625(C=C)	in nujol 3240(NH) 1642(C=C) 1275(C-O) 1240(C-O)	in KBr 3460(NH) 3000-2900(C-H) 1690(C=O) 1625(C=C)

Table 4 (cont.)

type of spectrum	substance M	substance M-HCl	mitragynine (Beckett, 1965)	mitragynine (Keawpradub, 1990)
^{1}H NMR δ values (ppm.)	in $CDCl_3$ (200 MH_z)	in D_2O (200 MH_z)	in $CDCl_3$ (60 MH_z)	in $CDCl_3$ (500 MH_z)
	7.9(1H,s,NH)	7.4(1H,s,H-17)	6.9(1H,s,NH)	7.7(1H,s,NH)
	7.4(1H,s,H-17)	6.9(2H,m,H-11, H-12)	6.4(4H,m, aromatic)	7.42(1H,s,H-17)
	7.0(1H,t,H-11)	6.5(1H,d,H-10)	3.8(3H,s, 22-OCH_3)	6.98(1H,t,H-11)
	J = 7.72 H_z	J = 7.3 H_z	3.6(6H,s,9-	J = 8.0 H_z
	6.9(1H,d,H-12)	3.7(3H,s,22-OCH_3)	-OCH_3;17-OCH_3)	6.88(1H,d,H-12)
	J = 7.44 H_z	3.6(3H,s,9-OCH_3)	1.1(2H,t,H-19)	J = 8.0 H_z
	6.5(1H,d,H-10)	3.5(3H,s,17-OCH_3)	0.8(3H,t,H-18)	6.45(1H,d,H-10)
	J = 7.26 H_z	3.0(6H,m,H-3,H-5, H-6,H-21)		J = 7.7 H_z
	3.9(3H,s,22-OCH_3)	2.4(3H,m,H-14, H-15,H-6)		3.9(3H,s,22-OCH_3
	3.7(6H,s,9-OCH_3; 17-OCH_3)	1.9(2H,m,H-19)		3.7(3H,s,9-OCH_3)
	3.0(6H,m,H-3,H-5, H-6,H-21)	1.3(2H,m,H-14, H-20)		3.6(3H,s,9-OCH_3)
	2.5(3H,m,H-14, H-15,H-6)	0.7(3H,q,H-18)		3.15(1H,d,H-3)
	1.75(3H,m,H-14, H-19)	J = 6.98 H_z		J = 12.0 H_z
	1.2(1H,t,H-20)			3.11(1H,t,H_α-5)
	J = 7.0 H_z			J = 12.0 H_z
	0.8(3H,t,H-18)			3.04(1H,d,H_β-5)
	J = 7.0 H_z			J = 12.0 H_z
				2.98(1H,d,H_α-21
				J = 12.0 H_z
				2.92(2H,m,H_β-6, H_β-21)
				2.53(2H,m,H_α-6, H-15)
				2.46(1H,t,H_β-14)
				J = 12.0 H_z

Table 4 (cont.)

type of spectrum	substance M	substance M-HCl	nitragynine (Beckett, 1965)	nitragynine (Keawpradub, 1990)
				1.78(2H,m,H-19)
				1.61(1H,d,H_{α}-14)
				J = 10.2 H_z
				1.19(1H,m,H-20)
				0.87(3H,t,H-18
				J = 7.2 H_z
^{13}C NMR	in $CDCl_3$	in D_2O		in $CDCl_3$
	50.283 MH_z	50.283 MH_z		67.8 MH_z
δ values	169(C-22),160	197.5(C-22),169		169(C-22),160.5
(ppm.)	(C-17),153(C-9)	(C-2),162(C-17)		(C-17),154(C-9)
	136(C-13),132	153(C-9),137		137(C-13),133.7
	(C-2),120(C-11)	(C-13),127(C-11)		(C-2),122(C-11)
	117(C-7),111	126(C-16),123		118(C-7),111.54
	(C-16),107(C-8)	(C-8),114(C-7)		(C-16),108(C-8)
	103(C-12),98	106(C-12), 103		104(C-12),99.74
	(C-10),61(C-17;	(C-10),62.5(C-17;		(C-10),61(C-17;
	$-OCH_3$),60(C-3)	$-OCH_3$),62(C-3)		$-OCH_3$),61(C-3)
	57(C-21),54(C-9;	55(C-9; $-OCH_3$,,		58(C-21),55(C-9;
	$-OCH_3$),52(C-5)	53(C-21), 52.5		$-OCH_3$),54(C-5)
	50(C-23;$-OCH_3$)	C-17; $-OCH_3$)		51(C-23;$-OCH_3$)
	40(C-20),39	51(C-23;$-OCH_3$)		41(C-20),39.95
	(C-15),29(C-14)	38(C-20),36		(C-15),30(C-14)
	23(C-6),18(C-19)	(C-15),26(C-14)		24(C-6),19(C-19)
	12(C-18)	23(C-6),19(C-19)		12.87(C-18)
		11(C-18)		

Table 4 (cont.)

type of spectrum	substance M	substance M-HCl	mitragynine (Beckett, 1965)	mitragynine (Keawpradub, 1990)
Mass spectra m/z (rel)	M^+ 398(28.87), 397(24.07), 269 (9.63), 200(7.72) 199(7.19), 186 (8.30), 129(6.88) 75(10.68)			M^+ 398(85.88), 397(79.07), 269 (23.24), 200 (27.47), 199 (21.30), 186 (26.29), 170 (5.42), 75(8.28) 28(5.00)

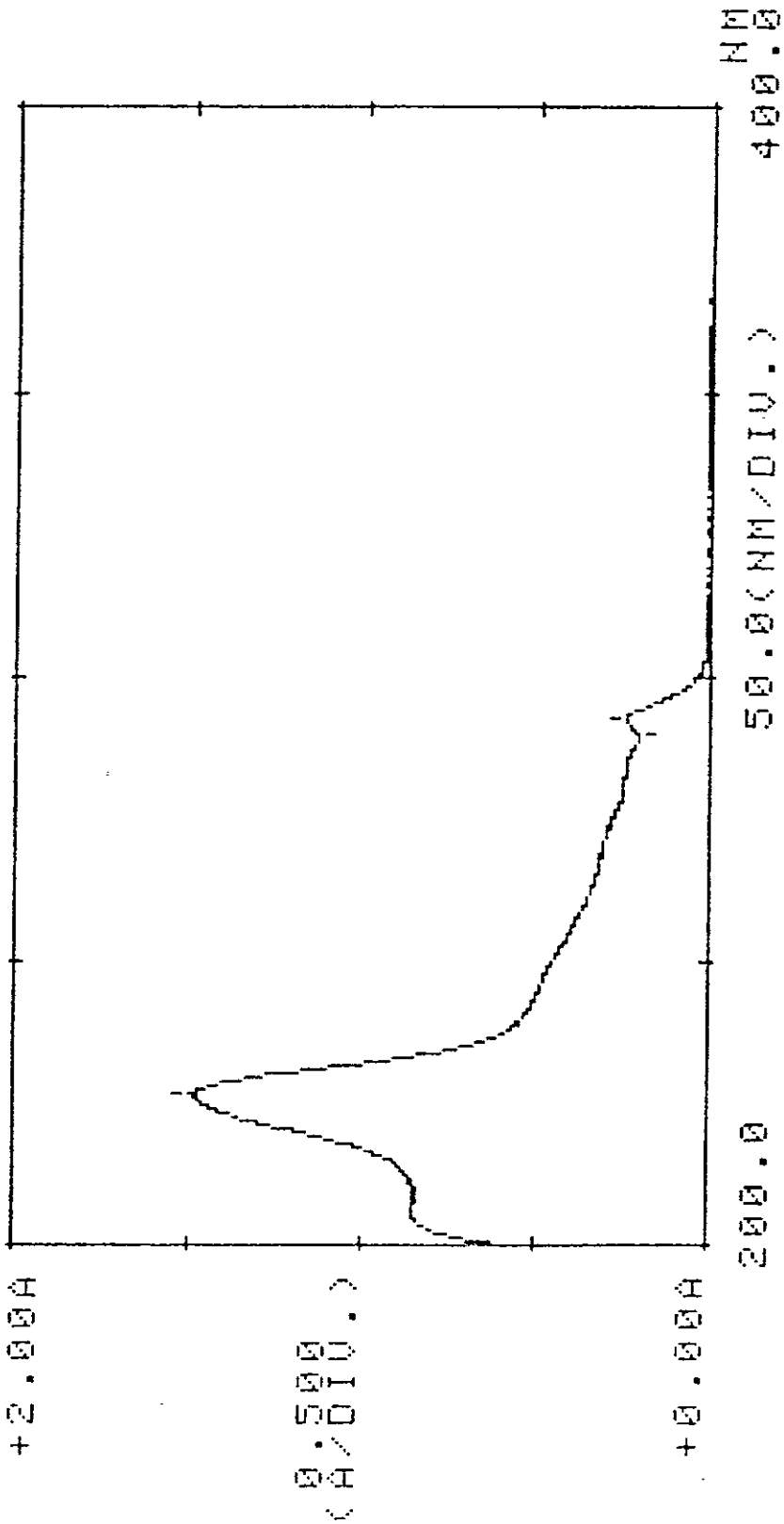


Figure 11 UV absorption spectrum of substance M in ethanol.

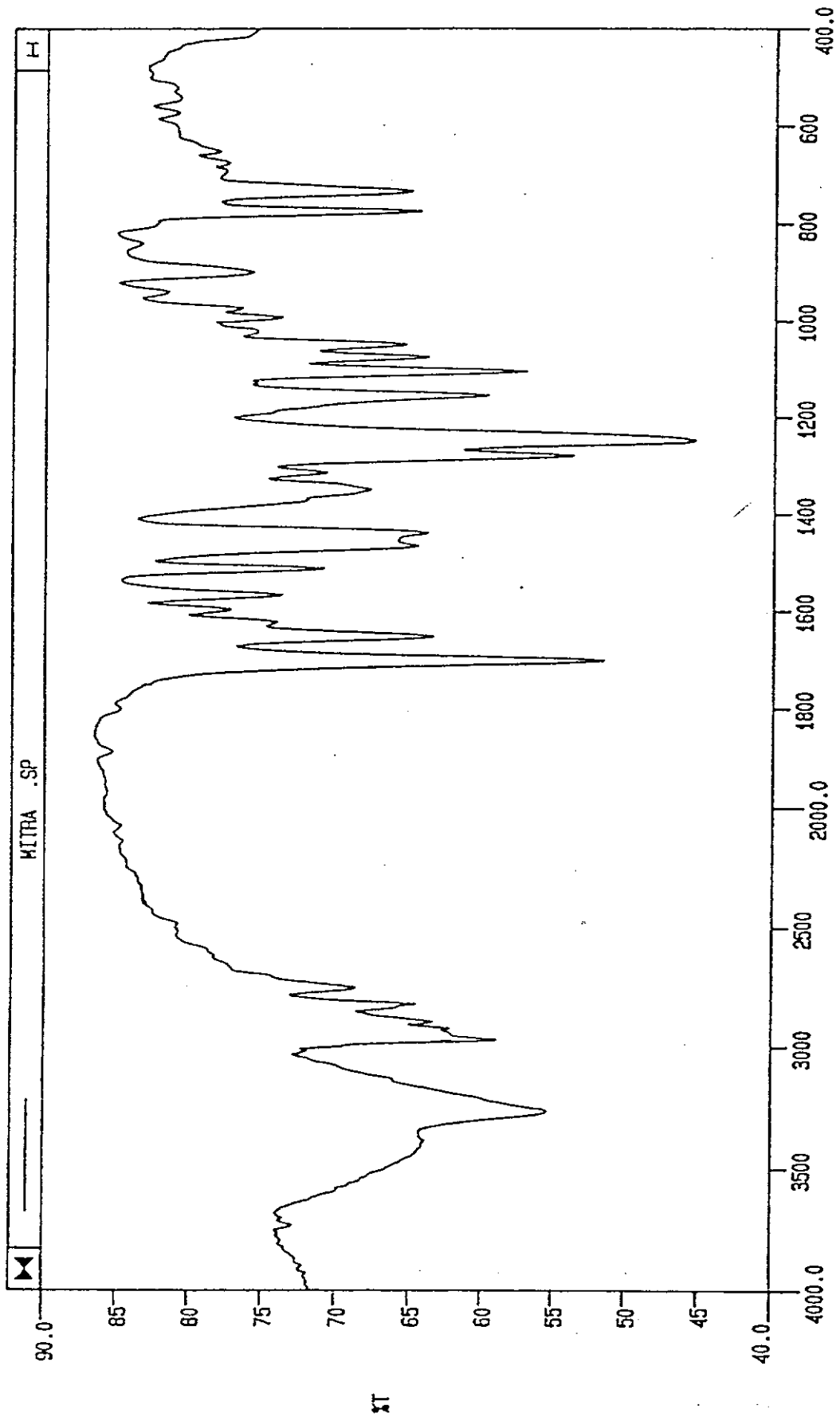


Figure 12 IR absorption spectrum of substance M in KBr pellet.

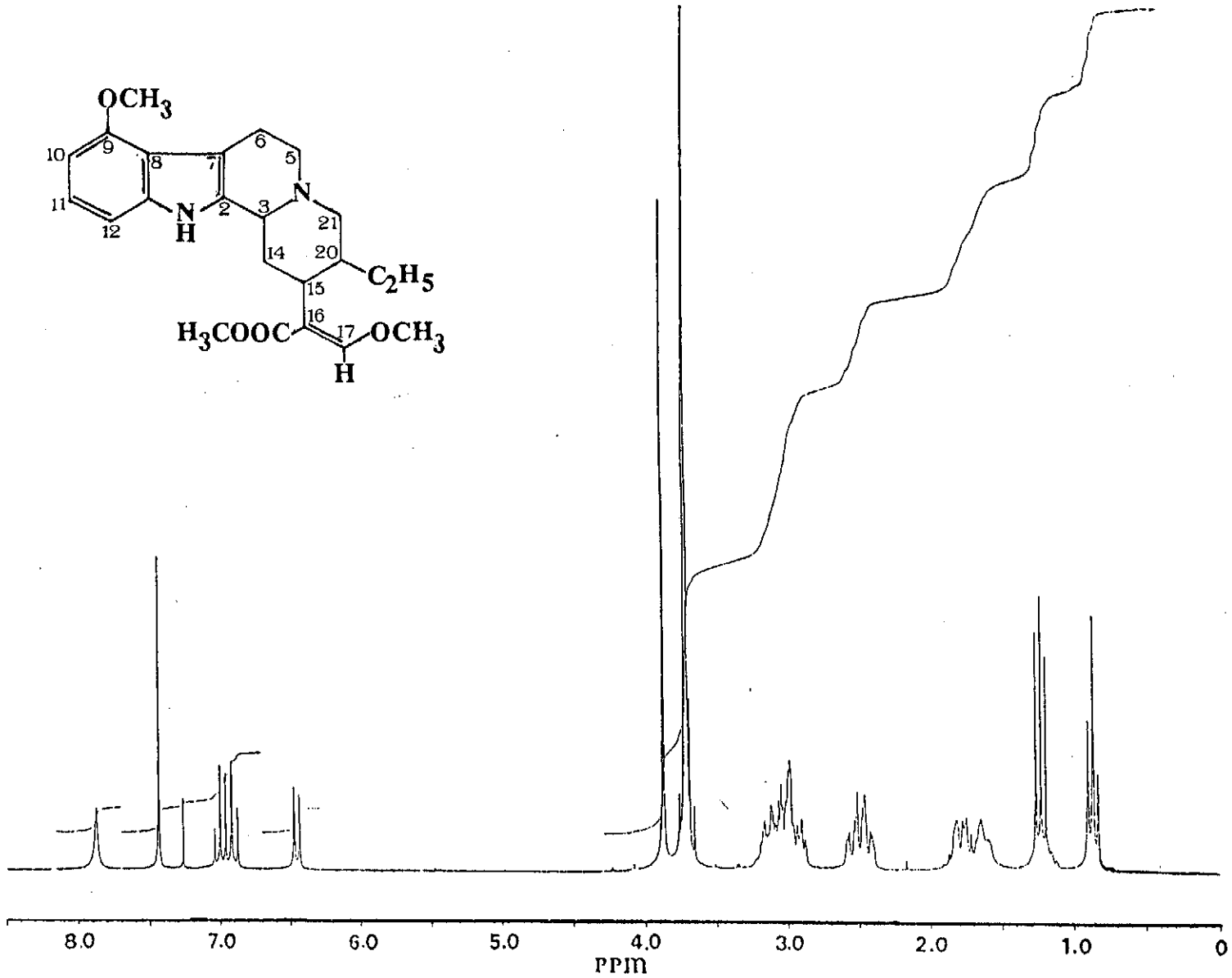


Figure 13 ^{1}H NMR spectrum of substance M in $CDCl_3$.

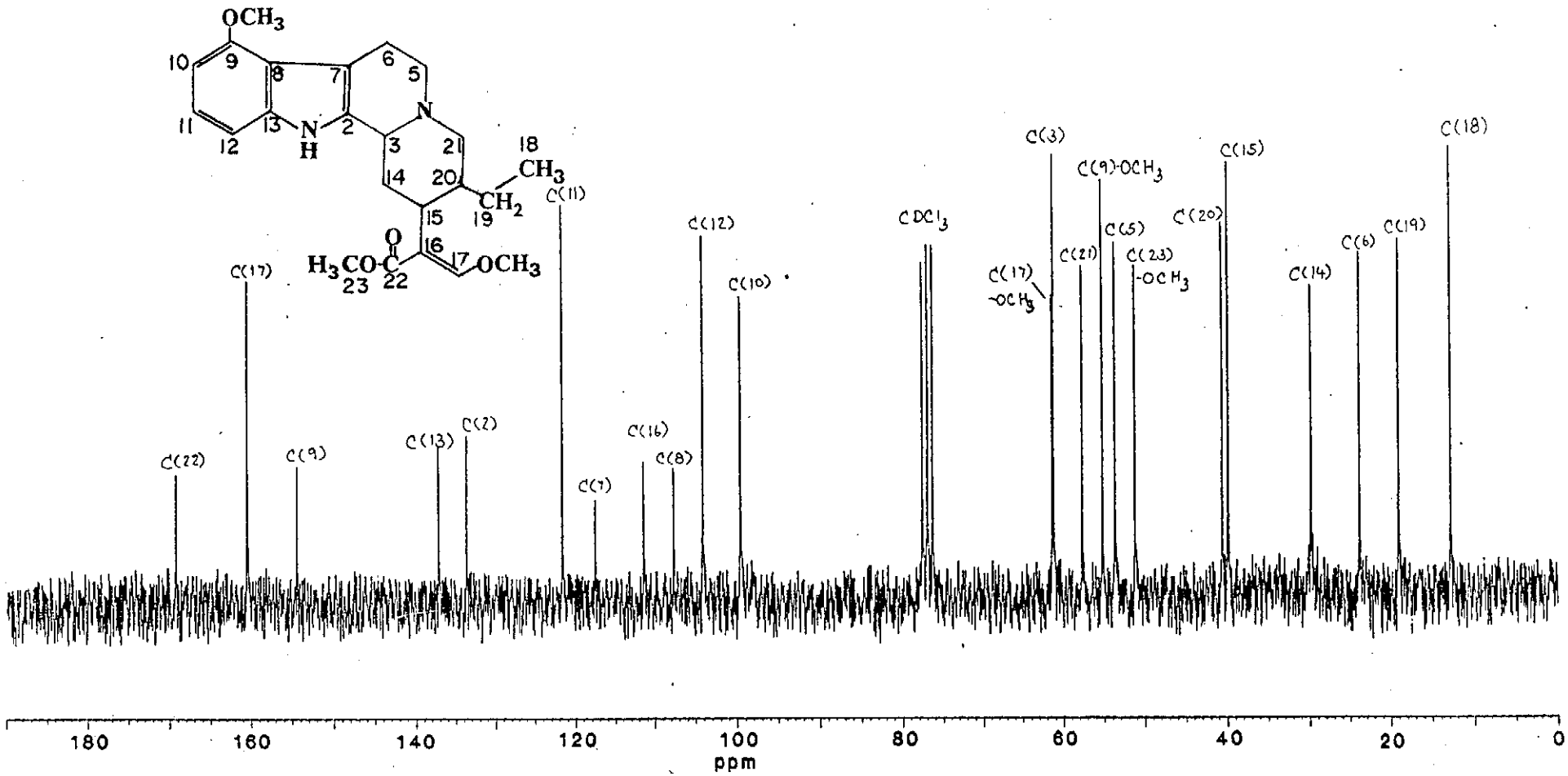


Figure 14 ^{13}C NMR spectrum of substance M in $CDCl_3$.

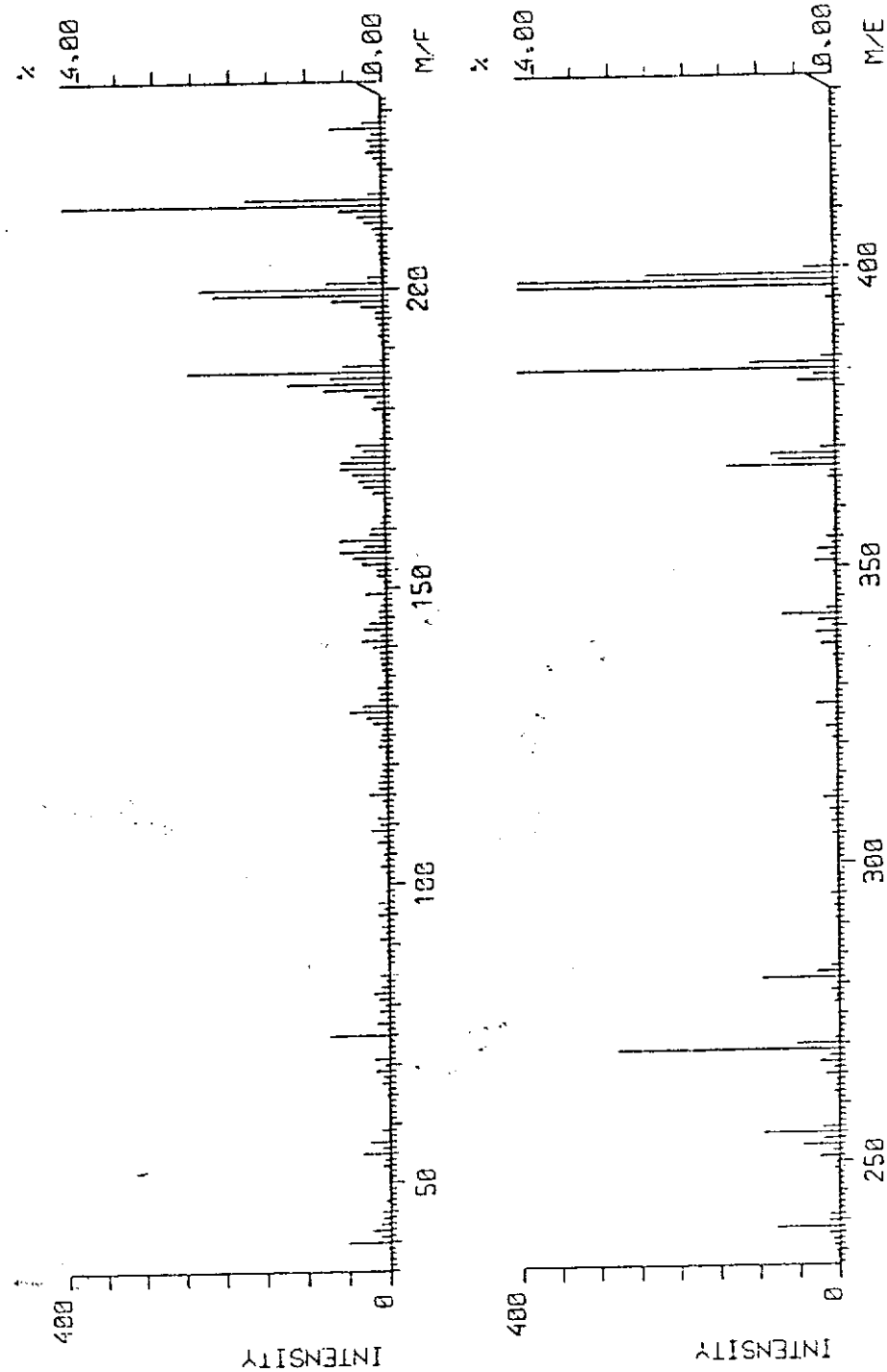


Figure 15 Mass spectrum of substance M.

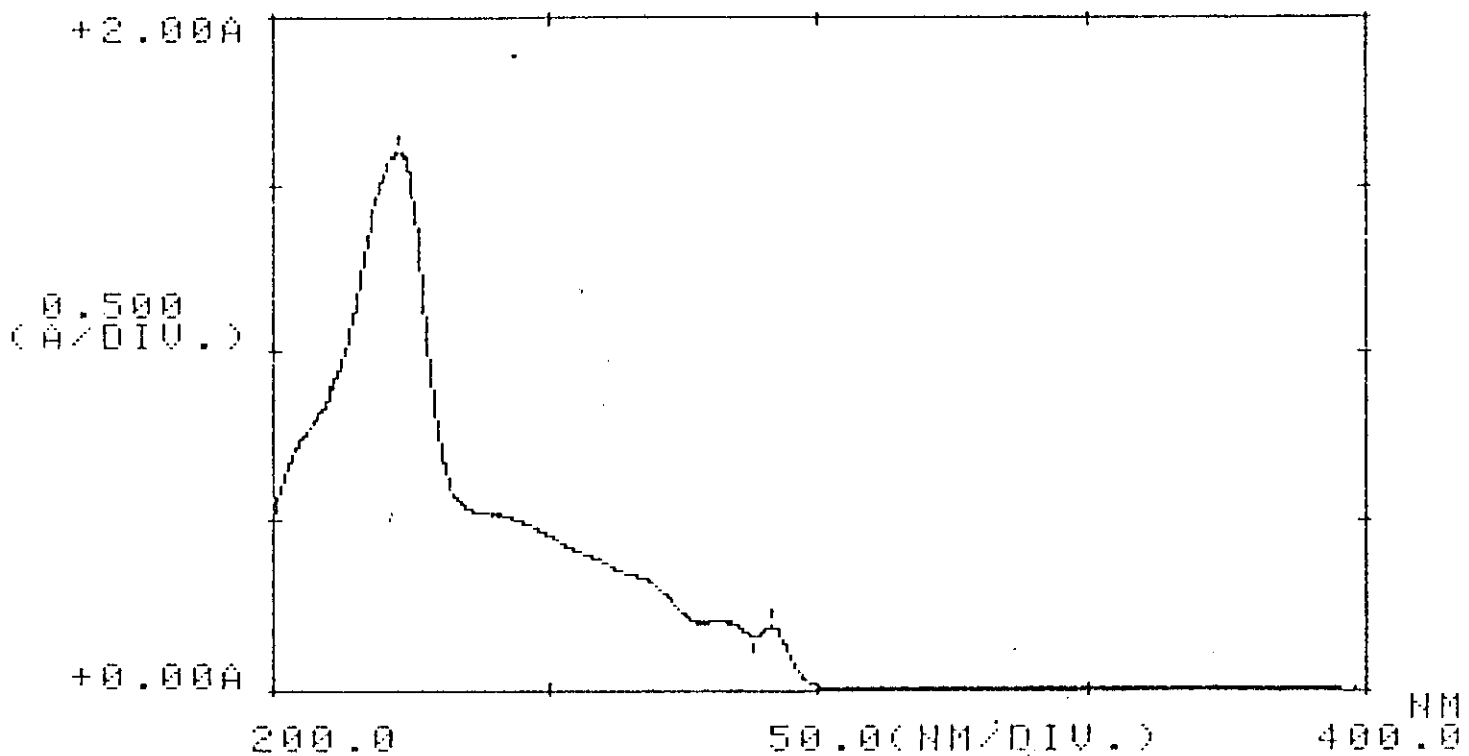


Figure 16 UV absorption spectrum of substance M-HCl in ethanol.

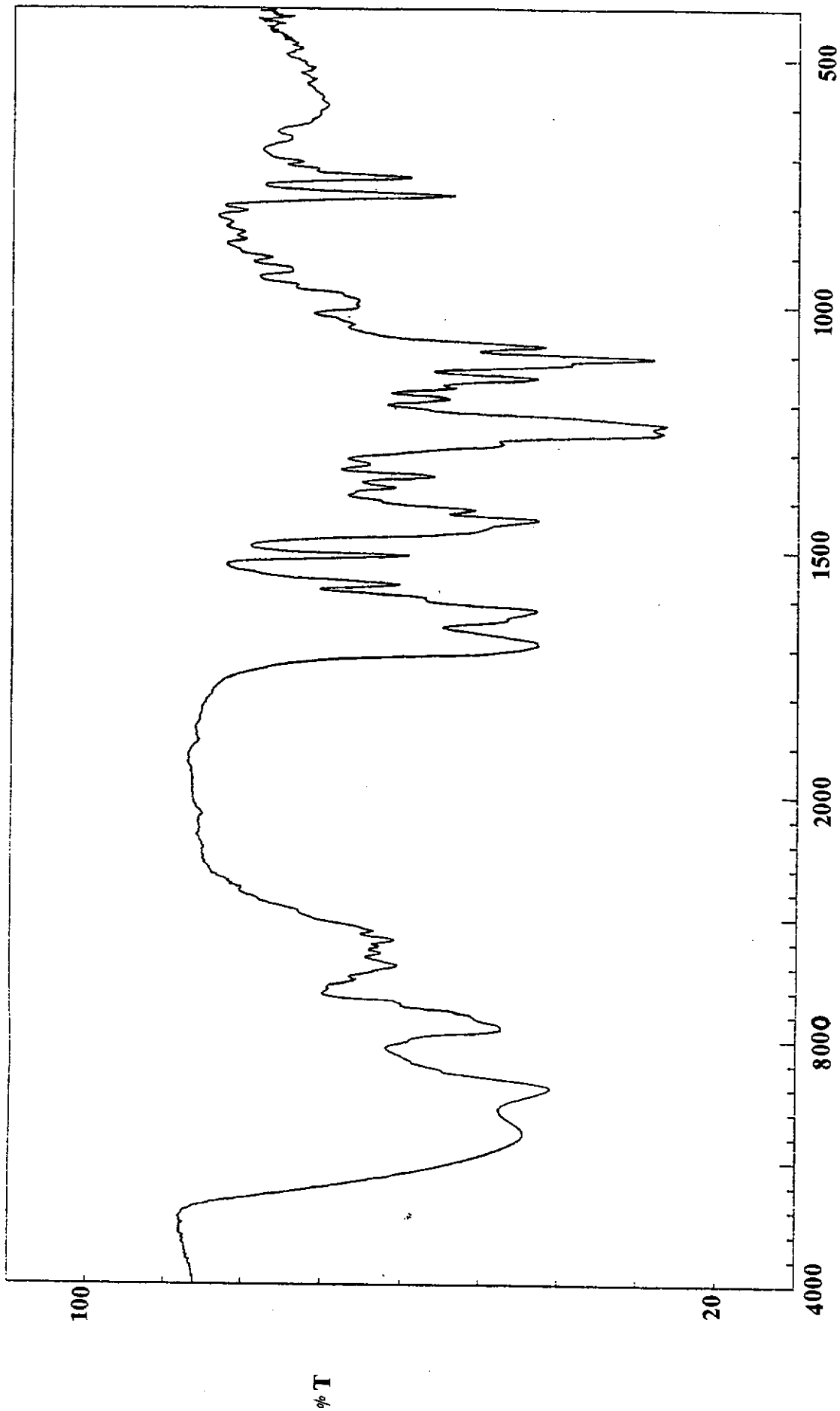


Figure 17 IR absorption spectrum of substance M-HCl in KBr pellet.

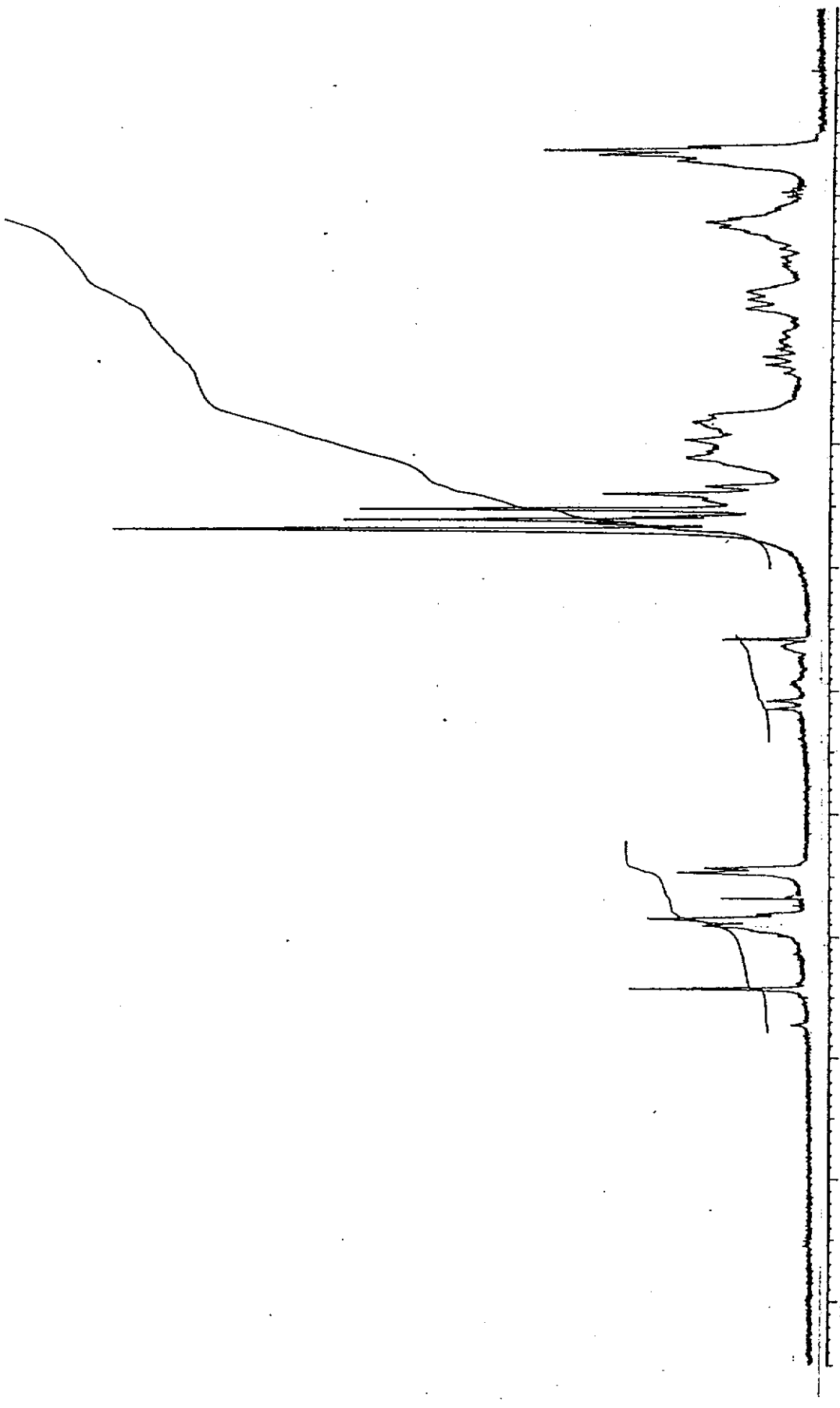


Figure 18 ^{1}H NMR spectrum of substance M-HCl in D_2O.

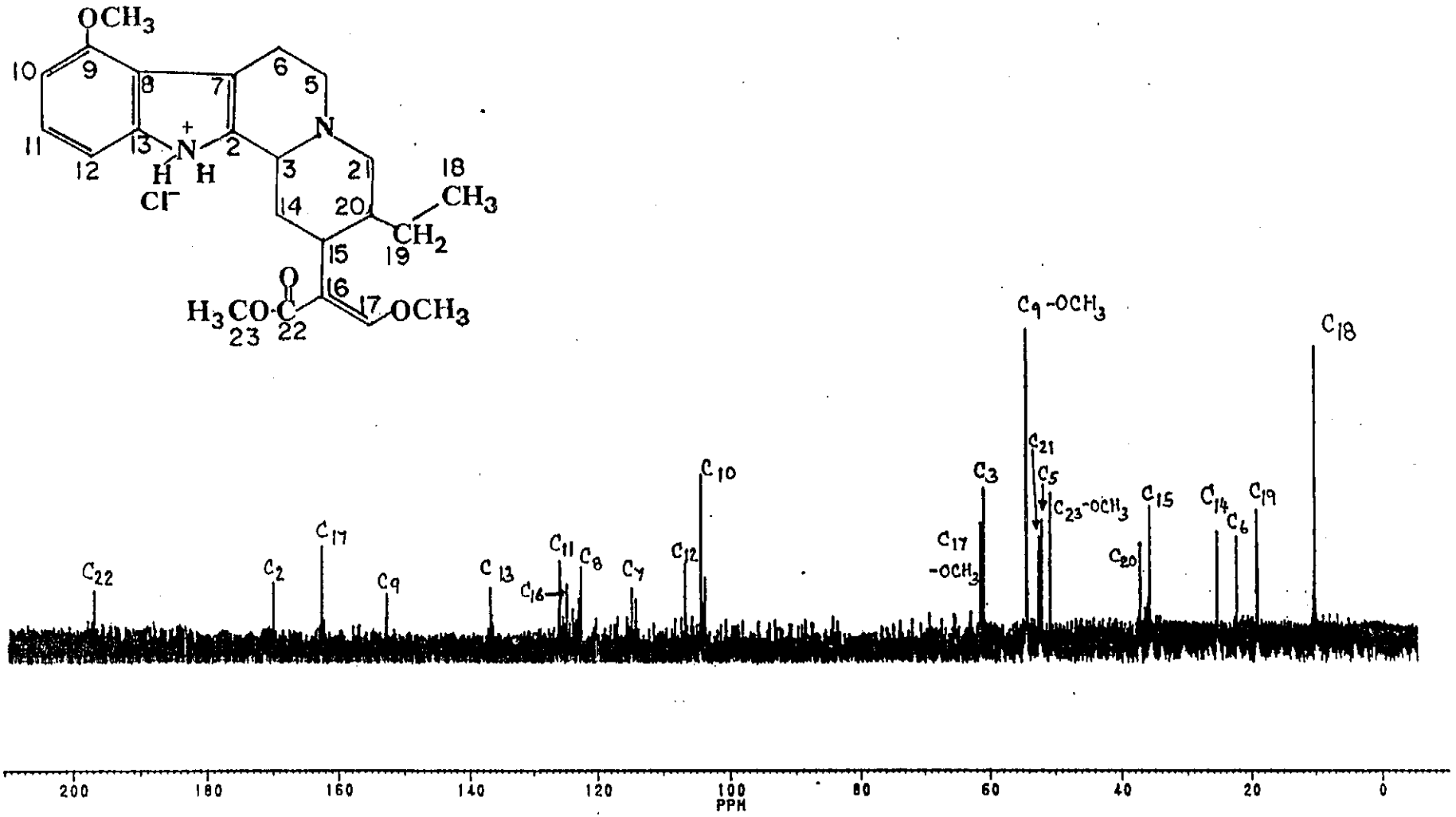


Figure 19 ^{13}C NMR spectrum of substance M-HCl in D_2O

II. Verification of mitragynine, crude extract, crude alkaloids and non-alkaloids on locomotor activity in mice after intraperitoneal injection or oral administration.

1. Intraperitoneal injection

1.1 Mitragynine hydrochloride

Mices were received substance M-HCl at doses 20, 50, 100, 150 and 200 mg/kg.body weight. AT dose 20 mg/kg.body weight, the substance M-HCl did not have any effect on the experimental animals. The mean locomotor activity of mice after receiving the substance were not significant different from those obtained at normal saline treatment at every periods; 30, 60 and 90 minutes after treatment(Figure 20, 21). The substance M-HCl strated suppressing the locomotor activity of experimental mice at dose higher than 20 mg/kg.body weight. The higher dose increased, the greater effect. The effects of the substance M-HCl would be steady after treatment at dose higher than 150 mg/kg.body weight(Figure 26).

At dose 50 mg/kg.body weight, the substance M-HCl decreased the locomotor activity of mice during the first thirty-minute period after treatment(Figure 22). This was significant different from those obtained at normal saline treatment(Figure 20). The effect was first observed at 2-3 minutes after treatment by a decrease in movement and slightly extension of body and limbs. However, it did not show the significant effect during the second and third thirty-minute periods after treatment(Figure 20, 22).

At dose 100 mg/kg.body weight, the substance M-HCl was clearly observed to have the effects on mice at the first and second thirty-minute periods after treatment(Figure 23). The mean locomotor activity obtained during the two periods were significant different from those of normal saline treatment (Figure 20). Likewise, the mice were nearly immobile and show

extension of body and limbs at 2-3 minutes after treatment.

At dose 150 and 200 mg/kg.body weight, the locomotor activity was significant reduced at 2-3 minutes after treatment and prolonged through the 90-minute periods (Figure 24, 25). The mean locomotor activities were significant different in all three thirty-minute periods from those of normal saline treatment(Figure 20). The spasm of limbs muscles were observed and prevented the movement of the animal. The extension of body and limbs were also demonstrated.

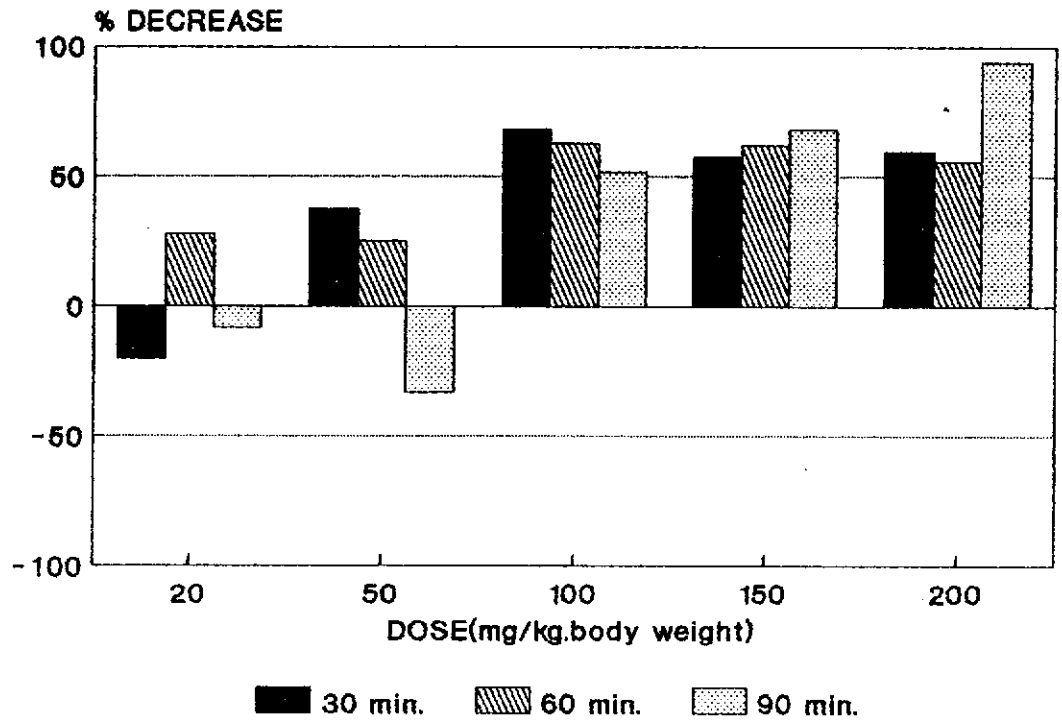


Figure 20 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and mitragynine treatment at doses 20, 50, 100, 150 and 200 mg/kg.body weight.

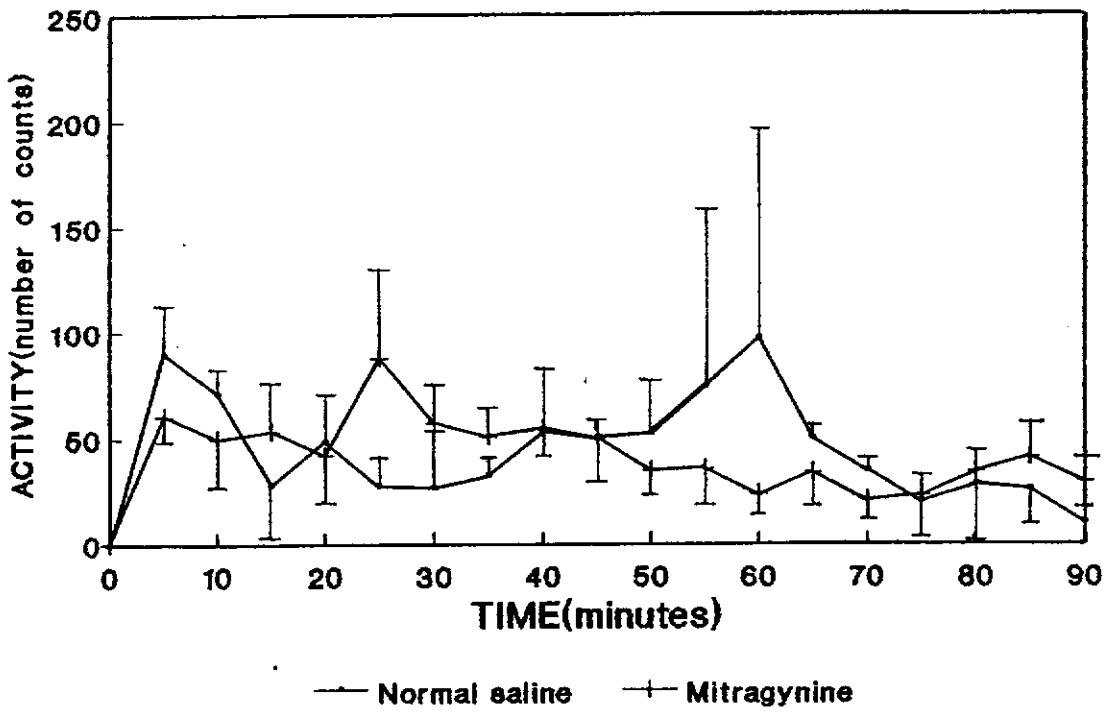


Figure 21 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 20 mg/kg.body weight) treatment through intraperitoneal injection.

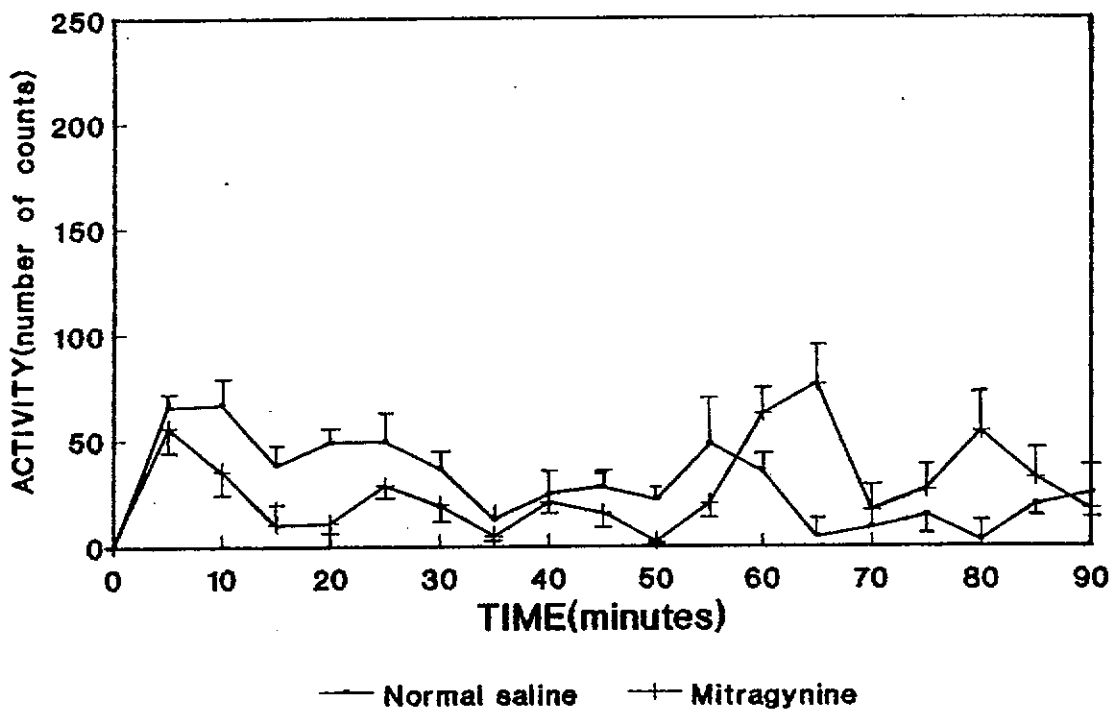


Figure 22 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 50 mg/kg.body weight) treatment through intraperitoneal injection.

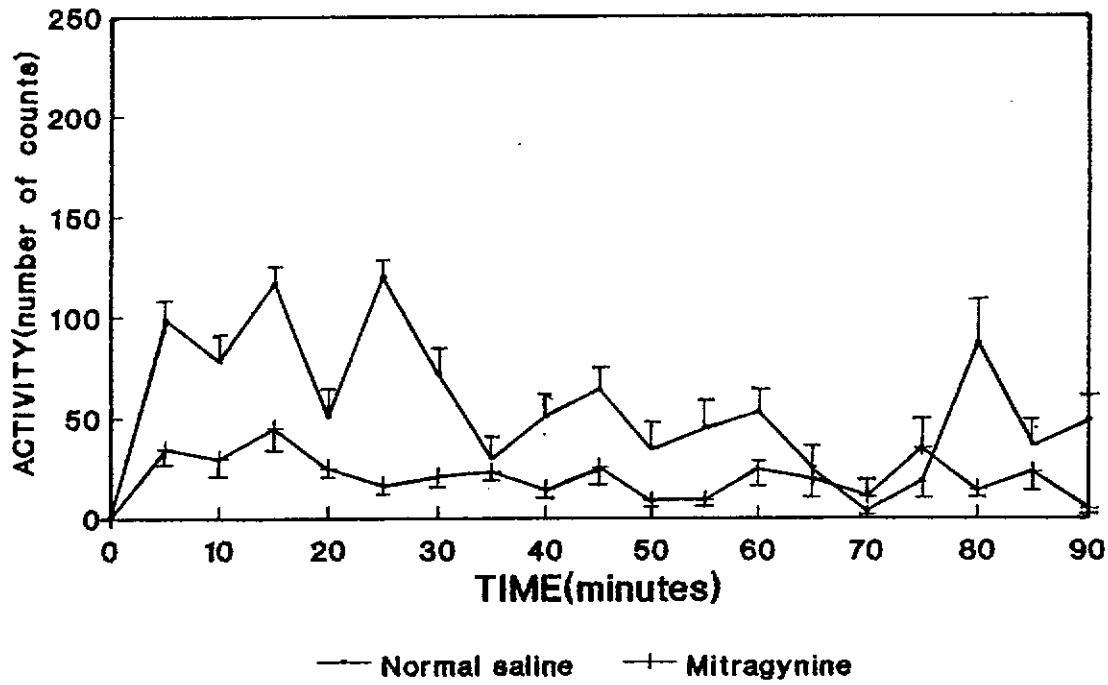


Figure 23 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 100 mg/kg.body weight) treatment through intraperitoneal injection.

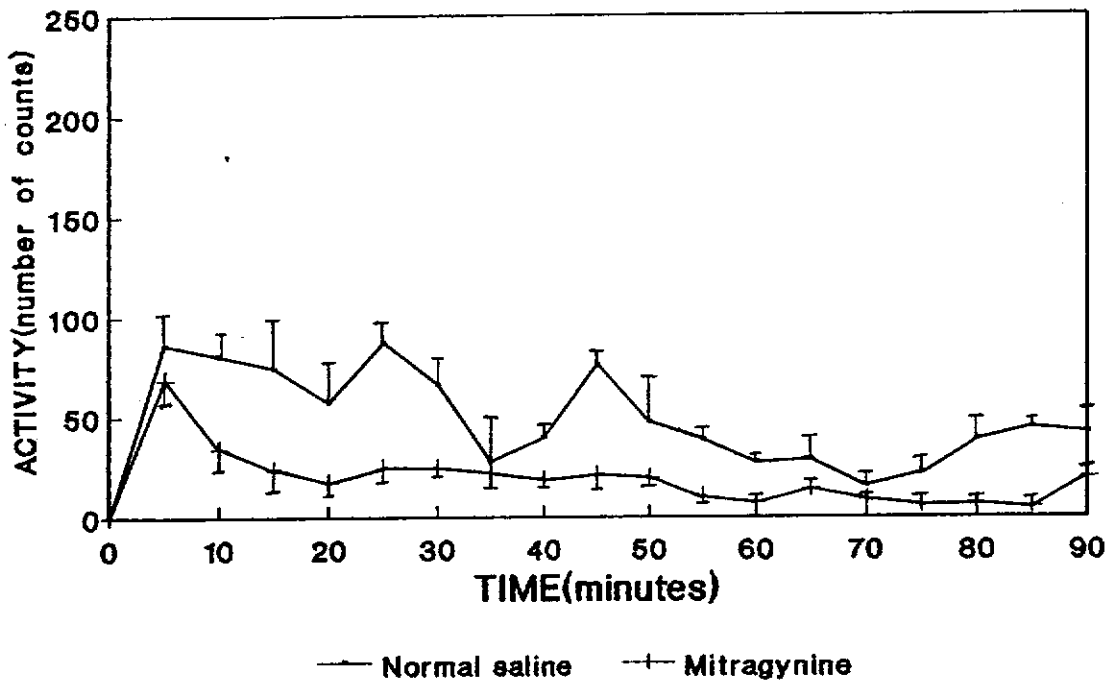


Figure 24 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 150 mg/kg.body weight) treatment through intraperitoneal injection.

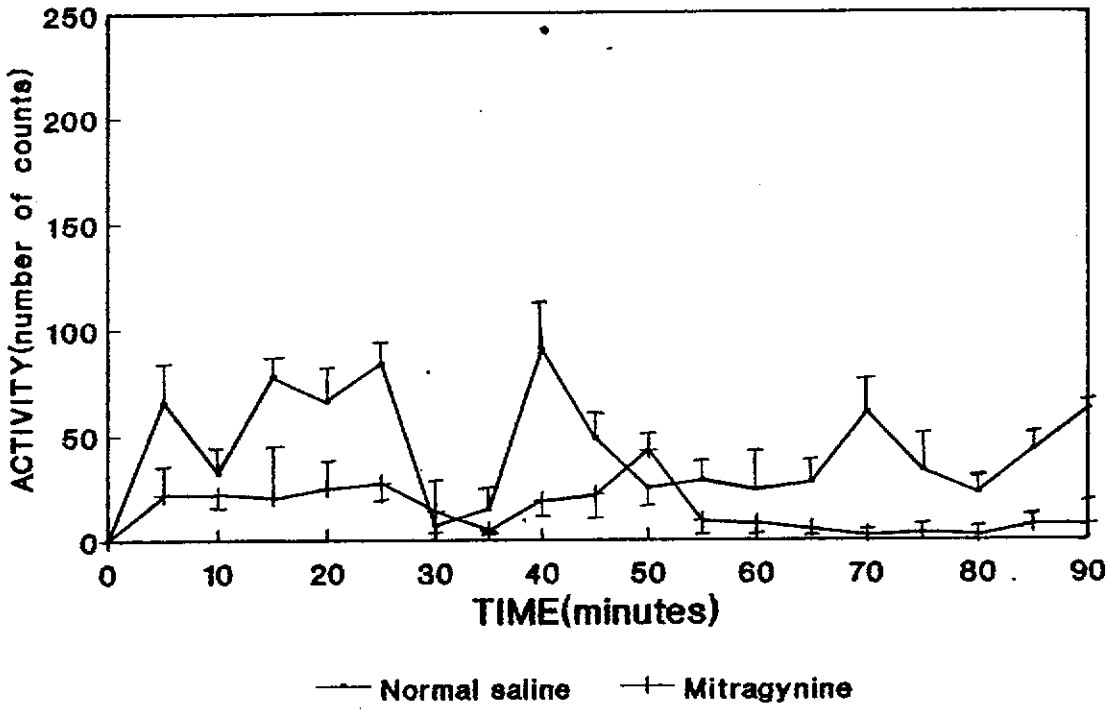


Figure 25 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 200 mg/kg.body weight) treatment through intraperitoneal injection.

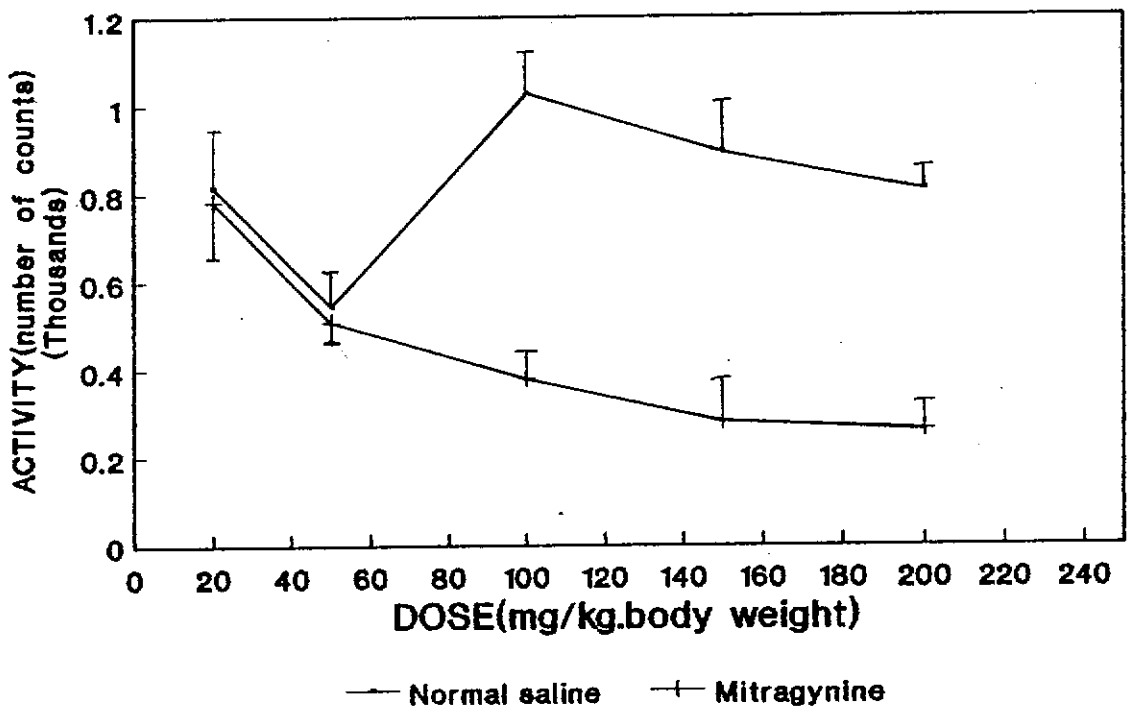


Figure 26 Dose response curve of mitragynine after intraperitoneal treatment.

1.2 Crude extract

The crude extract first demonstrated it effects at dose 10 mg/kg.body weight(Figure 28); the decrease of the activity was not significant different from that of the normal saline treatment. At higher dose, it strongly suppressed the activity until the lethal dose 50 mg/kg.body weight(Figure 31).

At dose 20 mg/kg.body weight, it demonstrated the significant effect at the first thirty-minute period(Figure 27, 29). The behavior changes were similar to those received substance M-HCl at 150 and 200 mg/kg.body weight. Convulsion was also observed in animals at 20 minutes until died within 30 minutes after treatment.

At dose 50 mg/kg.body weight, the crude extract produced similar effect to those received at dose 20 mg/kg.body weight (Figure 30) but they were stronger and caused all animal died within 15 minutes after treatment.

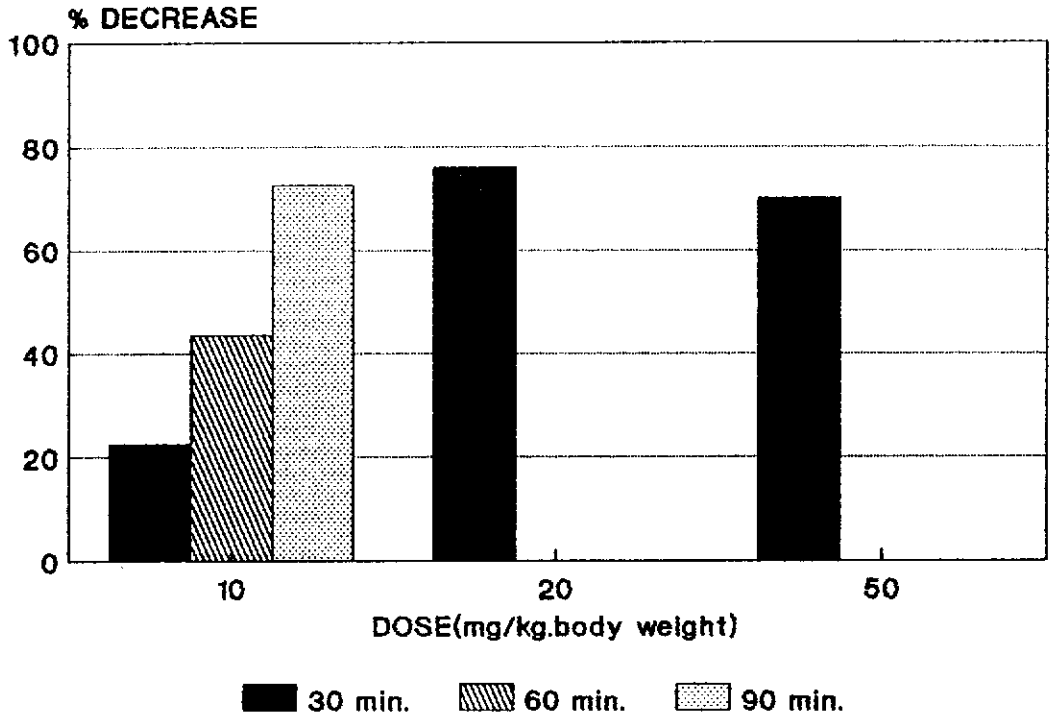


Figure 27 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and crude extract treatment at doses 10, 20 and 50 mg/kg. body weight.

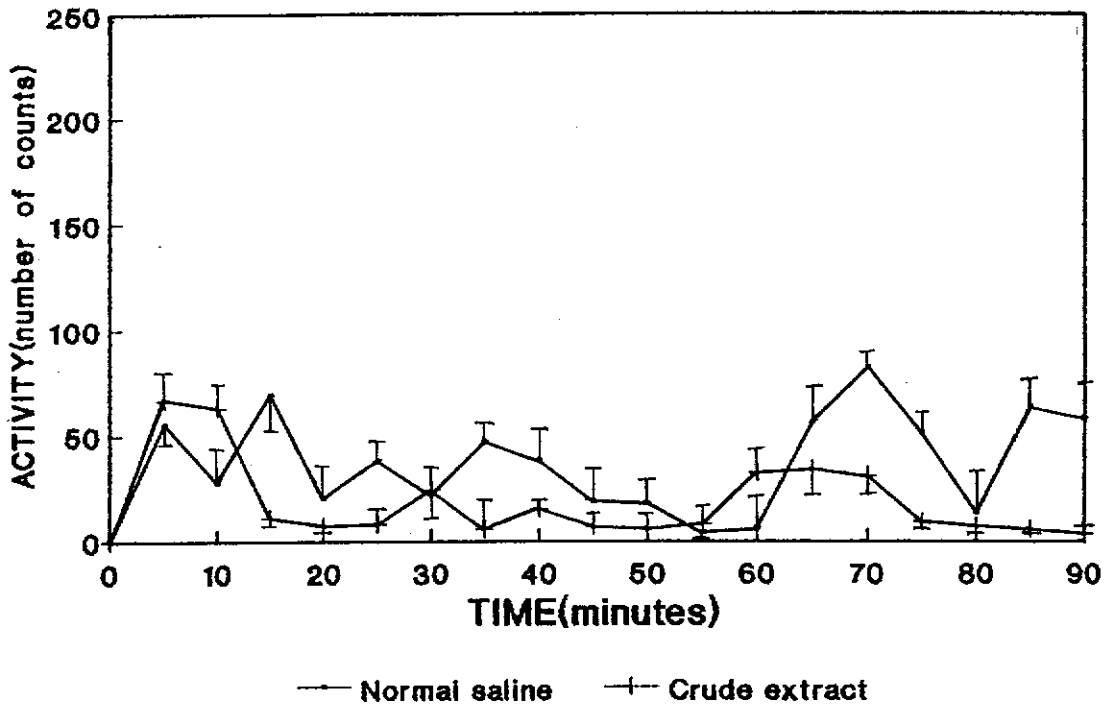


Figure 28 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude extract(dose 10 mg/kg.body weight) treatment through intraperitoneal injection.

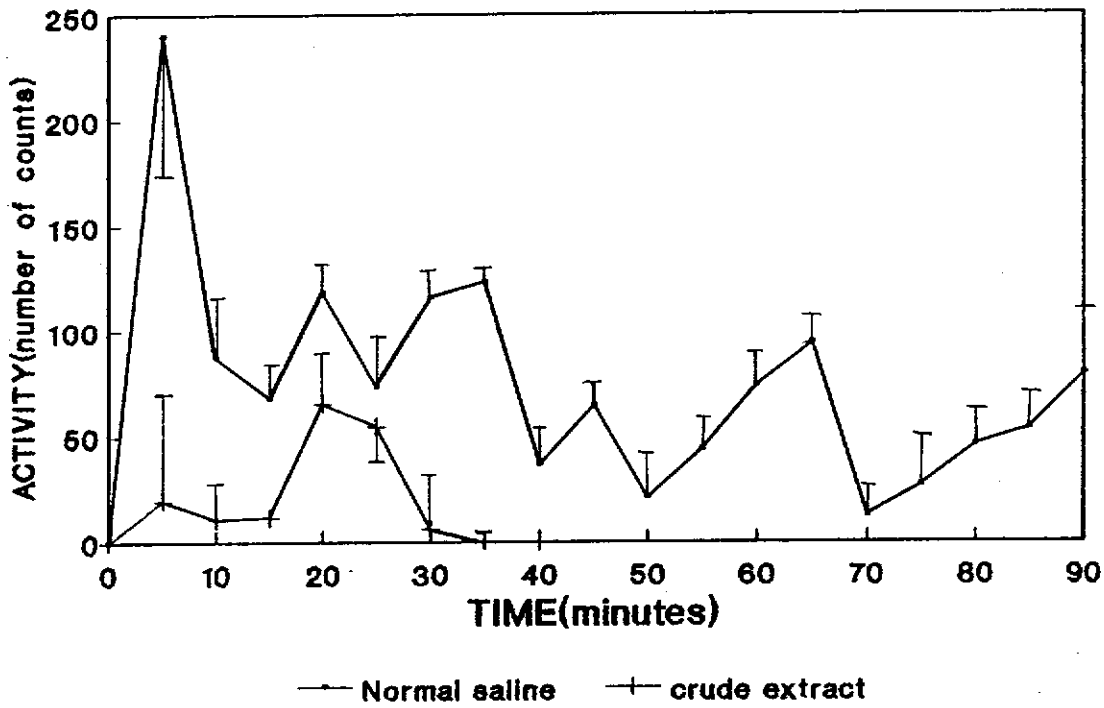


Figure 29 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude extract(dose 20 mg/kg.body weight) treatment through intraperitoneal injection.

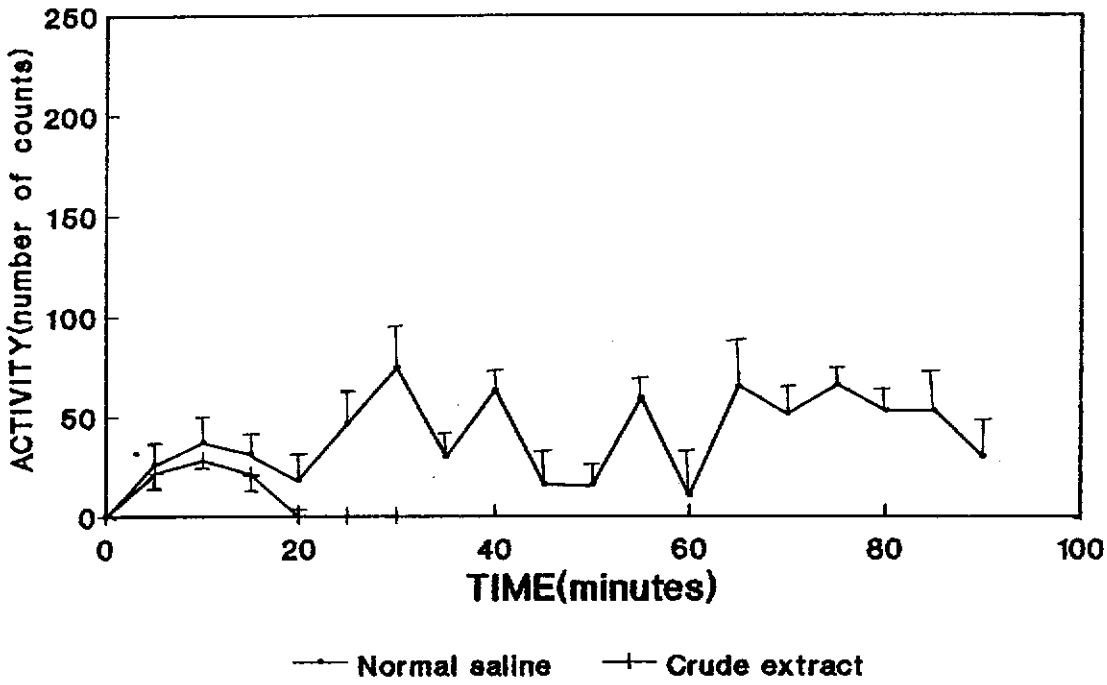


Figure 30 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude extract(dose 50 mg/kg.body weight) treatment through intraperitoneal injection.

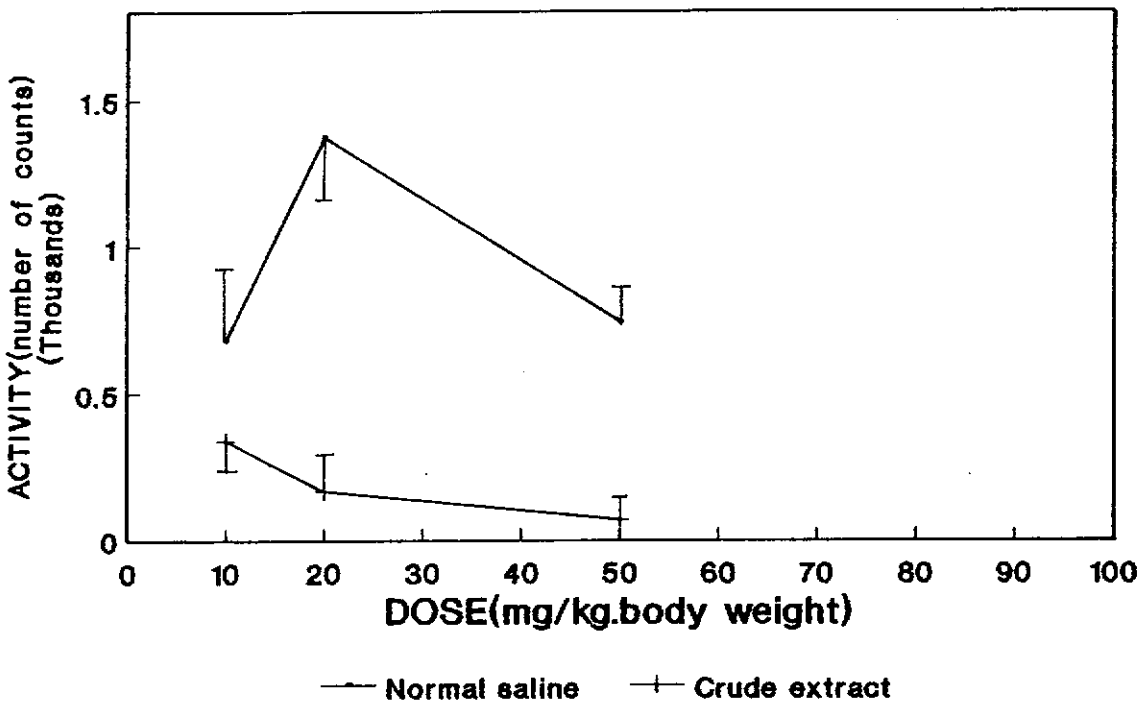


Figure 31 Dose response curve of crude extract after intraperitoneal treatment.

1.3 Crude alkaloids

The crude alkaloids suppressed the locomotor activity in mice at doses 50, 100, 150 and 200 mg/kg.body weight(Figure 34, 35, 36 and 37). At dose 50 mg/kg.body weight, the effect was strongly decreased to the minimum level similar to those of 100, 150 and 200 mg/kg.body weight(Figure 38). These were significant different from those obtained during the normal saline treatment at all time observed(Figure 32). The effect were first observed at 2-3 minutes after treatment. The animal show body and limbs extension with slight muscular spasm. However, the crude alkaloids did not have any effect at dose 20 mg/kg.body weight(Figure 32, 33).

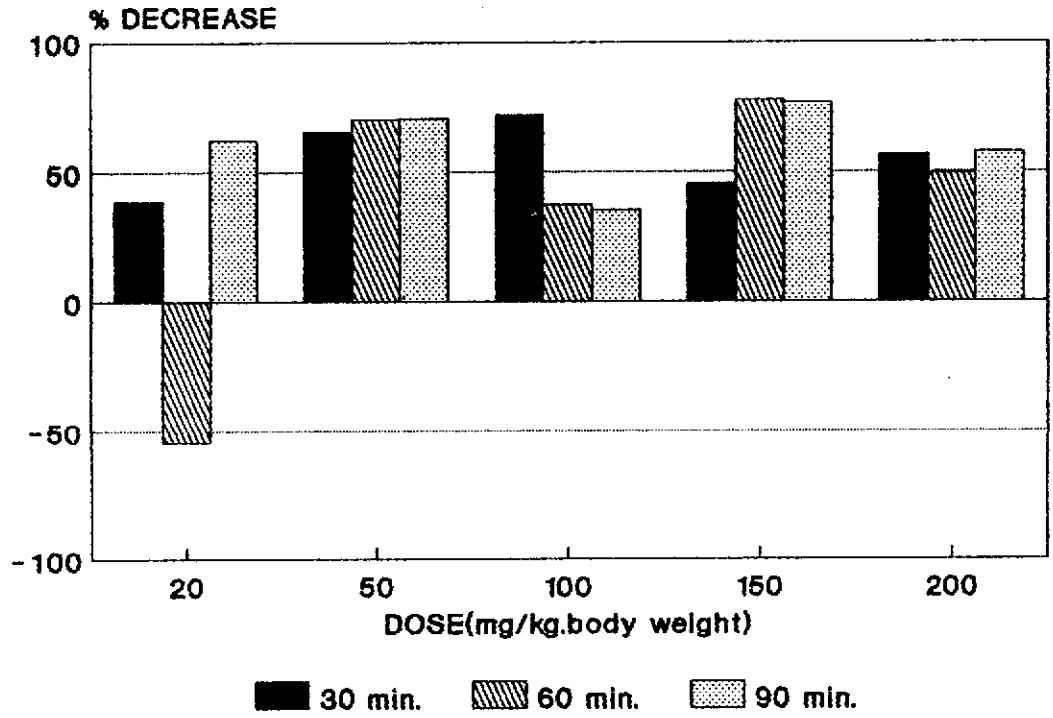


Figure 32 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and crude alkaloids treatment at doses 20, 50, 100, 150 and 200 mg/kg.body weight.

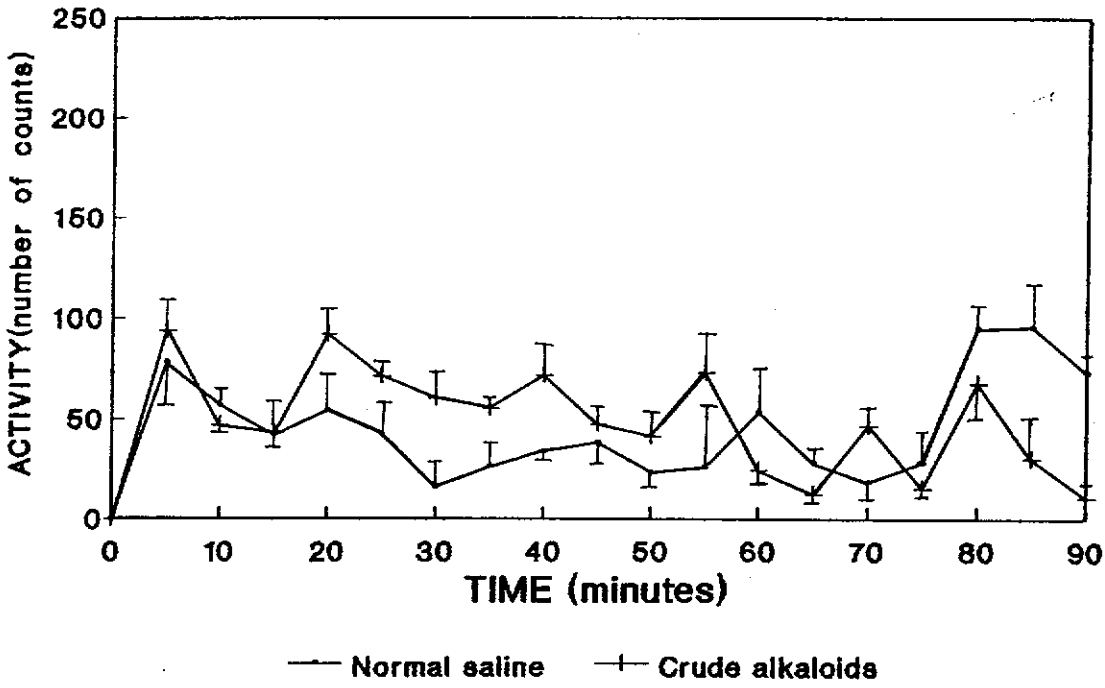


Figure 33 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude alkaloids (dose 20 mg/kg.body weight) treatment through intraperitoneal injection.

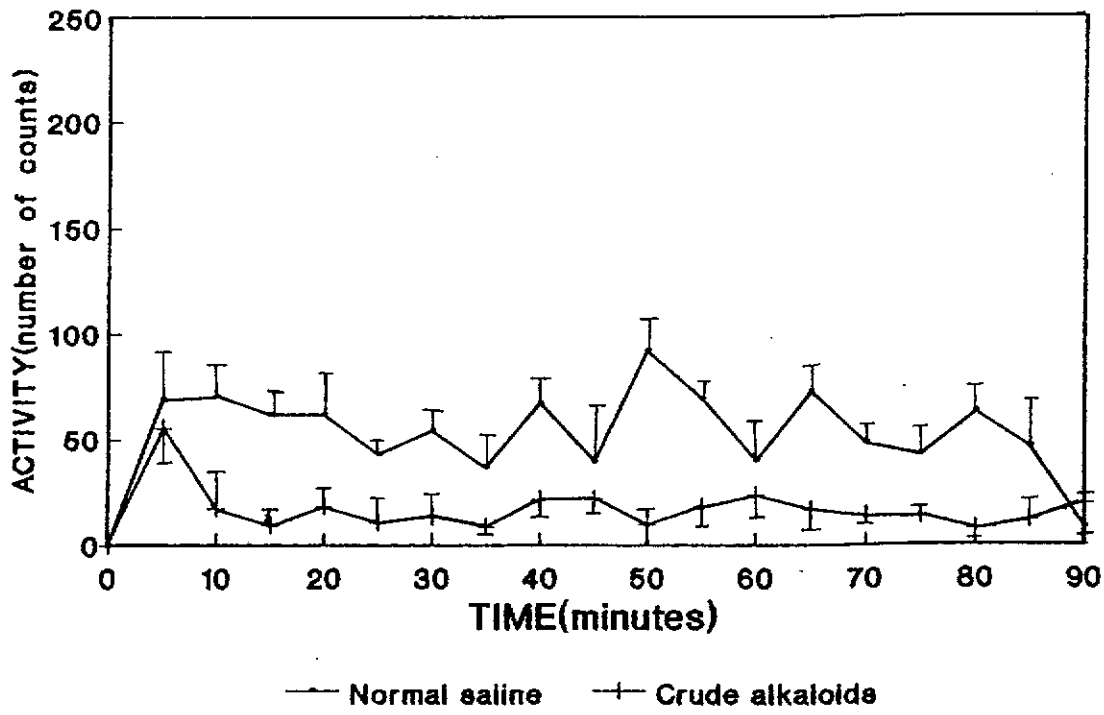


Figure 34 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude alkaloids (dose 50 mg/kg.body weight) treatment through intraperitoneal injection.

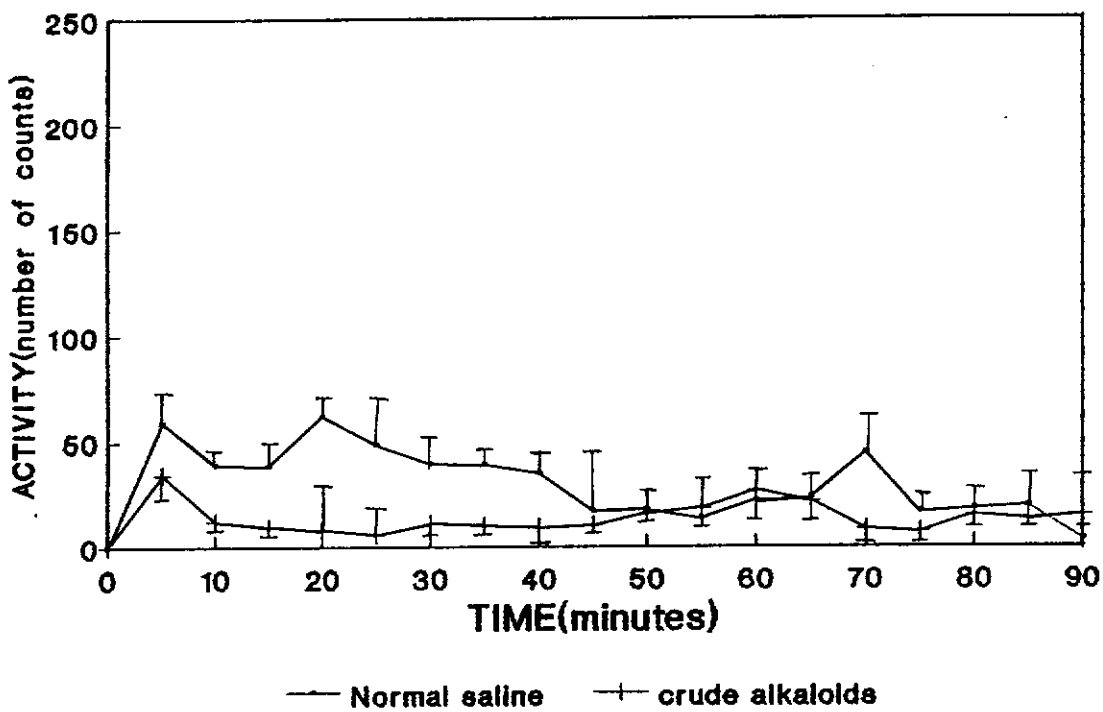


Figure 35 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude alkaloids (dose 100 mg/kg.body weight) treatment through intraperitoneal injection.

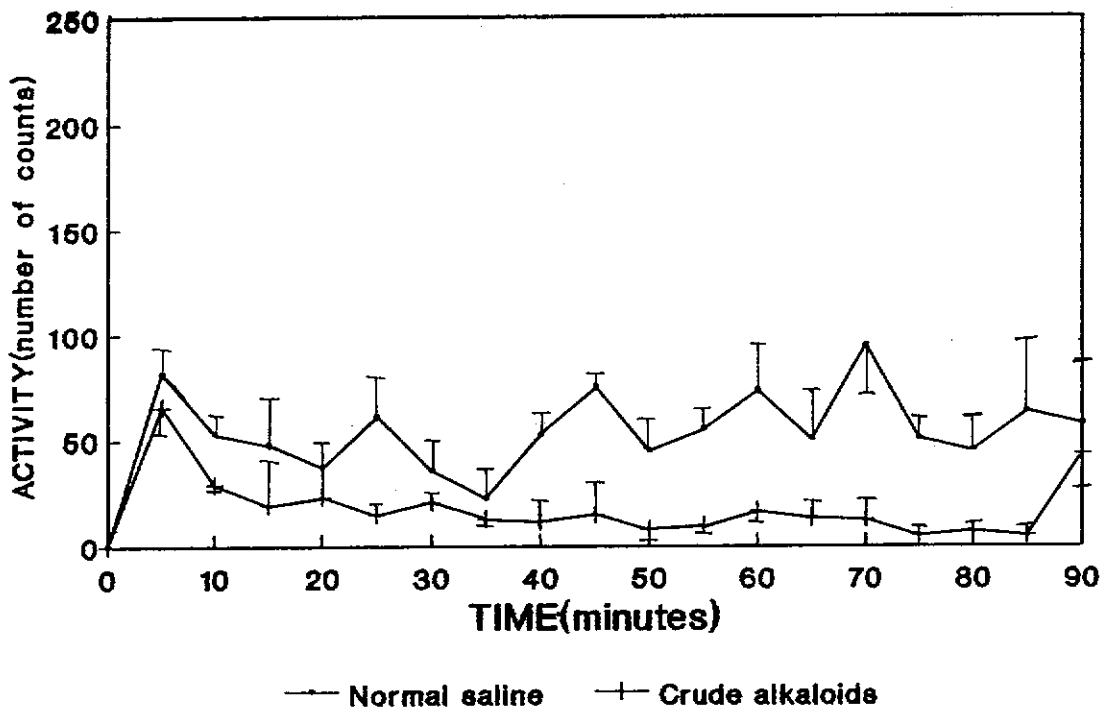


Figure 36 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude alkaloids (dose 150 mg/kg.body weight) treatment through intraperitoneal injection.

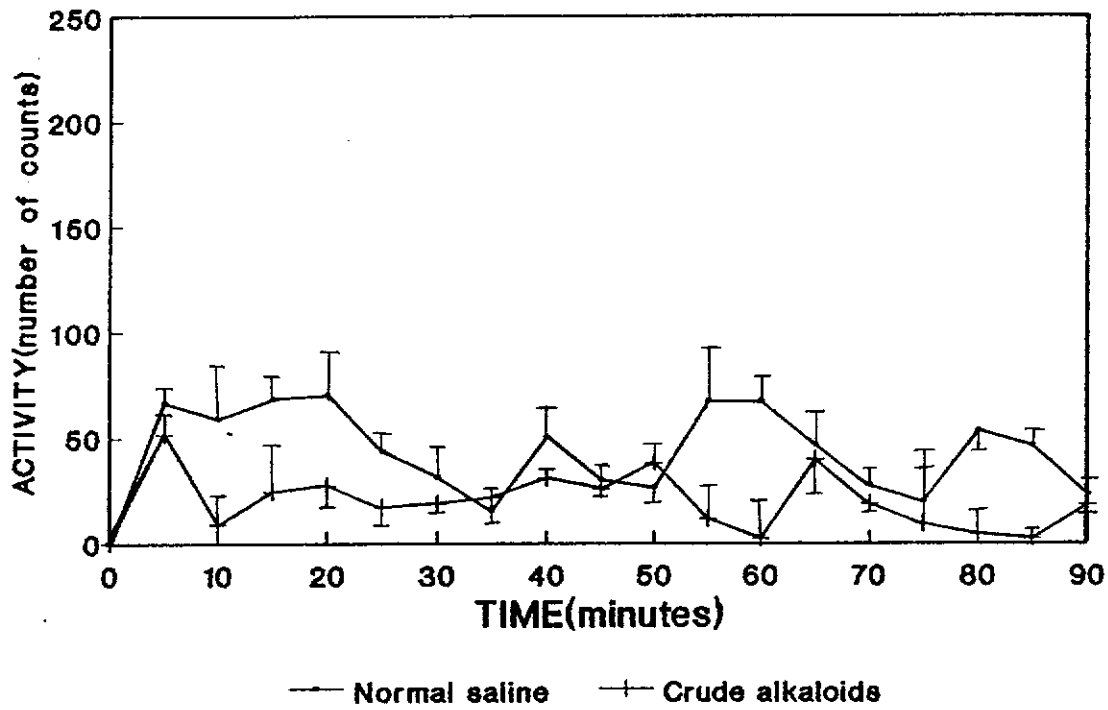


Figure 37 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude alkaloids (dose 200 mg/kg.body weight) treatment through intraperitoneal injection.

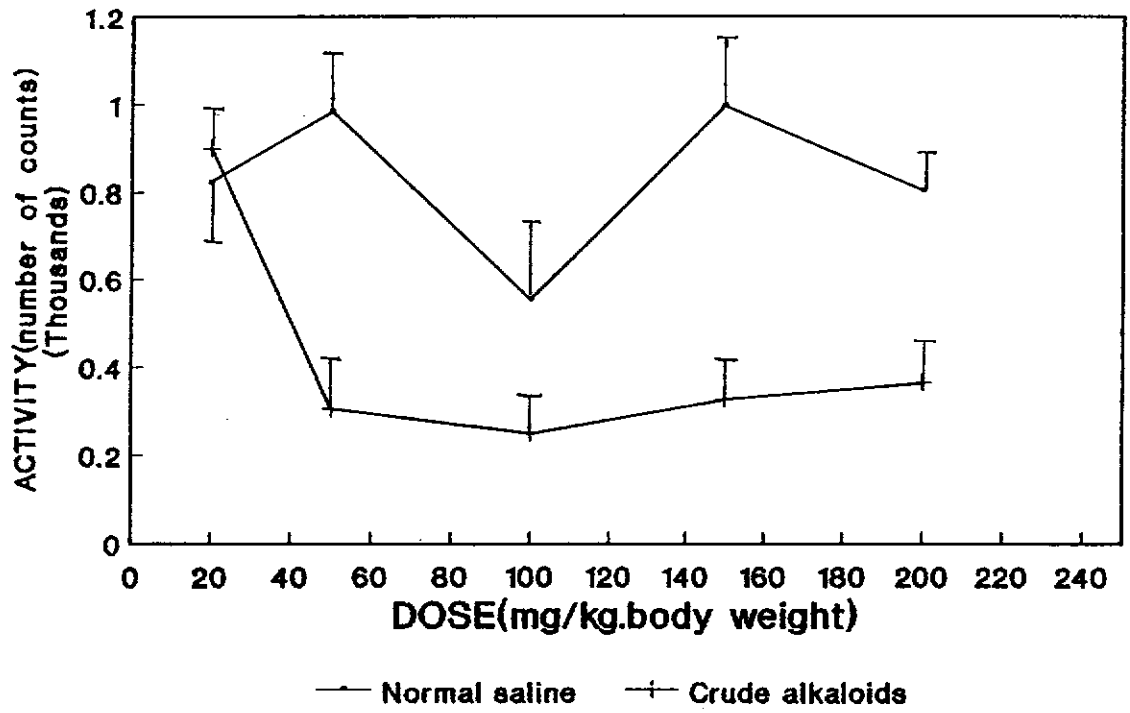


Figure 38 Dose response curve of crude alkaloids after intraperitoneal treatment.

1.4 Non-alkaloids

The non-alkaloids fraction strongly decreased the locomotor activity in mice at all doses applied (20, 50, 100 and 150 mg/kg.body weight; Figure 40, 41, 42 and 43). The effects were clearly observed at 2-3 minutes after treatment and persisted until the whole period of treatment. These effects were significant different from those obtained during the normal saline treatment(Figure 39). The mice was nearly immobile and shown strong body and limbs extension. The effect was strongly decreased at dose 50 mg/kg.body weigh and gradually decreased to the minimum level between dose 100 to 150 mg/kg.body weight (Figure 44). At dose 200 mg/kg.body weight, the non-alkaloids was not dissolved in normal saline solution.

When compared the highest mean locomotor activity responses of each treatment during the first thirty-minute , those of mitragynine hydrochloride, and the crude alkaloids were not significant different(Figure 45). The mean locomotor activity response of the non-alkaloids fraction was significant different from those of crude alkaloids and mitragynine hydrochloride (Figure 45). Likewise, that of crude extract was significant different from those of mitragynine hydrochloride, crude alkaloids and non-alkaloids(Figure 45).

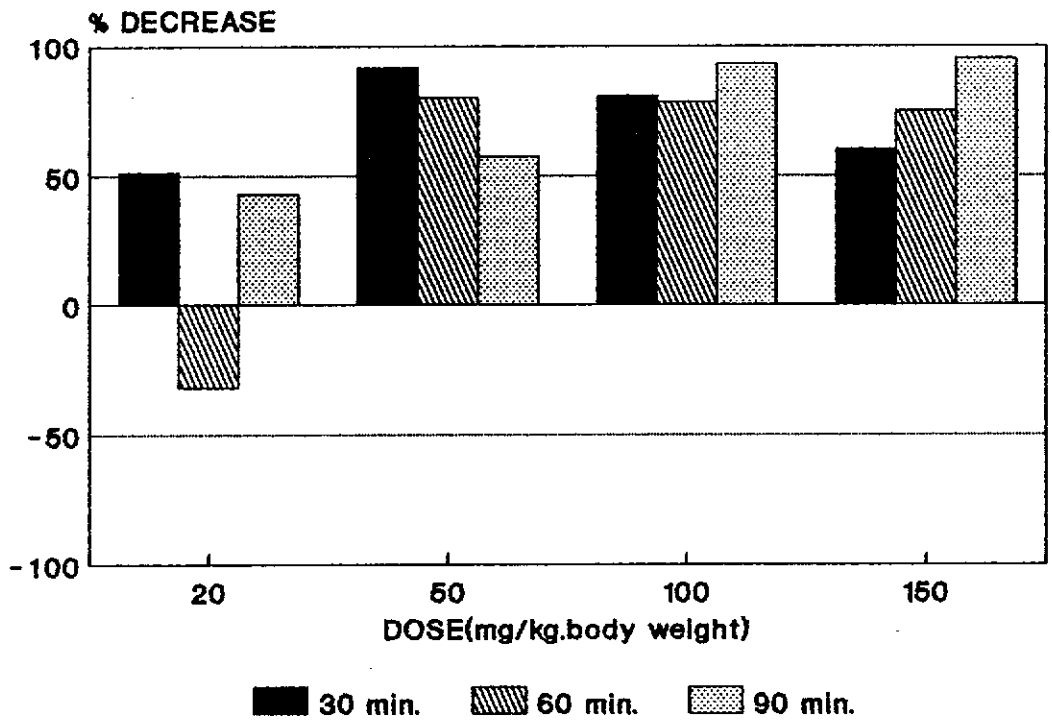


Figure 39 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and non-alkaloids treatment at doses 10, 20 and 50 mg/kg. body weight.

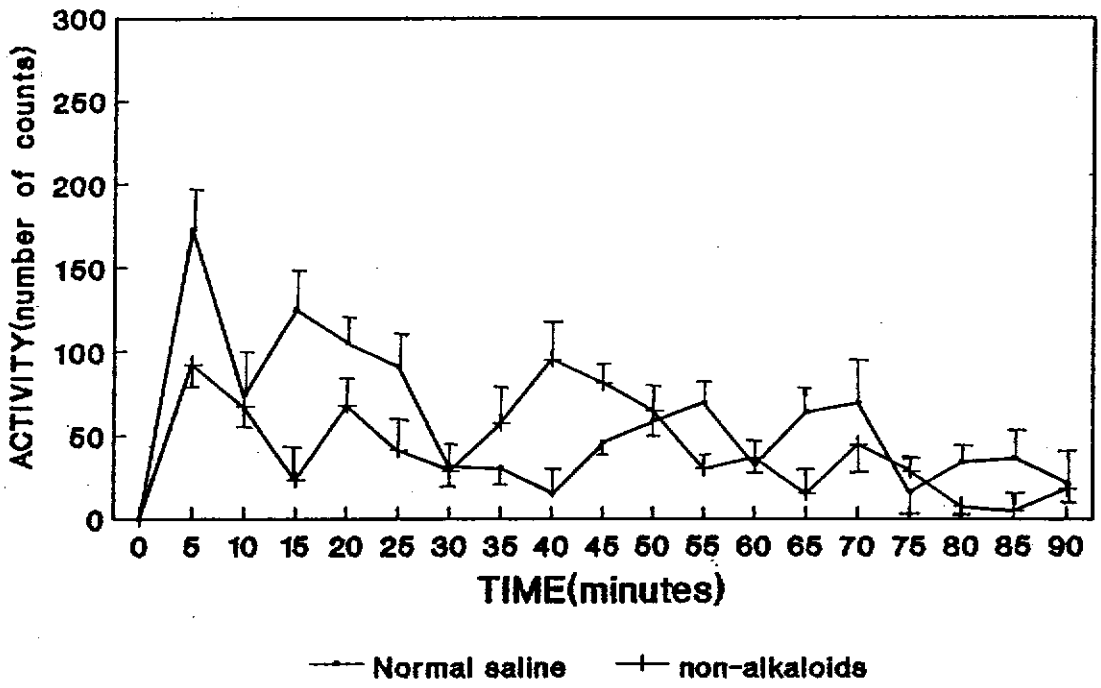


Figure 40 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and non-alkaloids(dose 20 mg/kg.body weight) treatment through intraperitoneal injection.

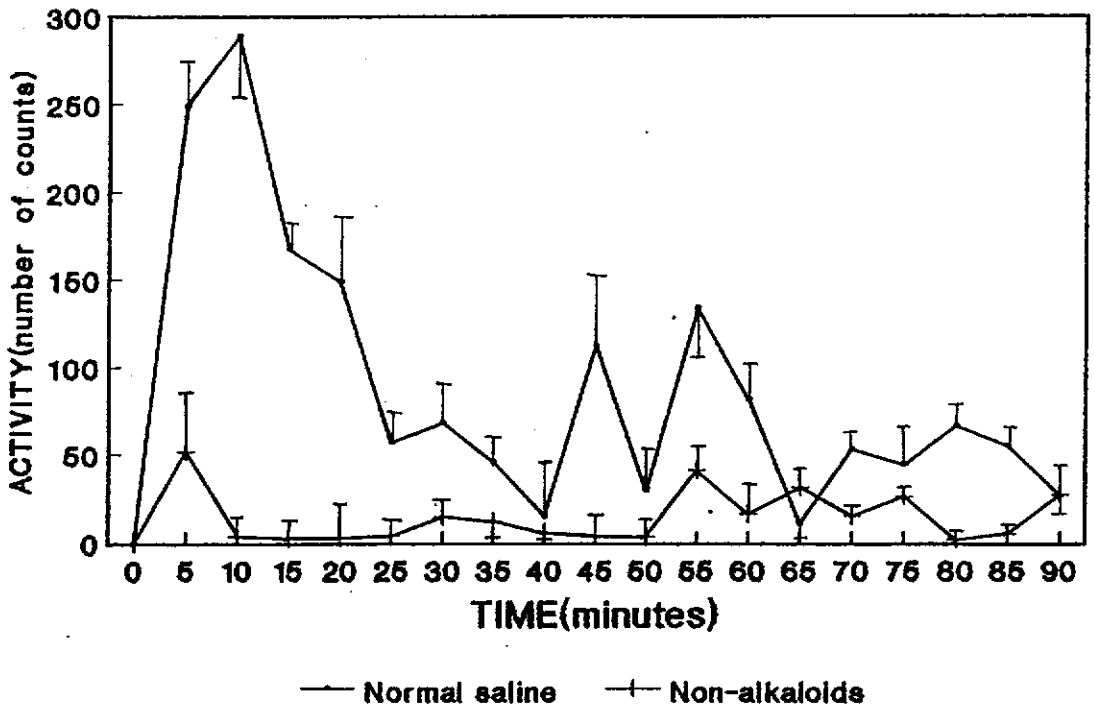


Figure 41 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and non-alkaloids(dose 50 mg/kg.body weight) treatment through intraperitoneal injection.

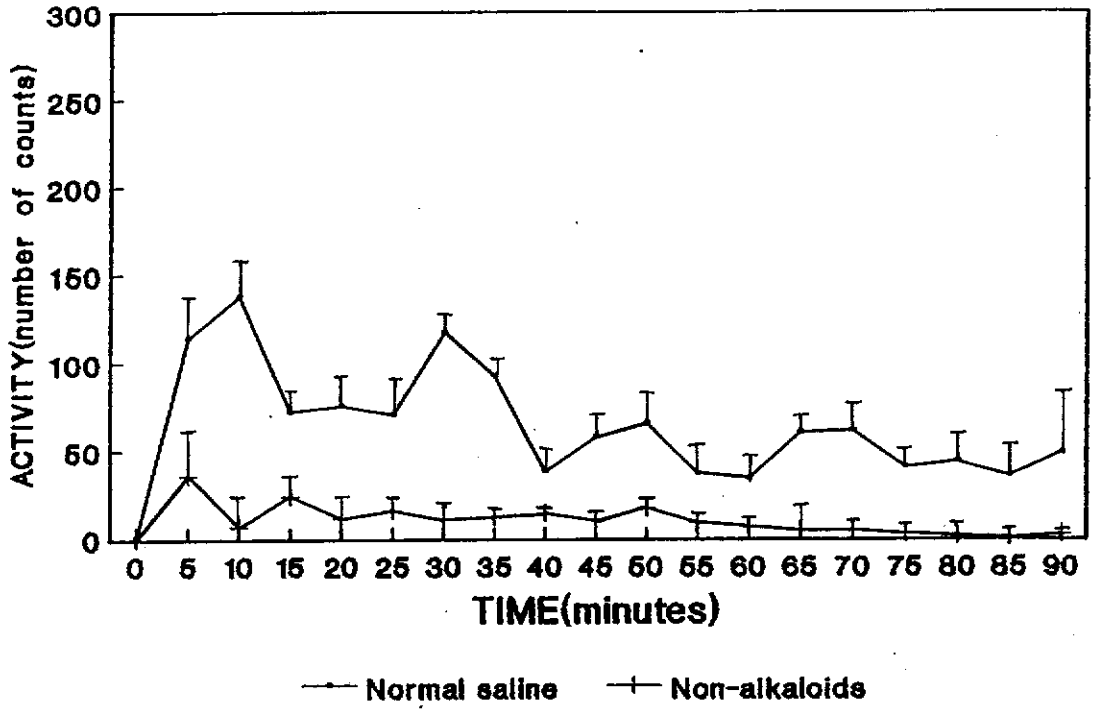


Figure 42 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and non-alkaloids(dose 100 mg/kg. body weight) treatment through intraperitoneal injection.

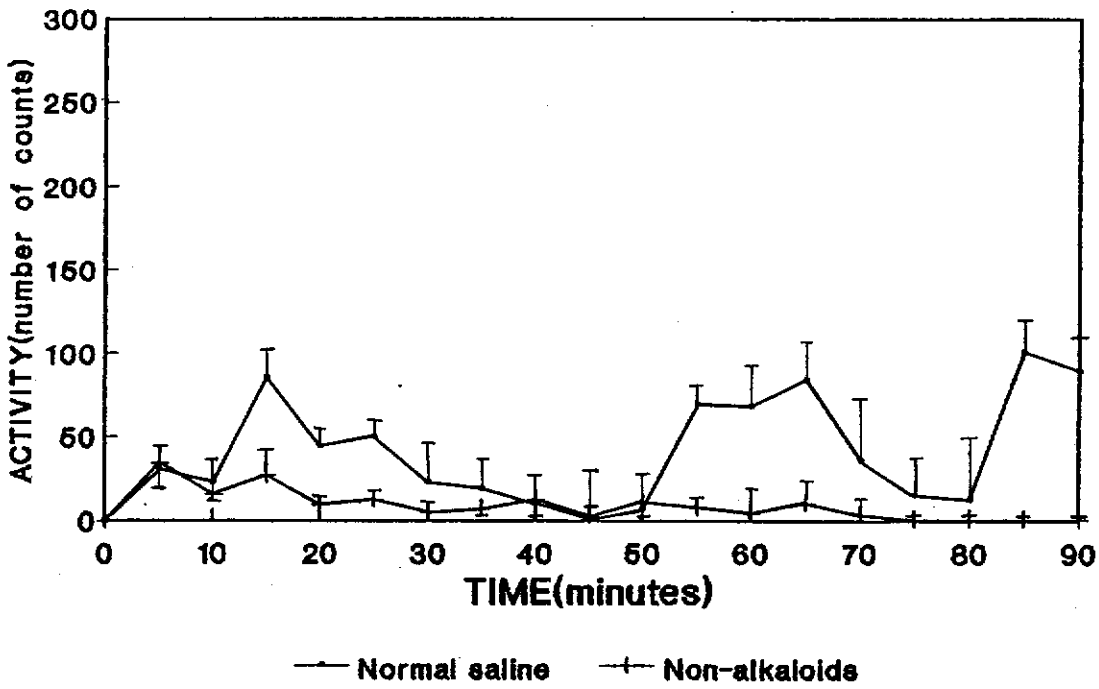


Figure 43 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and non-alkaloids(dose 150 mg/kg. body weight) treatment through intraperitoneal injection.

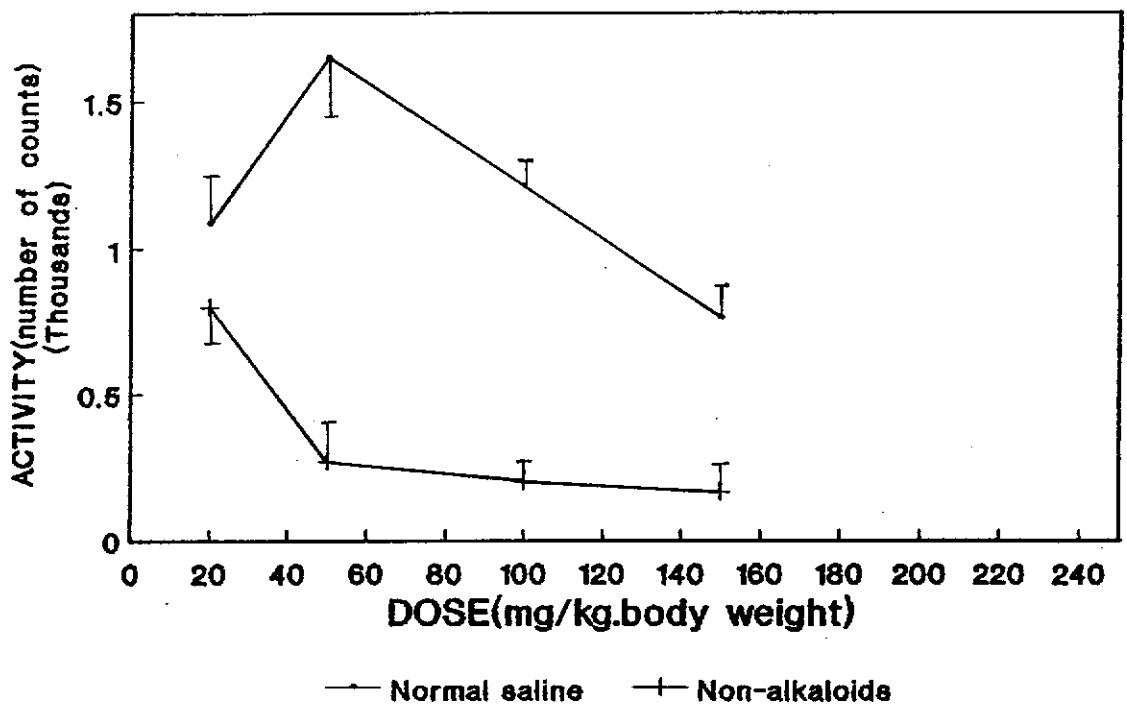


Figure 44 Dose response curve of non-alkaloids after intraperitoneal treatment.

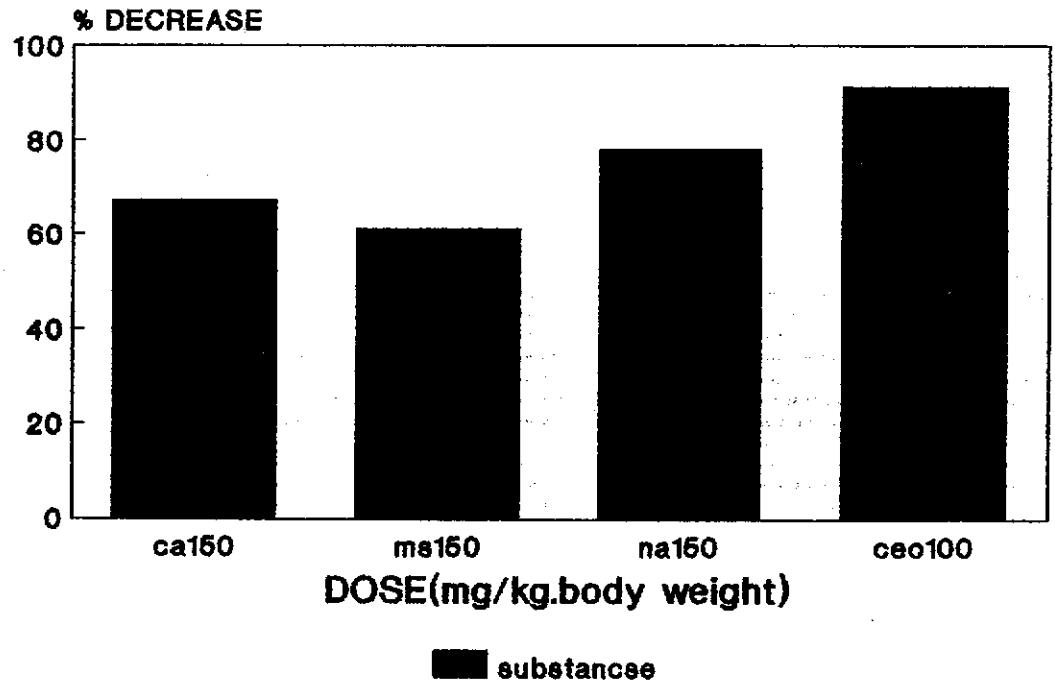


Figure 45 Histogram show % decrease of locomotor activity during 90 minutes after substances treatment through intraperitoneal injection.

2. Oral administration

2.1 Mitragynine hydrochloride

The mitragynine hydrochloride did not have significant effect on the experimental mice at doses 50, 100, 150 and 200 mg/kg.body weight after oral administration(Figure 47, 48, 49 and 50). However, it induced an increase in locomotor activity at doses 250 and 300 mg/kg.body weight(Figure 51 and 52). The effect was gradually increased from the dose 100 mg/kg.body weight until reaching the significant hightest response at dose 250 mg/kg.body weight(Figure 53). The effect was first observed at 4-5 minutes after treatment, the animals moved around the locomotor activity cage. No muscle spasm and body and limbs extension were observed in these experimental animals. At dose 250 and 300 mg/kg.body weight, the mean locomotor activity was significant different from that obtained after normal saline treatment during the whole peroids(Figure 46).

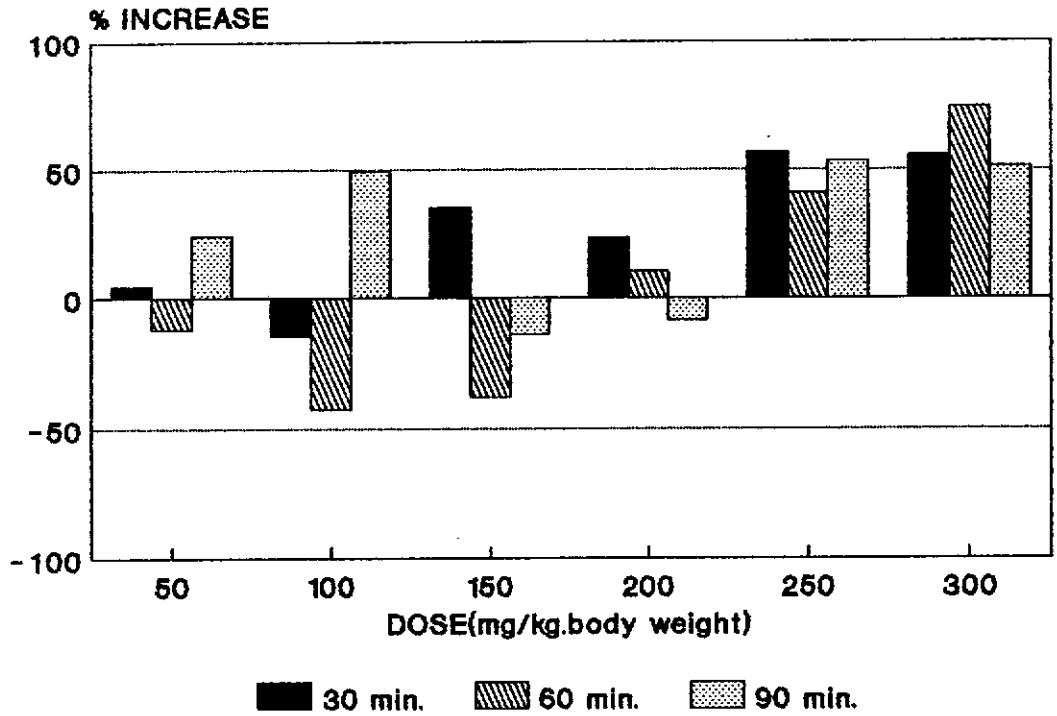


Figure 46 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and mitragynine treatment at doses 50, 100, 150, 200, 250 and 300 mg/kg.body weight.

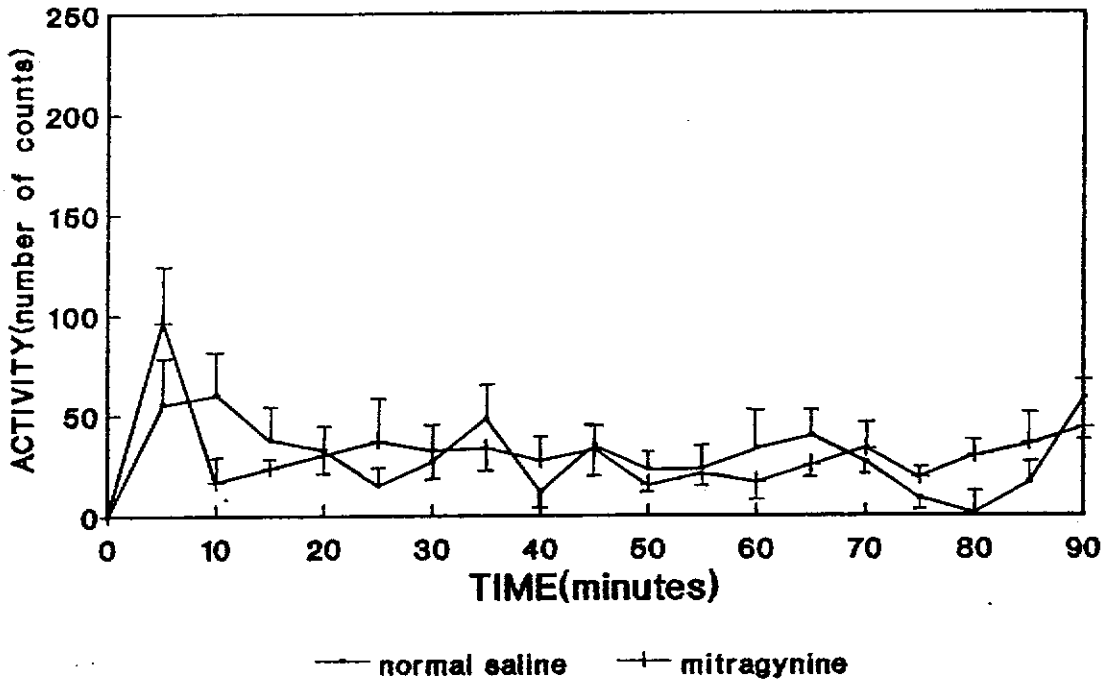


Figure 47 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine (dose 50 mg/kg.body weight) treatment through oral administration.

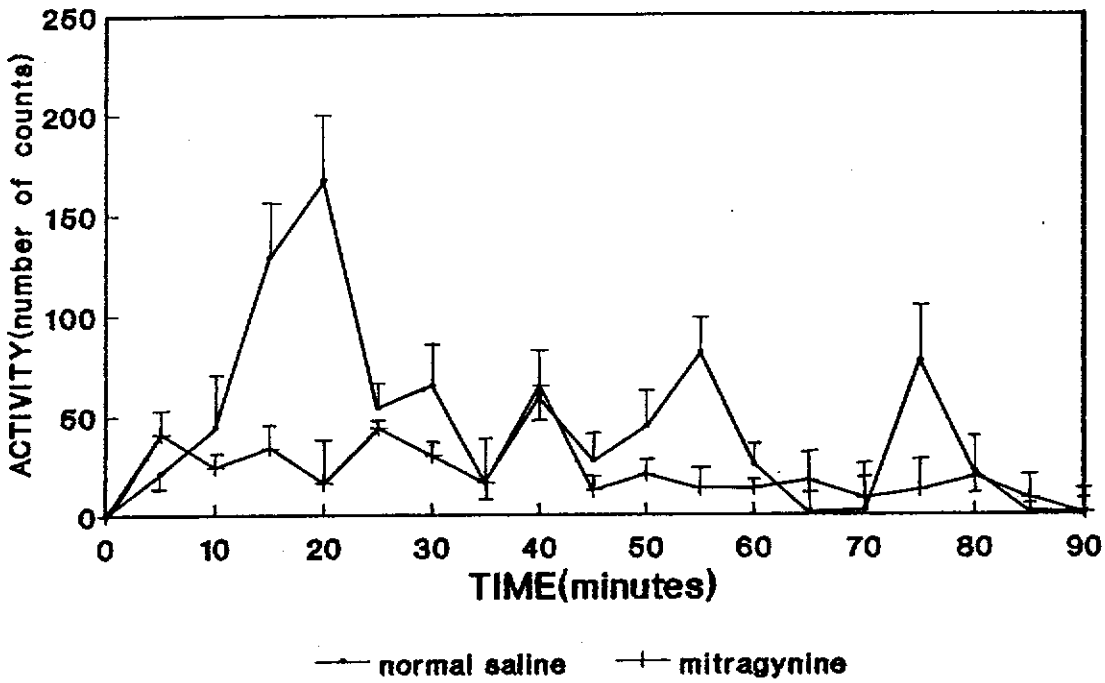


Figure 48 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine(dose 100 mg/kg.body weight) treatment through oral administration.

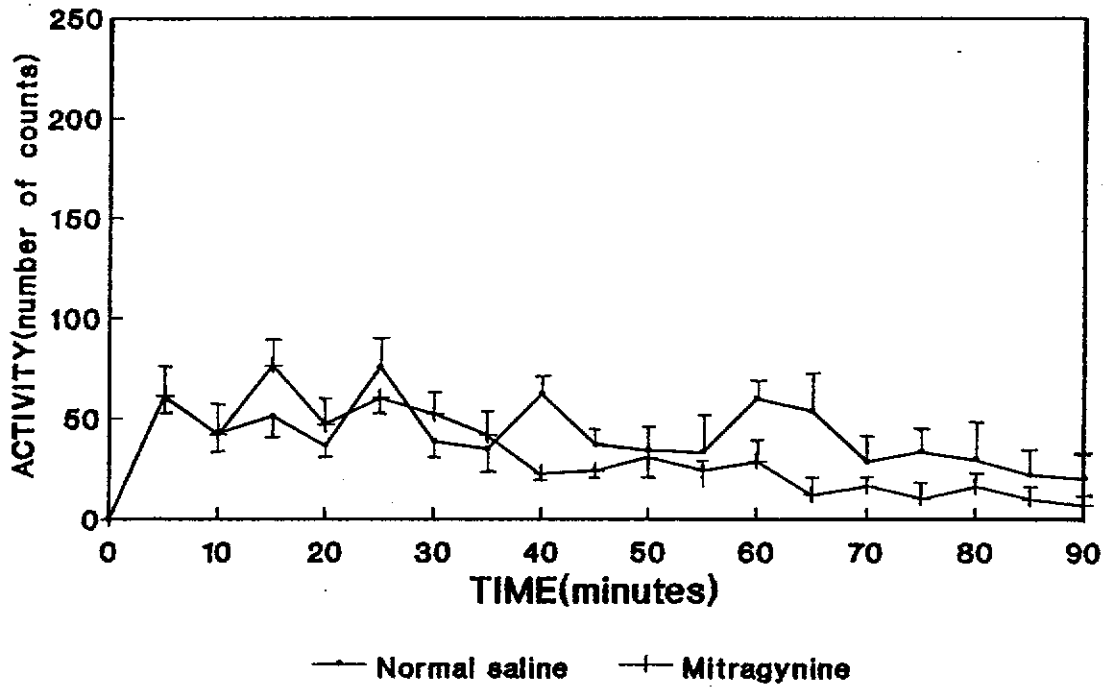


Figure 49 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine(dose 150 mg/kg.body weight) treatment through oral administration.

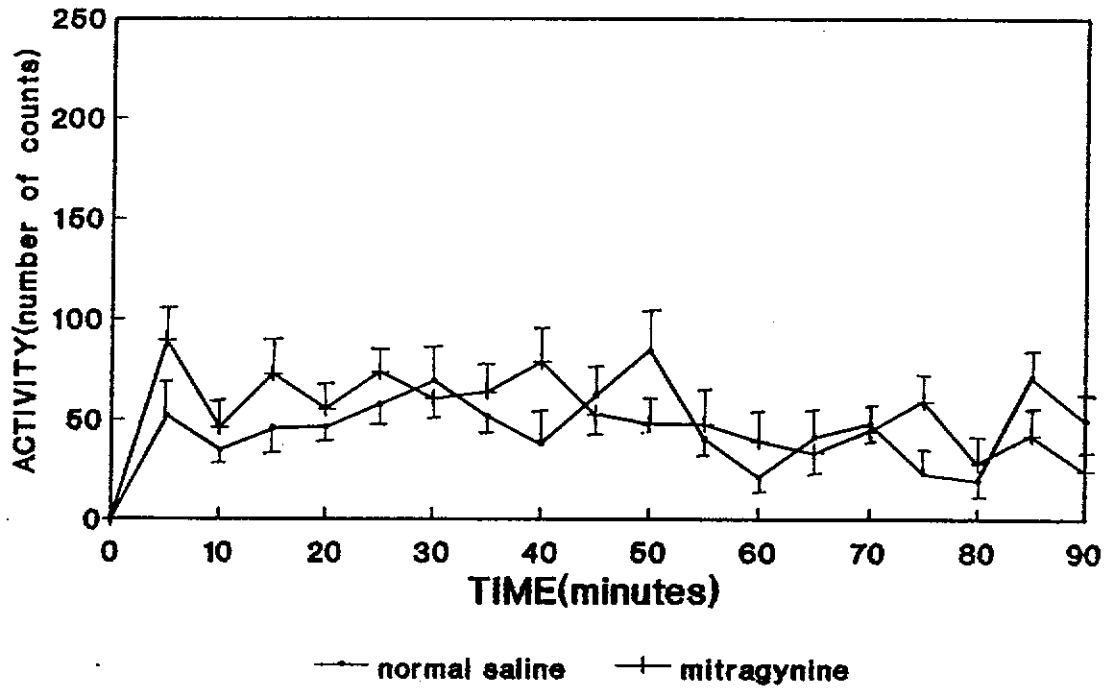


Figure 50 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine(dose 200 mg/kg.body weight) treatment through oral administration.

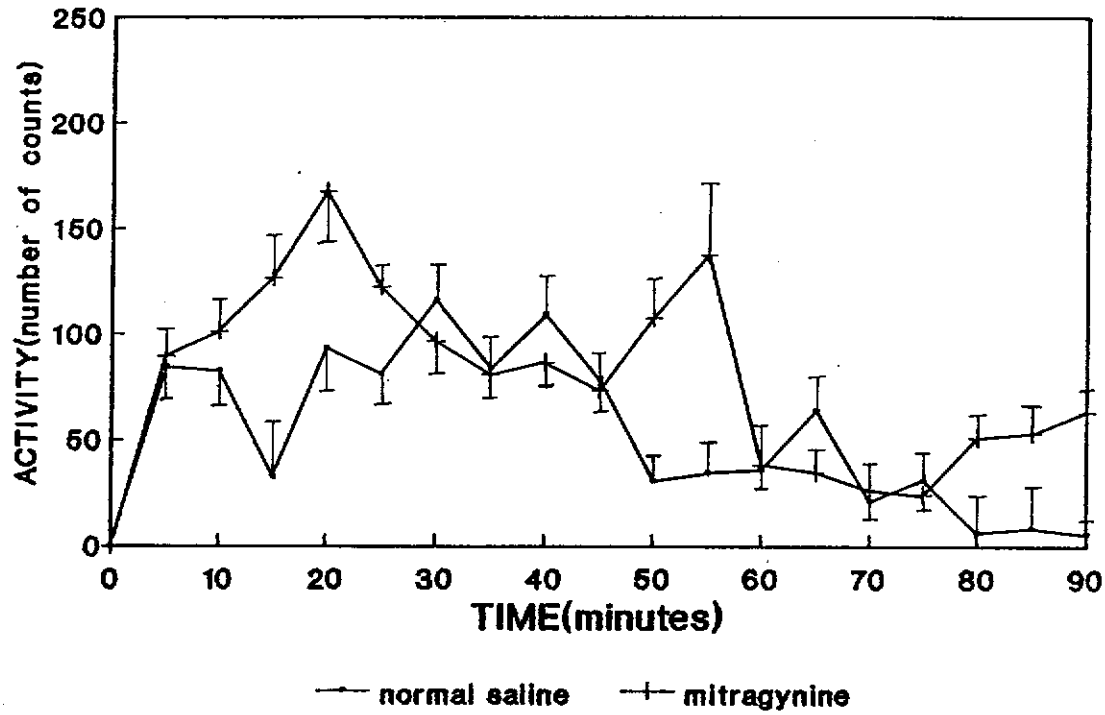


Figure 51 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine(dose 250 mg/kg.body weight) treatment through oral administration.

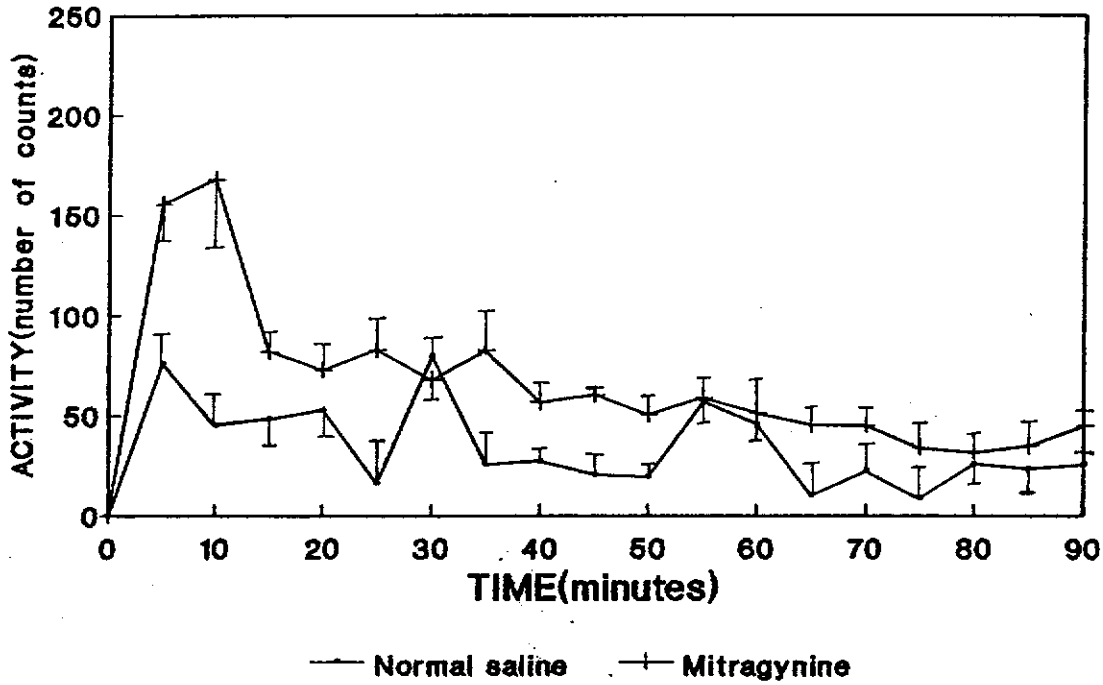


Figure 52 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and mitragynine(dose 300 mg/kg.body weight) treatment through oral administration.

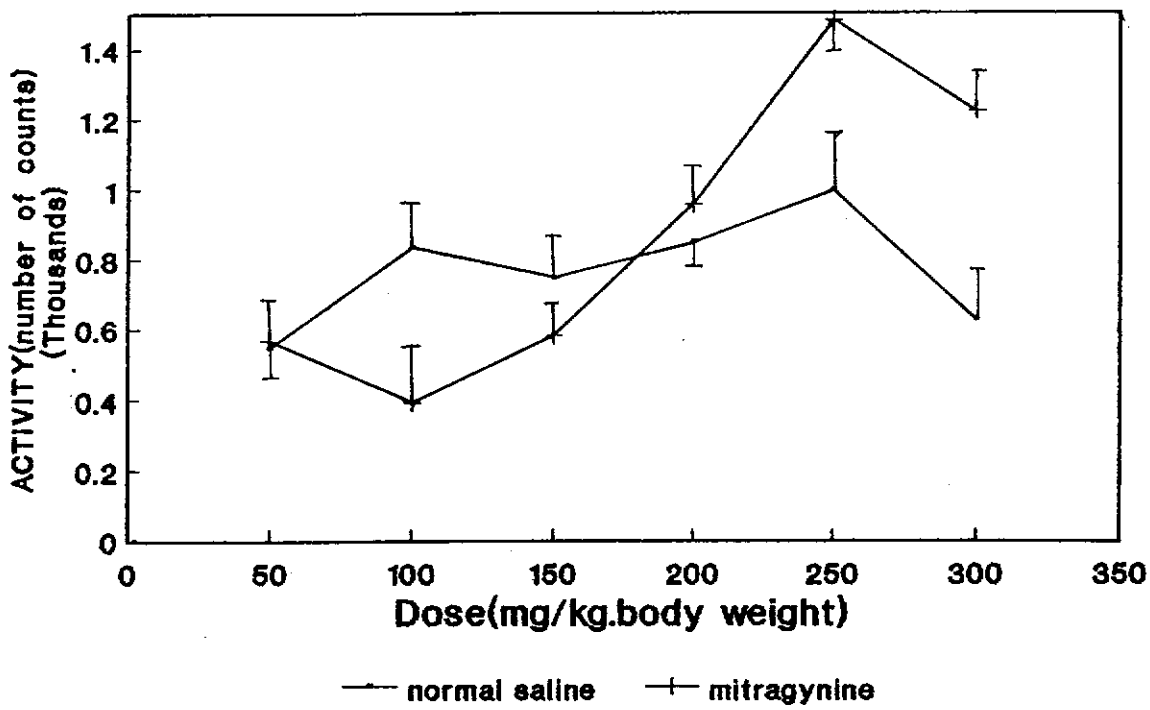


Figure 53 Dose response curve of mitragynine after oral administration.

2.2 Crude extract

The crude extract suppressed the locomotor activity after oral administration at dose 100 mg/kg.body weight during the whole periods after treatment(Figure 56). The animals show slightly depressed without muscle spasm or body and limbs extension. At dose 50 mg/kg.body weight the fraction did not have any effect(Figure 55). The administration of the crude extract could not have done exceeding 100 mg/kg.body weight due to the limited space of the stomach.

Because of the methode of oral administration, its were very difficult, and quantity of the crude alkaloids has not enough so I choose crude extract and mitragynine hydrochloride for test.

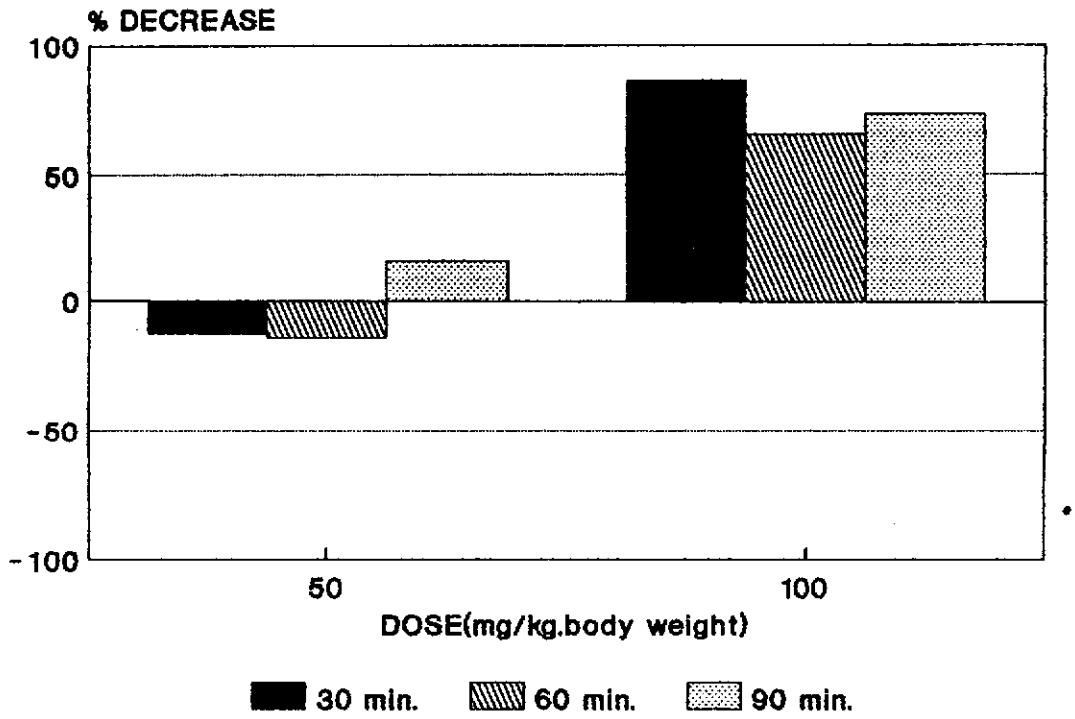


Figure 54 Histogram show the different of locomotor activity during 30, 60 and 90 minute period between normal and crude extract treatment at doses 50 and 100 mg/kg.body weight.

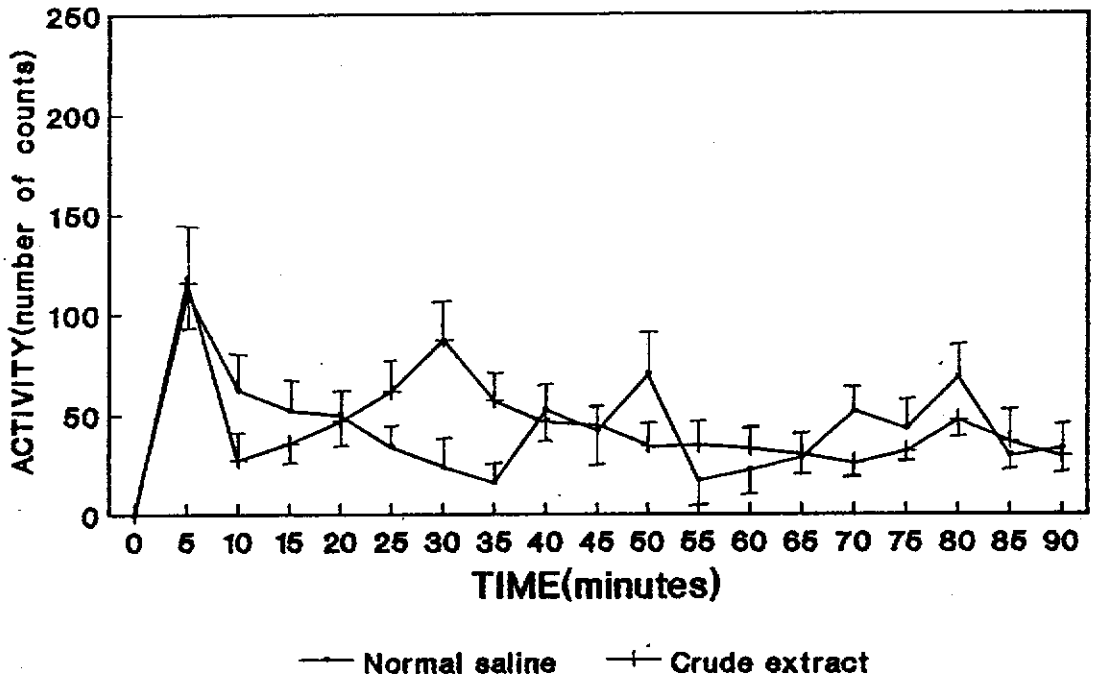


Figure 55 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude extract(dose 50 mg/kg.body weight) treatment through oral administration.

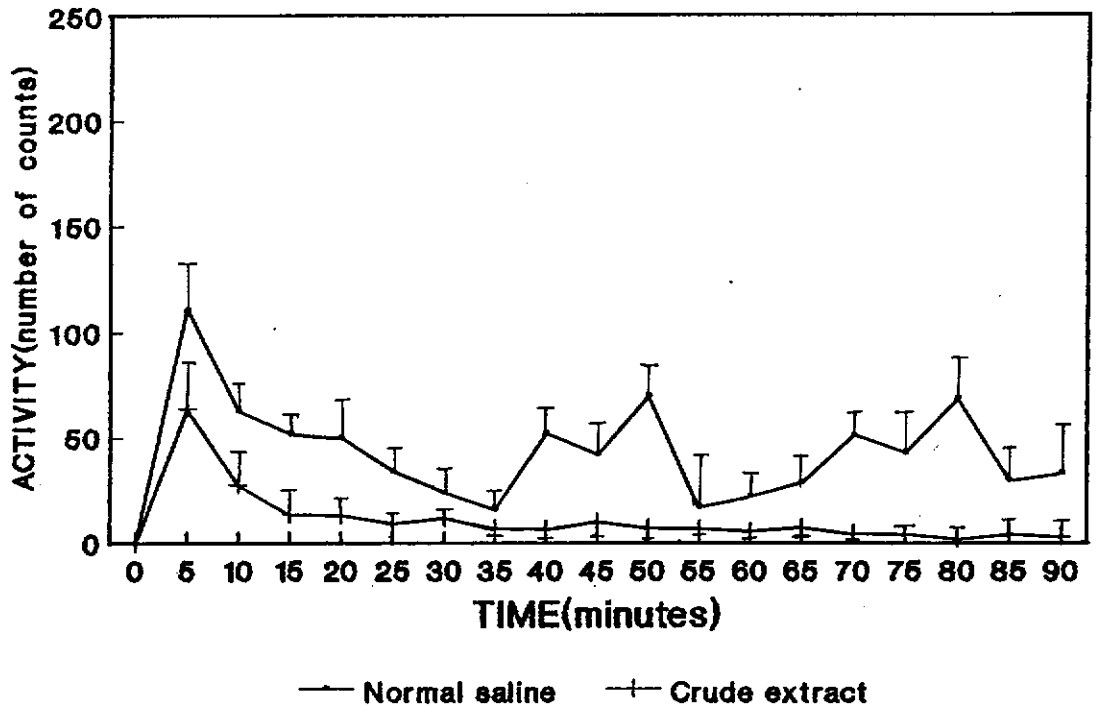


Figure 56 Mean ± S.E.M. of locomotor activity during 90 minutes period after normal saline and crude extract(dose 100 mg/kg.body weight) treatment through oral administration.

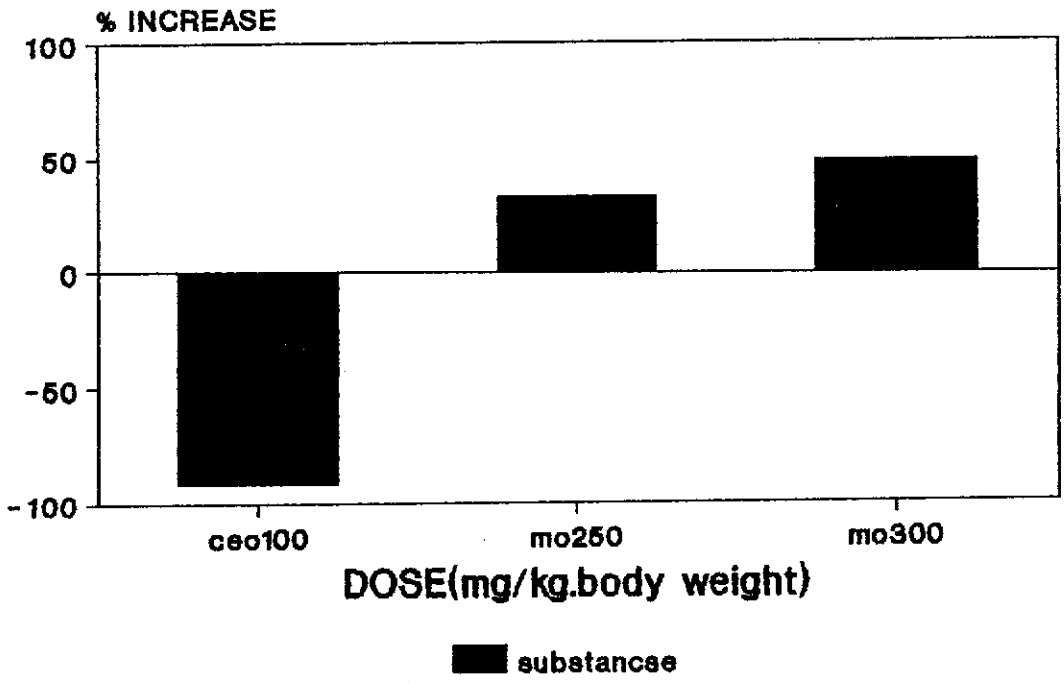


Figure 57 Histogram show % increase of locomotor activity during 90 minute after substances treatment through oral administration.

CHAPTER IV

DISCUSSION

In this study, the substance M, crude extract, crude alkaloids and non-alkaloids were prepared from the extraction of the dried leaves of the Mitragyna speciosa Korth. The physical and spectroscopic characteristics of the substance M were investigated to confirm its chemical structure. The effect of the substance M, crude extract, crude alkaloids and non-alkaloids on locomotor activity were later studied.

4.1 Chemical structure of the substance M.

The physical and spectroscopic charlacterristics (melting point, ultraviolet, infrared, NMR and mass spectrum) of the substance M when compared to those of previous reports (Beckett. 1965,1966 and Keawpradub. 1990) were similar(Table 4) Likewise, the preparation procedure of the substance M followed mainly those of Beckett(1965). These evidences convinced that, the substance M prepared in this study has chemical structure similar to the mitragynine.

4.2 Preparation of the substance M and substance M-HCl

The substance M or mitragynine prepared according to the procedure used in this study gave 0.25 percent yield. This is slightly lower than the original procedure of Beckett(1965) which offered 0.3 percent yield. The difference may be due to the chloroform extraction adopted in this study instead of ether of the original procedure. This indicated that the mitragynine may be dissolved in the ether better than the chloroform. However, the chloroform was used in this study for safety reason, the ether is highly volatile and inflammable. Other

modifications of the preparation of the mitragynine were also described by Beckett and Shellard(1966) which used sulfuric acid extraction of the crude extract instead of glacial acetic acid. His method, however, gave much lower yield; 0.025 percent. The fresh leaves were also used in the preparation in order to compared the alkaloidal products to those of the dried leaves. The fresh-leaves preparation gave only 0.022 percent yield. Thus, the procedure used in this study should be appropiated for preparation of mitragynine, it gave safety conditions and high yield of mitragynine.

4.3 Verification of the effects of the mitragynine, crude extract, crude alkaloids and non-alkaloids on the locomotor activity.

The animal locomotor activity monitoring system used in this study is reliable method and apparatus for the study of animal locomotor behavioral response after treatment with any test sample(George. 1988). The locomotor response will be the best evidence to indicate whether the animal is stimulated and depressed by the test substance.

After intraperitoneal injections of various doses of crude extract, crude alkaloids, non-alkaloids and mitrgynine depressive behavior responses were observed. The effect of the crude extract is the most potent(Figure 45), while the effect of crude alkaloids and mitragynine is not significant different and both effects was the weakest(Figure 45). The potency of the effect of non-alkaloids fraction was between those of the crude extract and the crude alkaloids and mitragynine fractions. This indicated the bioactive substances in the leaves of Mitragyna speciosa Korth. which induced the depressive effect should be contained in both alkaloids and non-alkaloids fractions. The mitragynine should be only the bioactive substance in the alkaloids fraction since the effect of the

crude alkaloids and mitragynine was not significant difference (Figure 45).

There are no pervious report described the chemical compositions of the non-alkaloids fraction obtained from leaves of Mitragyna speciosa Korth. However, study in other plants mentioned glycoside, protien/peptides, amines and resin in this fraction(George, 1988). The glycoside especially saponins were also reported to induce depressive effect(Wagner, 1983)

The direct evidence demonstrated the depressive effect of mitragynine after intraperitoneal administration, to our knowleage so far, has no previous report. Only fracments of data on this matter, however, it was said to have depressive effect in patient with the kratom addiction(Norakranphadung, 1966). Likewise, a slight decrease in spontaneous motor activity was observed in experimental dog after oral administration of mitragynine(Macko. 1972). Mitragynine solution(1 : 20,000 to 1 : 100,000) was also reported to cause a decrease in the amplitude of movements and a diminstration of the tone of the muscle in the isolated intestine(Grewal. 1932).

In this study, the mitragynine was found to increase the locomotor activity after oral administration at doses 250-300 mg/kg.body weight. This evidances directly indicated the stimulative effect of the mitragynine. This is the new finding since these is no previous report described on this matter. Only fracment of data demonstrated indirect evidences indicated the effect of mitragynine might concern the stimulation effect. This includes the mitragynine after intraperitoneal administration (dose = 18.4 mg/kg.body weight) was found to increase an exploratory behavior(Macko. 1972). However, finding in this study corresponds very well with behavioral changes reported in Thai kratom users(Suwanlert. 1975). The stimulant effects the desire to increase work output and

tolerance of the hot sunlight, was observed in those people after chewing the Kratom (Mitragyna speciosa Korth) fresh leaves or taking it as dried powder; the effects commenced after 5-10 minutes.

In conclusion, this study demonstrated the depressive effect of mitragynine, crude extract, crude alkaloids and non-alkaloids extracted from the leaves of Mitragyna speciosa Korth after intraperitoneal injection. At the same time, mitragynine was found to increase the locomotor activity, indicating the stimulative effect, after oral administration. These contradictory results of the mitragynine indicated the biotransformation of mitragynine within the gastrointestinal tract to metabolites, then the metabolites were absorped into the blood circulation to induce the effect. This corresponds very well with the stimulative response observed in those Kratom users after chewing fresh leaves or taking powder of the dried leaves of the Mitragynina speciosa Korth.

REFERENCES

Amattayakul, T. "The kratom leaves," J. Dep. Med. Sci., Thailand. 2(2) : 104 - 108 ; 1960.

Beckett, A.H., E.J. Shellard and A.N. Tackie. "The Mitragyna species of ASIA. Part IV : The alkaloids of the leaves of M.speciosa Korth," Planta Med. 13 : 241 - 245 ; 1965.

Beckett, A.H., E.J. Shellard. and A.N. Tackie. "The Mitragyna species of ASIA. Part VI : Oxindole alkaloids from the leaves of M.speciosa Korth," Planta Med. 14 : 266 - 276 ; 1966.

Field, E.J. "Mitragynine and mitraversine; two new alkaliods from species of Mitragyna." J. Chem Soc. 119 : 887 - 891 ; 1921.

George, D., Lutterodt and Abdul Maleque. "Effects on mice locomotor activity of a narcotic-like principle from Psidium Guajava leaves," J. Ethnopharmacol. 24 : 219 - 231 ; 1988.

Grewal, K.S. "Observations on the pharmacology of mitragynine," J. Pharmacol. Exp. Ther. 46 : 251 - 271 ; 1932.

Holmes, E.M. "Some medicinal products from the Straits settlements," Pharmaceu. J. 54 : 1095 - 1096 ; 1895.

Jansen, K.L.R. and C.J.P. Prast. "Ethnopharmacology of Kratom and Mitragyna alkaloids," J. Ethnopharmacol. 23 : 115 - 119 ; 1988.

Jansen, K.L.R. and C.J.P. Prast. "Psychoactive properties of Mitragynine(Kratom)," J. Psycho drugs. 20(4) : 455 - 457 ; 1988.

Joshi, B.S., Raymond-Hamet and taylor. N.I. Chem. and Ind. : 573 ; 1963.

Keawpradub, N. Alkaloids from the fresh leaves of Mitragyna speciosa (Korth.) Havil. Bangkok : A thesis submitted for the degree of Master of science in Pharmacy, Chulalongkorn University, 1990.

Macko, E., J.A. Weisbach and B. Douglas. "Some observations on the pharmacology of mitragynine," Arch. Int. Pharmacodyn. Therapie. 198(1) : 145 - 154 ; 1972.

Marcan, A. "Report of the government laboratory of Siam," Analist. 54 : 475 - 479 ; 1929.

Marcan, A. "Report of the government laboratory of Siam," Analist. 59 : 753 - 760 ; 1934.

Norakanphadung, P. Pramuan Khuamru Ruang Yaseptit Hai Thot. Bangkok : Thanyarak Hospital ; 1966.

Shellard, E.J. "The alkaloids of Mitragyna with special referance to those of M.speciosa Korth," Bul. Nar. 26(2) : 42 - 55 ; 1974.

Suwanlert, S. "A study of kratom eaters in Thailand," Bul. Nar. 27(3) : 21 - 27 ; 1975.

Thuan, L.C. "Addiction to *Mitragyna speciosa*," Proceedings of the Alumni Association, Malaya. 10(4) : 322 - 324 ; 1957.

Wray, L. "Notes on the anti-opium remedy," Pharmaceu. J. 78 : 453 ; 1907.

Wagner, H., S. Ott., K. Jurcic., J. Morton. and A. Planta Med. 48 : 136 ; 1983.

Zacharias, D.E., R.D. Rosenstein and E.A. Jeffrey Acta Cryst. 18 : 1039 ; 1964.

Zarembo, J.E. and others. "Metabolites of mitragynine," J. Pharm. Sci. 63 : 1409 - 1415 ; 1974.

CURRICULUM VISTA

NAME : SARINTORN CHALERMCHAIVINIJKUL

DATE OF BIRTH : OCTOBER 11, 1967.

PLATE OF BIRTH : BANGKOK, THAILAND

ADDRESS : 4263, SUKHUMVIT ROAD, BANGNA, PRAKHANONG, BANGKOK 10260

INSTITUTION ATTENED

1984 : MATTAYOM SUKSA 6 (LEVEL 12) COMPLETED AT SAINUMPUENG SCHOOL BANGKOK METROPOLIS.

1988 : DEGREE OF BACHELOR OF EDUCATION (MAJOR CHEMISTRY) SRINAKHARINWIROT UNIVERSITY BANGSAEN, THAILAND.

การทดสอบฤทธิ์ของไมตราจินีนและสารสกัดต่างๆ ที่ได้จากใบของต้นกระท่อม

บทคัดย่อ

ของ

สรินทร เฉลิมชัยวินิจกุล

เสนอต่อมหาวิทยาลัยศรีนครินทรวิโรฒ ประสานมิตร เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาเคมีชีวภาพ
กันยายน 2535

การศึกษาฤทธิ์ของสารสกัดส่วนต่างๆ 4 กลุ่มซึ่งแยกได้จากใบกระท่อม ได้แก่ สารสกัดดิบ สารอัลคาลอยด์รวม สารส่วนที่แยกอัลคาลอยด์ออกแล้ว และสารไมตราจินีนบริสุทธิ์ พบว่าสารทั้ง 4 กลุ่มดังกล่าวมีผลในทางระงับประสาทส่วนกลางทำให้การเคลื่อนไหวของหนูขาวลดลง โดยสารสกัดดิบเมื่อฉีดสารปริมาณ 20 และ 50 มิลลิกรัมต่อน้ำหนักตัวหนึ่งกิโลกรัมเข้าทางช่องท้อง มีผลทำให้หนูตายภายในเวลา 15-30 นาที และในระดับปริมาณสารที่เท่ากัน(150 มิลลิกรัมต่อน้ำหนักตัวหนึ่งกิโลกรัม) พบว่าสารสำคัญในการออกฤทธิ์ระงับประสาทประกอบด้วยสาร 2 กลุ่ม กลุ่มแรกคือสารส่วนที่แยกอัลคาลอยด์ออกแล้ว สามารถลดการเคลื่อนไหวของหนูได้ถึง 80 เปอร์เซนต์ กลุ่มที่ 2 คือกลุ่มของสารอัลคาลอยด์ซึ่งจากการทดลองสามารถสรุปได้ว่า สารสำคัญในการออกฤทธิ์ของสารกลุ่มนี้คือสารมิทราจินีนซึ่งเป็นสารอัลคาลอยด์หลักที่มีปริมาณมากที่สุด สามารถลดการเคลื่อนไหวได้ 60-70 เปอร์เซนต์ แต่เมื่อให้สารไมตราจินีนทางปากในปริมาณสูง 250-300 มิลลิกรัมต่อน้ำหนักตัวหนึ่งกิโลกรัมปรากฏผลตรงกันข้ามกับการฉีดสารเข้าทางช่องท้องคือ หลังให้สาร 4-5 นาที หนูจะมีการเคลื่อนไหวมากขึ้นกว่าปกติ 50 เปอร์เซนต์ ดังนั้นผลการทดลองสามารถสรุปได้ว่า ใบกระท่อมมีฤทธิ์ทั้งระงับและกระตุ้นประสาทส่วนกลาง

EVALUATION OF EFFECTS OF MITRAGYNINE AND DIFFERENT FRACTIONS EXTRACTED FROM LEAVES OF MITRAGYNA SPECIOSA KORTH.

AN ABSTRACT

By

SARINTORN CHALERMCHAIVINIJKUL

Presented in partial fulfillment of the requirments for the
Master of Science degree in Biological Chemistry
at Srinakharinwirot University
September 1992

Effects of crude extract, crude alkaloids, non-alkaloids and mitragynine prepared from the leaves of Mitragyna speciosa Korth on locomotor activity were investigated in mice. It was found that all substances, after intraperitoneal administration decrease significantly the animal activity. The crude extract produced the strongest effect, all animals dried within 30 minutes after injection at doses 20 and 50 mg/kg.body weight. The non-alkaloids fraction decreased the animal activity upto 80 percent while the crude alkaloids and the mitragynine produced more or less similar effect. However, the mitragynine after oral administration (250-300 mg/kg.body weight), increased the animal activity more than 50 percent, the onset of the substance is 4-5 minutes after injection. Thus, evidences from this study suggested that most of substances prepared from the leaves of Mitragyna speciosa Korth produced depressive effect. Only mitragynine produce stimulative effect after oral administration.