STUDY OF TOXIC CHEMICALS PRESENT IN MOSQUITO REPELLANTS

A DISSERTATION

SUBMITTED TO THE CENTRAL DEPARTMENT OF CHEMISTRY

IN PARTIAL FULFILLMENT FOR THE REQUIREMENT OF M. Sc. DEGREE IN CHEMISTRY

BY

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2012 (2069)

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M. Sc. Dissertation Entitled
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RECOMMENDATION LETTER

This is to certify that the present work in this dissertation entitled **"Study of Toxic Chemicals Present in Mosquito Repellants"** has been carried out by **Mr. Khem Raj Shrestha** as a partial fulfillment for the requirement of M. Sc. Degree in Chemistry. As far as our knowledge this type of work has not been submitted to this institute.

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ACKNOWLEDMENT

It is my great pleasure and honor to express heartiest gratitude to my supervisor Prof. Dr. Mohan Bikram Gewali, professor of Tribhuvan University and Co-Supervisor Dr. Kanti Shrestha, Senior Scientific officer, Nepal Academy of Science and Technology (NAST) for instilling me the quality of research, dedication and values of hard work. Their constant care, kind supervision, endless encouragement and expert guidance have been major driving force throughout my research work.

I would like to express my sincere gratitude to Associate Professor Dr. Kedar Nath Ghimire, Head of Central Department of Chemistry, T.U., Kirtipur, for providing me an opportunity to conduct this dissertation work. Similarly my sincere gratitude also goes to Prof. Dr. Tulsi Prasad Pathak, former Head of Central Department of Chemistry, T.U. for providing me such a golden opportunity to carry out the dissertation work.

I would like to acknowledge to Nepal Academy of Science and Technology (NAST) for providing all the laboratory facilities and excellent working atmosphere. I wish to express my indebtedness and gratitude to Dr. Dinesh Raj Bhuju, Chief of Science Faculty, NAST for his kind encouragement and guidance.

I am extremely thankful to Prof. Dr. S. P. Dhoubhadel, Prof. Dr. Krishna Manandhar, Prof. Dr. Mangala Devi Manandhar, Prof. J. K. Shrestha, Dr. K. K. Shrestha, Associate Prof. K. R. Palak, Dr. Sushan Joshi, Dr. S. K. Kalauni, Mr. Santosh Khanal, Dr. Bhoj Raj Panta (NAST), Dr. Sajan Lal Shaula (NAST), Dr. Gan Bahadur Bajrachaya (NAST) and Mrs. Tista Prasai (NAST), for their cooperation and guidance during my research.

I would like to thank all my friends especially Amrit Acharya, Prabin Shakya and Hem Prakash Karki, who being the closest friend laid uncountable dedication, constant support, inspiration and care during my research was a major source of strength to complete this research work.

I am extremely thankful to all the wonderful staff and friends of NAST by whom I have been surrounded and enjoyed my work. I am thankful to my colleagues Bijaya Laxmi Maharjan, Rita Mandal, Puspha Sharma, Reena Bayanju, Kamil Prajapati, Dinesh Maharjan, Ramesh Pun, Lawarensa Shrestha, and Madhu K.C. for their spontaneous co-operation and support.

Last but not the least, I would like to express special gratitude and thanks to my highly respected parents Mr. Laxmi Raj Shrestha and Mrs Sitadevi Shrestha, brother Dhurba Shrestha, Keshav Shrestha and sister Parwati Shrestha, Shanti Shrestha for their continuous support and encouragement.

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ABSTRACT

Household pests such as mosquitoes, houseflies, cockroaches etc are important vectors for communicable diseases like malaria, dengue etc. The mosquito repellants are widely used as the insecticides to control the mosquitoes in households. The active ingredients of the mosquito coil are pyrethroid previously called as pyrethrins. This dissertation evaluated the practice of use of different types of mosquito repellants in the selected population and analyzed the chemical constituents present in mosquito repellants with an effort to propose a standard protocol.

Altogether 120 respondents were selected randomly for the survey. A semi-structured questionnaire was prepared. In the chemical analysis, a total of six solid forms of repellants and three liquid forms of mosquito repellants were included. The solid repellants were extracted by using soxlet extraction and shaking extraction method. For soxlet extraction 95% ethanol was used as solvent whereas for shaking extraction method mixture of solution of formic acid and toluene in the ratio of 5:1 was used as solvent. The crude extracts obtained after the extraction was subjected to thin layer chromatography followed by column chromatography with a view to identify the compounds present in them. Gas generated by burning the coil of mosquito repellants was also analyzed. Gas chromatography coupled with mass spectroscopy (GC-MS) was conducted only for the residual solution obtained from smoke after burning the coil. The carbonyl content of the *Surya* mosquito coil and *Octagon Good Knight* mosquito coil samples after burning the respective mosquito coils were analyzed by dinitro-salisylic acid (DNS) method.

Among the respondents, 76% respondents were found to use the chemical agent as the mosquito repellants and rest of them uses net. Among the chemical repellant users, liquid was used by 70%, coil by 22%, mat by 6% and cream by 2% of respondent. In carbonyl compound analysis, *Octagon Good Knight* and *Surya* mosquito coil were found to contain 4.44mg/mL and 4.78 mg/mL of carbonyl compounds respectively. The GC-MS analysis shows that the residual solution of gas collected after burning the *Surya* mosquito coil and *Octagon Good Knight* mosquito coil samples were found to contain allethrin, palmitic acid, acridine, cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester, stearic acid, decanoic acid (carpic acid), octadecanoic acid, hexadecanoic acid, n-heptadecanol-1,3-pentanol-2,3-dimethyl, benzyl benzoate, anthraquinone-2-methyl and cholestadiene.

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ABBREVIATIONS AND ACRONYMS

DEET	:	N,N-diethylnetatoluamide
DMSO	:	Dimethyl Sulfoxide
DNS	:	Dinitro-Salicylic Acid
KAP	:	Knowledge, Attitude and Practice
MOAC	:	Ministry of Agriculture and Cooperatives
NMEP	:	Nepal’s Malaria Eradication Programme
PAHs	:	Polycyclic Aromatic Hydrocarbons
PPD	:	Plant Protection Directorate
λ_{max}	:	Wavelength at Maximum Absorbance
R_f	:	Retention Factor
USAID	:	United States Agency for International Development
TLC	:	Thin Layer Chromatography
UV	:	Ultra Violet

CHAPTER I

1. INTRODUCTION

1.1. Background

Mosquitoes, sand flies, houseflies, cockroaches, ants, fleas, etc are common house hold pests which are responsible vectors for malaria, dengue, yellow fever etc. Pesticides are the commonly used to control the pests. Insecticides repellants mostly used in household in daily life are considered as highly toxic chemicals which are also used in agriculture, medicine, industry and the household arena. Nearly all insecticides have the potential to disturb ecosystems, many are toxic to humans and others are concentrated in the food chain. Mosquito repellants are most common pesticides used in household. Mosquito coils are usually used on a daily basis to control mosquitoes in tropical areas and seasonally in subtropical and temperate areas.[1]

Traditionally, different substances such as the smoke, plant extracts, oils, tars, and muds were used to repel mosquitoes. Later with the development of insect repellant technology individual compounds were discovered and isolated leading to the formulation of new more efficient forms of mosquito repellants. The herbal extract from citronella plant, an Asian grass was the first effective substance used as mosquito repellant. Its repellance was accidentally discovered in 1901. Its effectiveness was limited due to its high volatility. The disadvantages of using citronella oil prompted researchers to study alternative synthetic compounds. The first synthetic compound discovered was dimethyl phthalate in 1929. This material showed a good level of effectiveness against certain insect species, but it was ineffective against some others. Two other materials Indalone and Rutgers 612 (2-ethyl-1,3-hexane diol) were developed as insect repellants. Like dimethyl phthalate, these materials had also certain limitations which prevented their widespread use. Since none of the available materials were ideal repellants, research enter into new synthetic materials continued which led to the discovery of synthesized DEET (N,N-diethylnetatoluamide). The use of DEET was limited by its high toxicity to humans. Later pyrethroid was discovered which was found to be less toxic than the formerly used repellants but highly toxic to pests.

Most repellant chemicals work by interfering with the mosquito's homing system. This homing system located on the antennae, which are made up of a number of chemical receptors. Research had shown that these chemical receptors are activated by lactic acid which naturally evaporates from the skin of warm-blooded animals. The mosquitoes have the innate ability to follow the lactic acid emissions to their source. When a repellant ingredient is applied to the skin or vaporized in environment, it inhibits the binding site of the lactic acid on the chemical receptors of mosquito's antennae which essentially repels the mosquito. [2]

1.2. Household insecticides and its hazards

The flower of Pyrethrums plant (*Chrysanthemum cinerariaefolium*, *C. coccineum*; family *Asteraceae*) produces natural chemical pyrethrins. The seed cases of Pyrethrums plant contain pyrethrins compounds; this plant was commercially grown to supply the insecticide. Pyrethrins are a pair of natural organic compounds namely pyrethrin I and pyrethrin II which have potent for insecticidal activity. Now a day’s new synthetic compounds Pyrethroid and chrysanthemic acids are used as mosquitos repellant which are the derivative of pyrethrins.They are produced industrially in a cyclopropanation reaction of a diene as a mixture of *cis* and *trans* isomers followed by hydrolysis of the ester, they are common in commercial products such as household insecticides and insect repellants, in the concentrations generally considered as harmless to human beings but can harm sensitive individuals.[3]

Pyrethroids are usually combined with piperonyl butoxide that inhibits the key activities of microsomal oxidase enzymes from clearing the pyrethroid from the body of the insect and thus the pyrethroid becomes lethal to the insects. Insects with certain mutations in their sodium channel gene may be resistant to pyrethroid insecticides.[5] Commercial pyrethroid insecticide used are allethrins, bifenthrin, cyfluthrin, cypermethrin, deltamethrin, permethrin, prallethrin, rasmethrin, phenothrin or sumithrin, tetramethrin, tralomethrin, transflutrin, imiprothrin. Among them allethrin, prallethrin, transfluthrin and d-allethrin are the major pyrethroid used in mosquito repellant.

Allethrins are a pair (*cis* and *trans*) of related synthetic compounds used in insecticides. Bifenthrin a 4th generation pyrethroid is insoluble in water, has high persistence in soil, it is the longest residual termiticide. Cypermethrin is fast-acting neurotoxin in insects, easily degraded on soil and plants but can be effective for weeks when applied to indoor inert surfaces, exposure to sunlight, water and oxygen accelerates its decomposition. Deltamethrin one of the safest, most popular and widely used pesticides is a synthetic pyrethroids, it classified as safe for mammals, but highly toxic to aquatic life, and therefore must be used with extreme caution around water.

Permethrin that works as a contact insecticide, cause the nervous system toxicity and leads to the death or knockdown (out of the air) of the insect. It is most effective against mosquitoes, flies, ticks, and chiggers. Permethrin has low toxicity in mammals. It poorly absorbed by the skin and rapidly inactivated by ester hydrolysis. Combination of permethrin treated clothing and skin application of a DEET based repellant creates a formidable barrier against Mosquito bites. Resmethrin is a pyrethroid insecticide with many uses, including control of the adult mosquito population. Phenothrin, also called sumithrin, is a synthetic pyrethroid that kills adult fleas and ticks; it has also been used to kill head lice in humans. Phenothrin is often used with methoprene, an insect growth regulator that interrupts the insect's biological life cycle by killing the eggs. Tralomethrin is also a potent insecticide that kills insects by modifying the gating kinetics of the sodium channels in neurons, increasing the length of time the channel remains open after a stimulus, thereby depolarizing the neuron for a longer period of time, this leads to uncontrolled spasming, paralysis, and eventual death. Transfluthrin used in the indoor environment against flies, mosquitoes and cockroaches is relatively volatile substance and acts as a contact and inhalation agent.

Researches has shown that mosquito coil smoke have many potential adverse health effects, but large populations in developing countries still use mosquito coils in their daily lives. In previous studies of various aspects of mosquito coil smoke, emissions of irritating and carcinogenic compounds and other pollutants have not been quantified, which precludes the use of emission rate data to predict pollutant concentrations in households and to quantify health risks. But due to lack of comparative data on the smoke emissions from different types of mosquito coils, no informative recommendations to consumers regarding the mosquito coil that have lower emissions of health-damaging pollutants can be provided. It is thus necessary to perform tests of coil emissions in a systemic manner.[4]

Pyrethrins are neurotoxins that attack the nervous systems of insects. When present in amounts not fatal to insects, they have an insect repellant effect. Pyrethrins are harmful to fish, birds, and mammals, including human beings. In human beings, pyrethrin irritates the eyes, skin, and respiratory systems, and may cause other harmful effects. Pyrethrins are gradually replacing organophosphates and organochlorides. The derivative compound of pyrethrin are called pyrethroid that are axonic poisons and it works by keeping the sodium channels open in the neuronal membranes of insects, the sodium channel is a small hole through which sodium ions are permitted to enter the axon and cause excitation. As the nerves cannot de-excite, the insect is paralyzed. Now days, the most active chemical component of mosquito repellant is pyrethroids. Among the pyrethroid: Alletrhin, Pralllethrin, d-Alletrhin, Transflutrin etc. are most common.[6] basically, three forms of mosquito repellant as coil, mat and liquid are available in market. Mosquito coils are the preferred anti mosquito products in low income countries. The mosquito coil is widely known as an efficient mosquito repellant. The major active ingredients of the mosquito coil are pyrethrins accounting for about 0.3–0.4% of coil mass .The remaining components of mosquito coil are organic fillers, binders, and dyes.[7,4]

When a mosquito coil is burned, the insecticides evaporate with the smoke, which prevents the mosquito from entering the room. It is believed that Pyrethroids are of low chronic toxicity to humans and low reproductive toxicity in animals, although headache, nausea, and dizziness were observed in male sprayers exposed to 0.01-1.98 $\mu g/m^3$ pyrethrins for 0.5-5 hr. No carcinogenic and mutagenic effects have been found for these insecticides. The lowest lethal oral dose of pyrethrum is 750 mg/kg for children and 1,000 mg/kg for adults. But the combustion of the remaining materials besides active chemical generates large amounts of sub-micrometer particles and gaseous pollutants. These sub-micrometer particles can reach the lower respiratory tract and may be coated with a wide range of organic compounds, some of which are carcinogens or suspected carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) generated through incomplete combustion of biomass (mosquito coil base materials).

Researchers have found that the gas phase of mosquito coil smoke contains some carbonyl compounds with properties that can produce strong irritating effects on the upper respiratory tract like formaldehyde and acetaldehyde. Because coil consumers usually use mosquito coils for at least several months every year, cumulative effects from long-term exposure to the coil smoke may also be a concern. Burning one mosquito coil releases the same amount of particulate matter (PM2.5) as burning 75 – 137 cigarettes. Also, the emission of formaldehyde from burning one coil can be as high as that released from burning 51 cigarettes. The bioaccumulation and biomagnifications of active chemical from mosquito repellant are found to be deposited and metabolized in lung, liver and kidney. Epidemiologic studies have shown that long- term exposure to mosquito coil smoke is associated with asthma and persistent wheeze in children.[8,3]

Toxicological effects of mosquito coil smoke on tested rats were found to be focal deciliation of the tracheal epithelium, metaplasia of epithelial cells, and morphologic alteration of the alveolar macrophages. In addition, the levels of total protein and lecithin and the activities of lactate dehydrogenase, acid phosphatase, and ß-glucuronidase in the lung-lavage fluid were found significantly higher.[9,2]

However, due to the lack of effective implementation, exact quantification, open trade of import, there may be chances of vulnerable condition if not control in time. Moreover, there is no governing body of the government to regulate the threshold limit of active ingredient content in pesticides such as mosquito repellant. There is no authentic protocol on chemical and biological analysis of mosquito repellant. Therefore this study will evaluate knowledge, attitude and practice regarding the mosquito repellant user and physiochemical properties of mosquitoes and their active compound and help to set standard quality protocol recommendation to formulate the national policy regarding pesticides for regulating or control the quality of pesticides produced by the industry operated under private or government sectors[10].

CHAPTER II

2. OBJECTIVE OF THE STUDY

2.1. General objective

This study will survey the use of the mosquito repellants and analyze the chemical constituents of different mosquito repellants.

2.2. Specific objectives

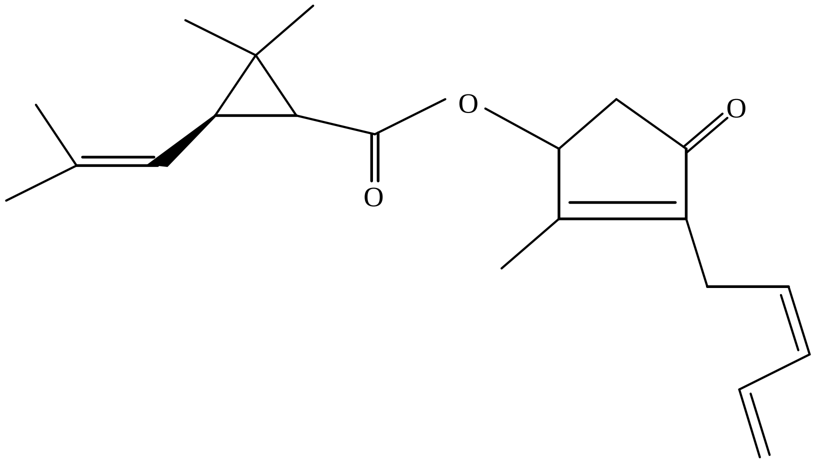
1. To evaluate the practices regarding the mosquito repellant users of Kathmandu valley.
2. To analyze the chemical constituents of the mosquito repellants.

CHAPTER III

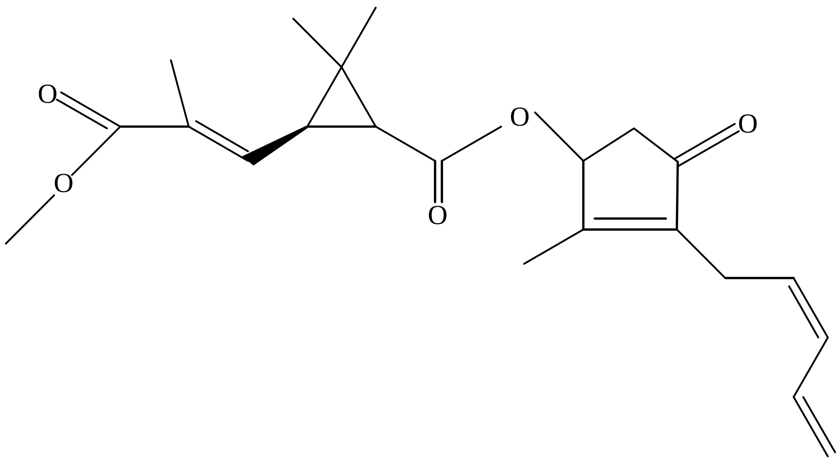
3. LITERATURE REVIEW

3.1. General structure of Pyrethrin and Pyrethroid

Pyrethrins are naturally occurring chemicals found in the flower *Chrysanthemum cinerariaefolium* and *C. coccineum* of family Asteraceace. Pyrethrins are pair of natural organic compounds that have potent insecticidal activity. The chemical structures of pyrethrins (pyrethrin I and pyrethrin II) are shown below

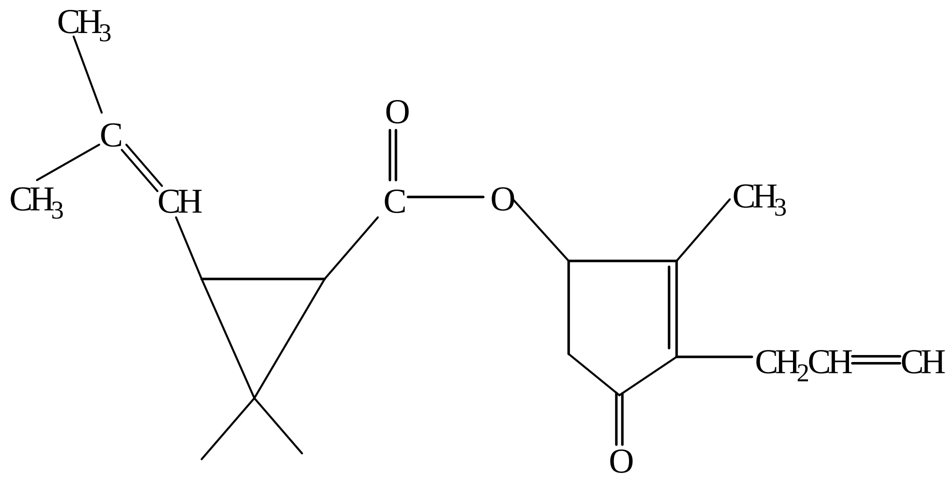


Pyrethrin I

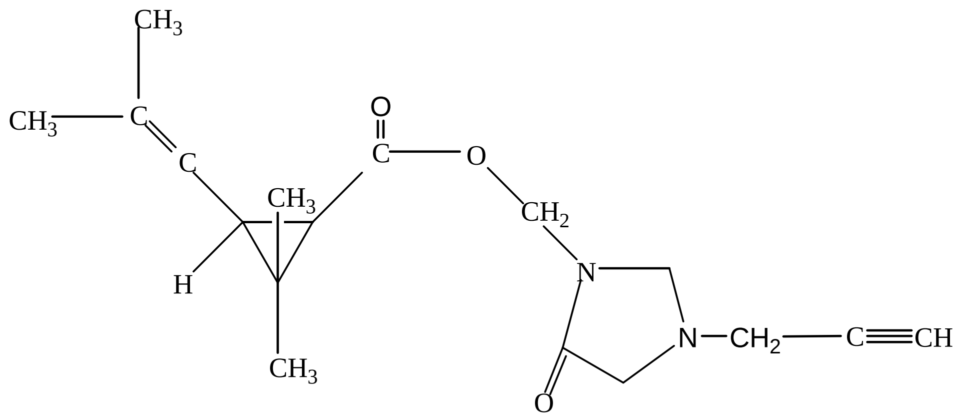


Pyrethrin II

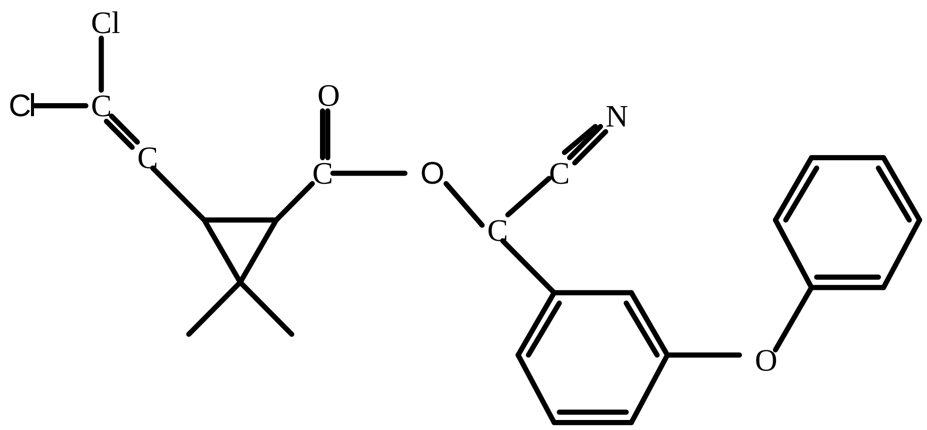
Pyrethroids are synthetic chemical compounds similar to the natural chemical pyrethrins. The *chrysanthemic acid* is produced industrially in a cyclopropanation reaction of a diene as a mixture of *cis*- and *trans* isomers followed by hydrolysis of the ester, they are common in commercial products such as household insecticides and insect repellants, these products are generally considered as harmless to human beings but can harm sensitive individuals.[8 5] They are usually broken apart by sunlight and the atmosphere in one or two days. There are all about 13 commercial pyrethroid insecticides which are allethrin, bifenthrin, cyfluthrin, cypermethrin, deltamethrin, permethrin, prallethrin, rasmethrin, phenothrin or sumithrin, tetramethrin, tralomethrin, transflutrin, Imiprothrin. The structures of different pyrethroid are as follows.



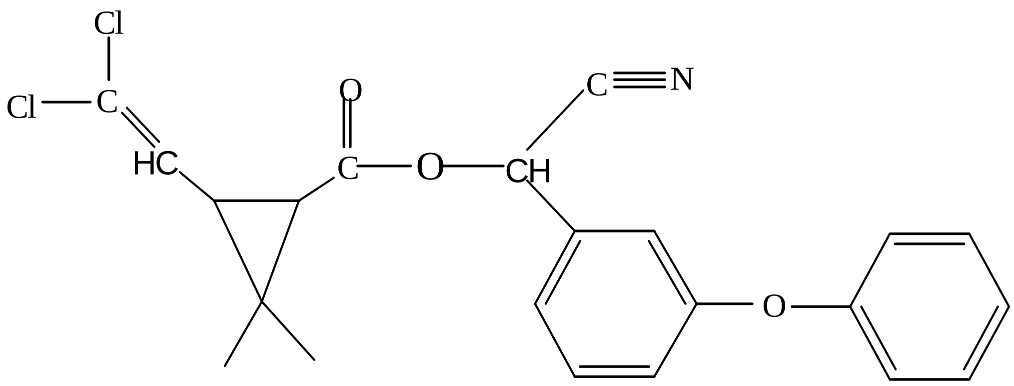
Allethrin



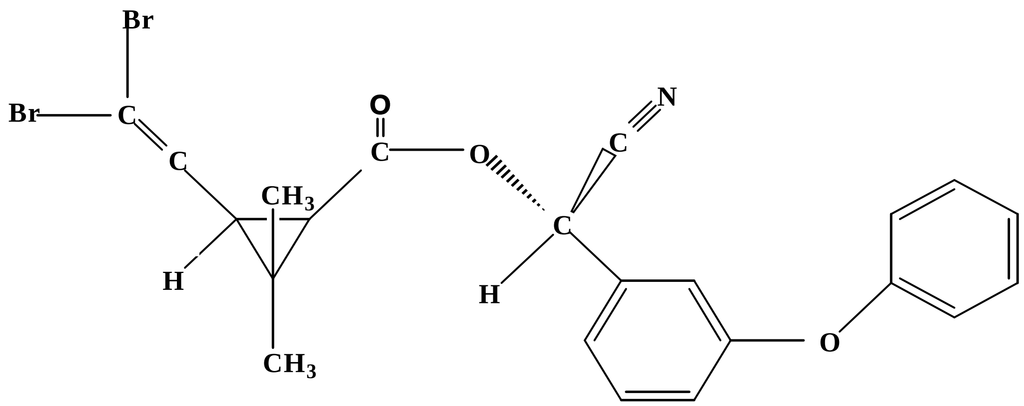
Imiprothrin



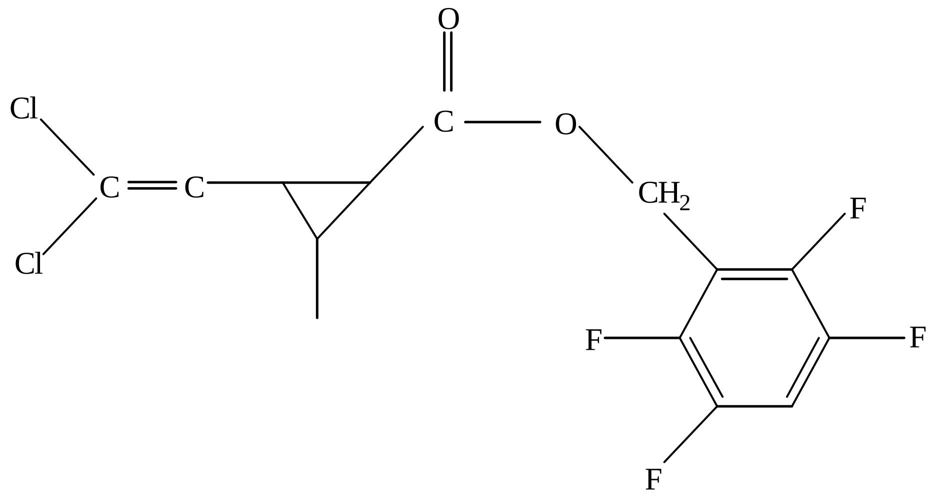
Cyfluthrin



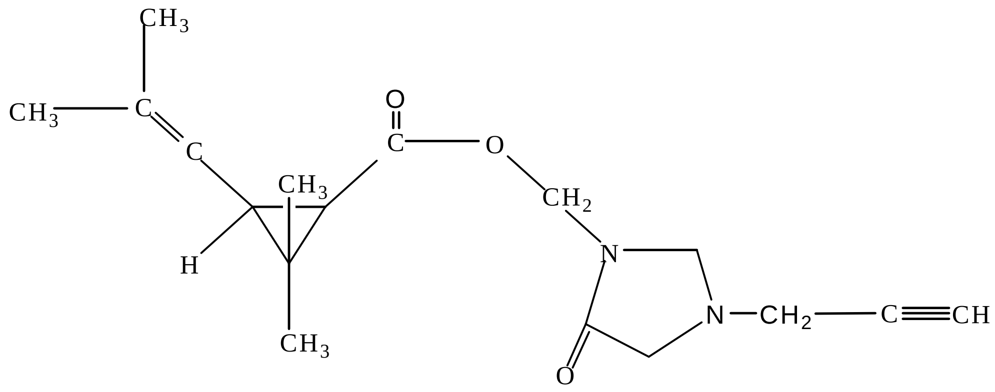
Cypermethrin



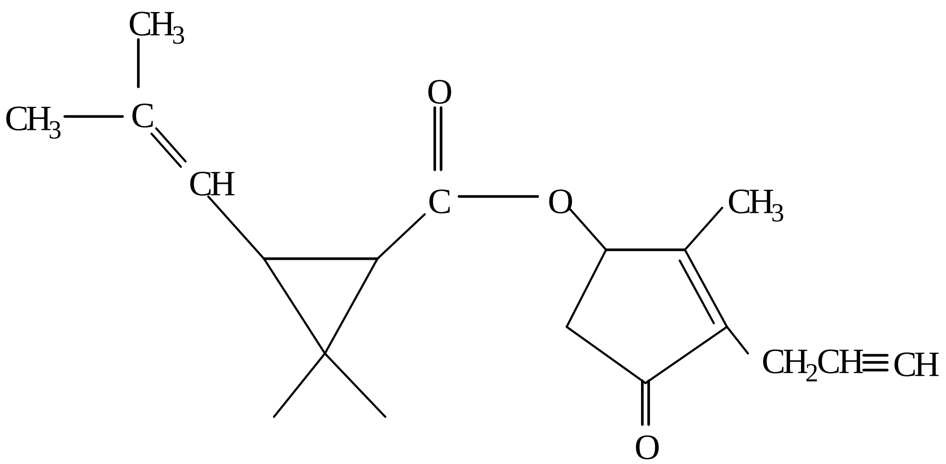
Deltamethrin



Transfluthrin



Imiprothrin



Prallethrin

3.2. Mosquito repellant

Mosquito repellant have been used for more than 40 years in view of their wide availability, and consequently accounting for 25% of the world insecticide market. Its use and demand is increasing day by day, their use has risen dramatically over the past 10 years in India. Mosquito coils, considered as the most efficient repellant, is the preferred mosquito repellant in low income countries. The major active ingredients of the mosquito coil are pyrethrins (now Pyrethroid), accounting for about 0.3–0.4% of coil mass and remaining components are organic fillers, binders, dyes, and other additives capable of smoldering.

3.3. Mosquito coils and health effects

Insecticides like mosquito coils made up of toxic chemicals posed Toxicological, Ecological effects. Toxicological effects of mosquito coil smoke focal deciliation of the tracheal epithelium, metaplasia of epithelial cells, and morphologic alteration of the alveolar macrophages.[13] Epidemiologic studies have shown that long- term exposure to mosquito coil smoke is associated with asthma and persistent wheeze in children.[14,12] Mosquito coil use (relative to non-use) (1.27; 0.99 to 1.62; p=0.058) had an impact on the respiratory health of male adults in Hong Kong.

Researchers from the University of California- Riverside analyzed mosquito coils in Jakarta and Bandung, Indonesia, in addition to others in Southern California. The mosquito coils purchased in the US contained octachlorodipropyl ether, known as S-2, a substance not registered for any legal use in the US. The packaging did not indicate S-2 was an ingredient. Use of those coils likely exposes those around it to bis (chloromethyl) ether, or BCME, an extremely potent lung carcinogen. However, an improved mosquito coil consists of sawdust, coconut shell flour, and pyrethroid compound and potato starch as a carrier of an insecticide.[15]

The active ingredients in mosquito that is pyrethrium or synthetic pyrethroid along with other constituents such as organic fillers, binder, fungicides and other additives in the coils. When burned releases about 70 % of its originally containing Cadmium, Cromium, lead, and about 99% of allethrin.[19]

Indoor pyrethroid exposure is of considerable magnitude in India and other countries including the United States due to widespread use of pyrethroid-based repellants to control pests such as mosquitoes and cockroaches because of their high insecticidal and low mammalian acute toxic effects. Severe toxicity of pyrethroids has not been found in developed countries, it appears to be common in developing countries because of their extensive and intensive use for agricultural and domestic purposes. However, the chronic toxic effects of these compounds in humans are not yet reported.

The GC-MS report in previous studies of the mosquito coil smoke have qualitatively identified sixteen compounds including allethrin, benzene, toluene, xylene, o-tertbutylphenol, eicosyne and several polycyclic aromatic hydrocarbons (PAHs such as benzopyrene and benzofluoranthene).[5,20] The smoke contained three polycyclic aromatic hydrocarbons classified by the United States Environmental Protection Agency (USEPA) as probable human carcinogens benzopyrene, benzo fluoranthene, and benzofluoranthene. The researchers also found relatively high concentrations of volatile organic compounds such as benzene, a neurotoxicant and carcinogen that can affect bone marrow with chronic exposure.

3.4. Pesticide use in Nepal

The modern use of pesticides dates back to 1867, when *Paris green* was first used to manage *Colorado potato* beetle. After that, various inorganic or plant originated pesticides came into existence. The successful discovery of the use of 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane or dichloro-diphenyl-trichloroethane (DDT) by the Swiss scientist Paul Hermann Müller in 1939 opened the flood gates worldwide for more and more pesticide synthesis and use, especially for the control of agricultural pests and vector-borne diseases like mosquito.

Pesticide use is still a common practice for the control of insect pests worldwide, and Nepal is no exception. Although in recent years the use of toxic chemicals for health purposes has reduced drastically, on the other hand it is continuously increasing in the field of agriculture and household. Pesticides were introduced in Nepal in 1950s with the aim of controlling of pest for a resettlement program at the southern plains of the country. Nepal’s Malaria Eradication Programme (NMEP) initiated the use of pesticides in Nepal. Subsequently, in November 1952, DDT was introduced followed by *Paris green*, *Gammexene* and *nicotine sulfates* in 1955 by Ministry of Health (MOH), Government of Nepal (GON). These pesticides were imported under the support of USAID, Japan International Cooperation Agency (JICA), British ODA, World Health Organization (WHO) and Danish International Development Agency (DANIDA). In 1993 more insecticides were imported by NMEP as grant assistance for controlling black fever ('*Kalajar*'). Most of the pesticides used at that times were mostly from the *organochlorine* group. Similarly, the Department of Agriculture (DOA) started to apply chemical pesticides in the agricultural sector for pest control purposes from 1956. The chronological order of different groups of pesticides introduced in Nepal is: 1950s-*organochlorines;* 1960s-o*rgano phosphates*; 1970s - *carbamates*; 1980s - *synthetic pyrethroids*.

Allethrin: It is yellow to amber colored viscous liquid with a mild or slightly aromatic odor. It may decompose when exposed to heat or light. The chemical name is 2-methyl-4-oxo-3-(2-propenyl)-2-cyclopenten-1-yl-2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropanecarboxylate with CAS number of 584-79-2 (allethrin); 42534-61-2 (d-allethrin).

Transfluthrin: It is one of the best-tested insecticidal agents, and has been incorporated in mosquito liquid products against flying insects since 1996. In regard to its structure, toxicology and principle of action on insects' nerves, Transfluthrin is regarded as one of the fast-acting pyrethroids with low persistency. Transfluthrin is highly selective. Low quantities are exceptionally powerful against hygiene, health and material pests in the indoor environment. The excellent knock-down action of Transfluthrin at an extremely low concentration permits its use especially in products to combat flies, mosquitoes and cockroaches.

Prallethrin: It is the synthetic pyrethroids have 0.87%w/w solution for insecticidal action. Pure prallethrin in kerosene is used for the indoor amateur against the mosquito only. The liquid formulation is presented in a sealed polyethylene container to be inserted into a specifically desigened electrical heater. Prallethrin has the common name α-methyl-4-oxo-3-9prop-2-ynyl)-cyclo-pent-2-enyl and IUPAC name is *cis*; *trans*-2; 2-dimethyl-3-(2-methyl prop-1-enyl) cyclo propane carboxylate.

CHAPTER IV

4. MATERIALS AND METHODOLOGY

In this study, both field survey and laboratory experiments were carried out to achieve the objectives of the study. This chapter provides stepwise explanation of the methods applied in this research. The details of each step of the used methodology are described in the following sub-sections.

4.1. Survey analysis

Survey analysis was done in different places of Kathmandu and Lalitpur. For the study, people from different professions were selected. The study focused the uses of different types of mosquito repellants. A semi-structured questionnaire was developed and primary data was collected from the respondent by interviewing them.

4.2. Experimental

Nine different varieties of mosquito repellants were purchased from the market, of which six were in solid form and three in liquid form. The collected mosquito repellants samples were *Ocatagon Good Knight* mosquito coil, *Surya* mosquito coil, *Fumikilla* mosquito coil, *Kingtox* mosquito coil, *Super mat*, *Goodknight mat* where as *Goodknight Advanced*, *All Out Double Power* and *Mortin* were liquid samples.

4.2.1. Extraction

Extractions of active ingredients from the samples were done by soxhlet extraction as well as shaking extraction for solid samples where as liquid samples were directly analyzed by TLC followed by column chromatography.

4.2.2. Soxhlet extraction

Ten gm of powder was employed for soxhlet extraction at 95% methanol for 6 hours. The solvent was evaporated in Rota evaporator under reduced pressure to yield semi-solid product. This viscous methanol extract was then mixed with charcoal powder and heated repeatedly until color of coil samples disappeared, then it was left to dry for 1 day. Then the crude weight of different sample was determined.

4.2.3. Shaking extraction method

Ten gm of pulverized mosquito coil was taken in volumetric flask and mixed with toluene and 99% formic acid mixture in the ratio of 5:1, and then it was shaken vigorously for half an hour. The mixture was filtered to remove the unwanted material present in the coil, and then filtrate was mixed with the charcoal and warmed to remove the color of the coil. It was then left to dry 1 day. The crude weights of different samples were then determined.[34]

4.2.4. Determination of chemicals by TLC

TLC for the crude substance was performed using precoated TLC glass plates covered with silica gel 60 F_{252} (E.Merck, FRG), which was activated at 110°C for 30 min just before use. On these plates, 2µl aliquots of the 5mg/mL acetone solutions of crude sample were spotted and run under the solvent system hexane: benzene: acetone in the ratio of 9:2:4. R_f value of the different samples were determined. The visualization of spot was determined by placing the TLC plate in iodine chamber and UV light showing the yellow color spot.[18]

4.2.5. Determination of chemicals by column chromatography

The fractions of all samples both solid and liquid were subjected to the column chromatography. The length and diameter of sintered column used were 40 cm and 2 cm respectively. The silica gel (25 gram silica) in the form of slurry was packed to the height of 30cm. The slurry was prepared by mixing hexane with silica. The slurry was applied to the column and eluted subsequently with the solvents of increasing polarity in the order of hexane, acetone and methanol. A number of fractions were collected and their natures on TLC were determined.

4.2.6. Characterizations of chemicals

Repeated column chromatography followed by preparative TLC technique was employed for the detection of the compounds. The pure spotted compounds were characterized by comparing R_f value of pyrethroids compounds.

4.3. Analysis of chemical compounds present in smokes residue after the burning of mosquito coil

The samples obtained from burning the mosquito coils were dissolved in water and toluene to prepare the aqueous and non–aqueous solvents respectively which is further described below. The protocol as provided by Liu et al [5] was followed.

4.3.1. Method of sample preparation

A glass box with a dimension of $30\times14.5\times23$ cm^3 was taken. On one side of the box, two holes with a diameter of 1 cm and 10 cm apart was made. One hole was used as the inlet for the fresh air and another hole was used as outlet for the burnt mosquito smoke. The outlet was connected to a Buchner flask having toluene as solvent through a delivery tube. The delivery tube was dipped in the solvent taken. A mosquito coil weighing 13 gm was burned inside the glass box. The Buchner flask was connected to the suction, which created reduced pressure in the flask and due to which smoke moved smoothly and mixed thoroughly with the toluene solvent in the flask. Similar procedure was repeated with solvent water. After complete burning of the coil the solution was made to 100 mL by adding respective solvent (toluene/water). Two most abundantly used coils namely, *Octagon Good Knight* mosquito coil and *Surya* mosquito coil were burned in the box and two solvent toluene and water were used correspondingly.

4.4.2. Determination of chemicals by column chromatography

The toluene and water fraction (10mL) was subjected to column chromatography as described in section 4.2.5. The solvent system was as hexane, ethyl acetate and methanol.

4.4.3. Characterization of chemicals

Repeated column chromatography, and co-TLC, techniques were employed for the detection of the compounds as described in section 4.2.4.

4.4.4. Estimation of the carbonyl compound

The sample obtained from combustion of single coil was dissolved in toluene solvent preparing making the final volume to 100 mL. It was then analyzed for the concentration of carbonyl compounds by dinitro-salicylic acid (DNS) method in which 1 mL of prepared sample was taken in a test tube and to this 3 mL of DNS solution was added. Then the mixture was subjected to heat in a water bath at 100^0C for 10 minute and cooled in cold water. The color developed in the mixture solution was analyzed for carbonyl concentration by using spectrophotometer, taking absorbance at A_{540}. The results were compared with plotting standard curve by using different concentration of acetaldehyde solution as standard.

4.4.5. Gas Chromatographic analysis of smoke sample

The chemical constituents present in the sample obtained after burning mosquito coil were analyzed by GC-MS (Shimadzu-QP-2010 plus, Japan). The chromatographic column was 30 meter in length with diameter of 0.25mm coated with a 0.25-μm film was used. The GC oven temperature programmed was as follows $300^{o}C$, hold for 2 min, $150^{o}C\ min^{-1}$ to $250^{o}C$, hold for 6 min. Helium was used as a carrier gas at a constant flow of 1.2 mL min^{-1}. The temperatures of injector, transfer line, manifold and trap were set at 250, 290, 120 and 250 °C, respectively. The ion trap mass spectrometer was operated in the electron-impact mode, with ionization energy of 70 eV.

CHAPTER V

5. RESULTS AND DISCUSSION

5.1. Analysis of the data from the respondent

In order to find out which type of mosquito repellants were used by the Kathmandu valley, a semi-structured questionnaire (Appendix I) was prepared. In which 120 respondents responded the questionnaire. On the survey analysis of the respondents, most of them were found to use chemical repellants in Kathmandu valley. Almost 76% of the total users were found to use chemical repellants like liquid, mat, coil, cream and only 24% were found to use the net. Out of 76% of chemical repellant users, 70% respondents used liquid mosquito repellants, 22% used mosquito coils, 6% used mats and 2% used cream as mosquito repellants, respectively as shown below in pie chart.

Figure 3: **Percentage of the repellant users and non-users**

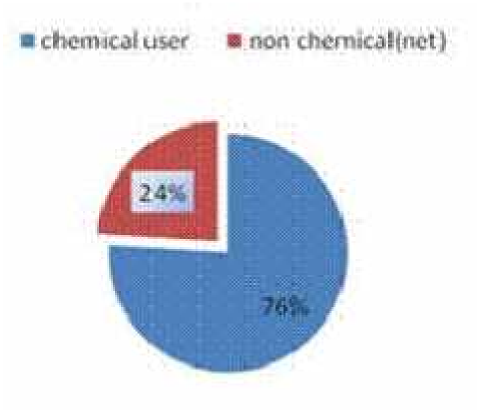
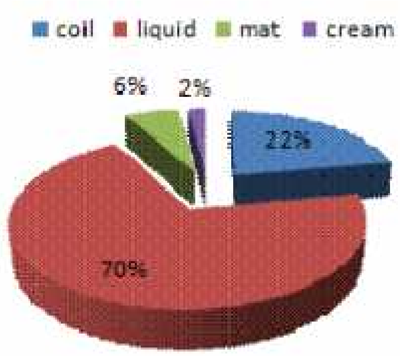


Figure 4: **Distribution of the repellants used by the respondents**



5.2 Chemical analysis of mosquito repellants samples

5.2.1 Coil sample

5.2.1.1 Soxhlet extraction

From ten gram of pulverized solid sample after soxhlet extraction, the weight of crude extract obtained were 0.12 gm (1.2%), 0.16 gm (1.6%), 0.11 gm (1.1%) 0.10 gm (1.0%), 0.09 gm (0.9%), and 0.12 gm (1.2%) from *Surya* mosquito coil, *Octagon Good Knight* mosquito coil, *Kingtox* mosquito coil, *Fumikilla* mosquito coil, *Super mat* and *Goodknight mat* respectively as shown in Table 1.

Table 1: Weight of crude extracts obtained by soxhlet extraction

S.N.	Name of sample	Crude extract weight (in gm)	Percentage (%)
1	*Surya* mosquito coil	0.12	1.2%
2	*Octagon Good Knight* mosquito coil	0.16	1.6%
3	*Kingtox* mosquito coil	0.11	1.1%
4	*Fumikilla* mosquito coil	0.10	1.0%
5	*Super mat*	0.09	0.9%
6	*Goodknight mat*	0.12	1.2%

5.2.1.2 Shaking solvent extraction method

From 10 gm of pulverized solid sample after shaking method the weights of crude extracts obtained were 0.06 gm (0.6%), 0.06 gm (0.6%), 0.11 gm (0.1%), 0.03 gm (0.3%), 0.09 gm (0.9%) and 0.04 gm (0.4%), from *Surya* mosquito coil, *Octagon Good Knight* mosquito coil, *Kingtox* mosquito coil, *Fumikilla* mosquito coil, *Super mat* and *Goodknight mat* respectively as shown in Table 2.

Table 2: Weight of crude extracts obtained by shaking solvent extraction.

S.N.	Name of sample	Crude extract weight (in gm)	Percentage (%)
1	*Surya* mosquito coil	0.06	0.6%
2	*Octagon Good Knight* mosquito coil	0.06	0.6%
3	*Kingtox* mosquito coil	0.11	0.1%
4	*Fumikilla* mosquito coil	0.03	0.3%
5	*Super mat*	0.09	0.9%
6	*Goodknight* mat	0.04	0.4%

5.2.2 Liquid sample

Among liquid mosquito repellants, three samples namely *All out Double powder*, *Goodknight advanced active* and *Mortin* were collected and analyzed. On successive analysis by TLC followed by column chromatography elutant no 21-30 show distinct spot on prepared TLC plate under solvent system hexane:benzene:acetone::9:1:1 having R_f value 0.52, 0.43 and 0.43 respectively as shown in Table 3.

Table 3: TLC analysis of fractions obtained from column chromatography of different liquid samples

SN	Sample (Brand)	Fraction	TLC Solvent system	R_f
1	*All Out Double Powder*	5% acetone in hexane	Hexane:benzene:acetone 9 : 1 : 1	0.52
2	*Goodknight Advanced Active*	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.43
3	*Mortin*	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.43

5.2.3 Solid sample

Sample of soxhlet extraction

From ten gm of pulverized powder sample, after soxhlet extraction TLC was employed followed by repeated column chromatography and preparative TLC for the detection of the compounds. In which fraction 21-30 gave distinct two spots on TLC. The fraction having single spot was concentrated under reduced pressure in which pale yellowish color was obtained. TLC followed by column chromatography results from the 21-30 fraction of surya mosquito coil have R_f value 0.42 and 0.48 in solvent system hexane:benzene:acetone::9:2:4 as shown in Table 4.

Similarly for the samples *Octagon Good Knight* mosquito coil, *Kingtox* mosquito coil, *Fumikilla* mosquito coil, *Goodknight mat and Super mat* same procedures were employed. The details of fraction, R_f value and solvent systems are shown in Table 4.

Table 4: TLC analysis of fractions obtained from column chromatography of different solid samples

SN	Sample (Brand)	Fraction	TLC Solvent system	R_f
1	*Surya* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.42 0.48
2	*Octagon Good Knight* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.53
3	*Kingtox* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.40 0.48
4	*Fumikilla* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.63 0.71
5	*Goodknight* mat	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.52
6	*Super* mat	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.43 0.48

Sample of shaking extraction

Solvent extraction was done by taking 10 gm of pulverized sample. After removal of the solvent, TLC followed repeated column chromatography and preparative TLC techniques for the detection of the compounds. Fraction 21-30 gave distinct two spots on TLC. The fraction having distinct spot was concentrated under reduced pressure which pale yellowish color. TLC followed by column chromatography results from the 21-30 fraction of *Surya* mosquito coil have R_f value 0.44 and 0.49 in solvent system hexane:benzene:acetone::9:1:1 as shown in Table 12. Similarly for the samples *Octagon Good Knight* mosquito coil, *Kingtox* mosquito coil, *Fumikilla* mosquito coil *Goodknight* mat and *Super* mat same procedures were employed. The details of fraction, R_f value and solvent systems are shown in Table 5.

Table 5: TLC analysis of fractions obtained from column chromatography of different solid samples

SN	Sample (Brand)	Fraction	TLC Solvent system	R_f
1	*Surya* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 1 : 1	0.44 0.49
2	*Octagon Good Knight* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 1	0.52
3	*Kingtox* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.40 0.47
4	*Fumikilla* mosquito coil	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.63 0.71
5	*Goodknight* mat	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0.52
6	*Super* mat	5% acetone in hexane	Hexane:benzene:acetone 9 : 2 : 4	0..39 0.51

In the study conducted by Ogierman and Silowiecki[18] the solvent system used was also n-hexane-benzene and acetone as the mobile phase in silca gel for separation of pyrethroid compound.They obtained two spot having R_f values 0.40 and 0.48. The two R_f value denotes the *cis* and *trans* isomers of the pyrethroid compounds.[18] The similar R_f values were also found in this study which indicated the presence of pyrethroid compounds in the sample of mosquito coils.

5.2.4. Analysis of smoke (gas) generated by burning the mosquito repellant coil:

The solution of residue in toluene and water separately collected after burning the sampled coil (*Surya* mosquito coil, *Octagon Good Knight* mosquito coil) in a box as mentioned in section 4.3.1 was subjected to the column chromatography. The protocol given by Weili l et al was followed.[5]

In water extract *Surya* mosquito coil sample, fraction 21-30 gave a single spot with minor other spots on TLC with Rf values 0.42 and 0.56 in EtOAc:hexane::3:9 solvent system where as in and *Octagon Good Knight* mosquito coil sample, fraction 21-30 gave two spots on TLC with R_f values 0.43 and 0.54 in EtOAc:hexane::3:9. As shown in Table 6.

Similarly in water extract *Surya* mosquito coil sample, fraction 21-30 gave a single spot on TLC with Rf values 0.51 in EtOAc:hexane::3:9 solvent system where as in *Octagon Good Knight* mosquito coil sample, fraction 21-30 gave spot on TLC with R_f value 0.54 in EtOAc:hexane::3:9. As shown in Table 7.

Table 6: TLC analysis of fractions obtained from column chromatography of gas residues in water extract

SN	Sample (Brand)	Fraction	TLC Solvent system	R_f
1	*Surya* mosquito coil	1%ethyl acetate in hexane	Ethyl acetate: hexane 3 : 9	0.42 0.56
2	*Octagon Good Knight* mosquito coil	1%ethyl acetate in hexane	Ethyl acetate: hexane 3 : 9	0.43 0.54

Table 7: TLC analysis of fractions obtained from column chromatography of different gas residues in toulene extract

SN	Sample (Brand)	Fraction	TLC Solvent system	R_f
1	*Surya* mosquito coil	1%ethyl acetate in hexane	Ethyl acetate: hexane 3 : 9	0.51
2	*Octagon Good Knight* mosquito coil	1%ethyl acetate in hexane	Ethyl acetate: hexane 3 : 9	0.54

Table 8: Carbonyl compound analysis by DNS method

SN	Sample (Brand)	mg/mL
1	*Surya* mosquito coil	4.44
2	*Octagon Good Knight* mosquito coil	4.78

The carbonyl compound was measured by using dinitro-salicylic acid (DNS) method as recommended by Miller(1959).[32] A study conducted in Taiwan, aliphatic aldehydes were reported to be found in mosquito coil smoke. The volatile aldehydes present in smokes are hazardous to the health.[6]

5.3. GC-MS analysis of smoke sample

Table 9: Chemicals present in *Surya* mosquito coil from water extract

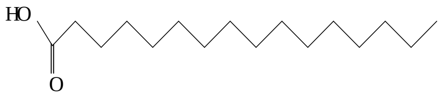
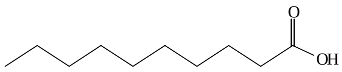
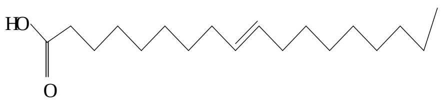
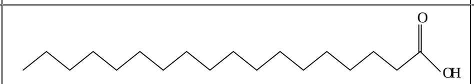
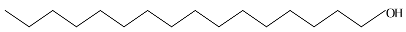
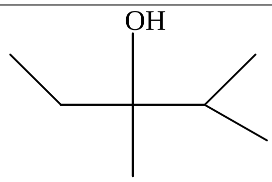
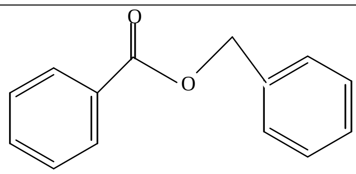
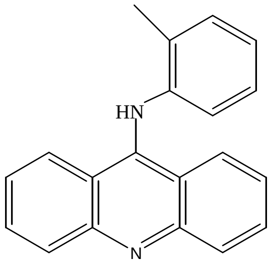
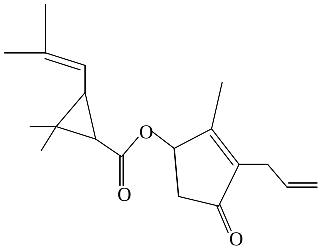
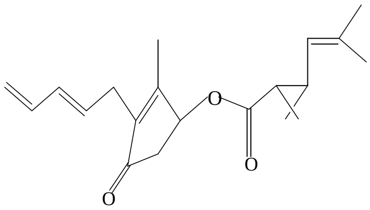
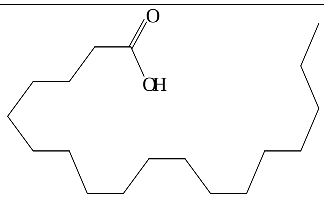
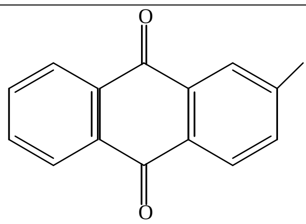
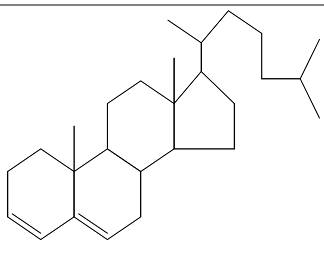
S.N.	**Retention Time in GC**	**Molecular ion peak m/z**	**Structure**	**Compound**
1	17.97	270		Palmitic acid
2	18.21	172		Decanoic acid
3	19.18	296		9-Octadecanoic acid methyl ester
4	19.33	298		Octadecanoic acid
5	19.44	256		n-Heptadecanol-1

Table 10: Chemicals present in *Octagon Good Knight* mosquito coil from toluene extract

S.N.	**Retention Time in GC**	**Molecular ion peak m/z**	**Structure**	**Compound**
1	14.58	116		3-pentanol-2,3-dimethyl
2	15.32	212		Benzyl Benzoate

3	15.58	284		Acridine
4	17.54	302		Allethrin
5	17.78	328		Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester
6	18.09	284		stearic acid
7	18.14			Anthraquinone-2-methyl
8	19.99	368		Cholestadiene

From GC-MS analysis of the water extract of the *Surya* mosquito coil, the major constituent were found to be palmitic acid, decanoic acid (carpic acid), 9-octadecanoic acid methyl ester, octadecanoic acid and n-heptadecanol-1. These compounds are primarily used for imparting pleasant odor [33].

Similarly, in GC-MS the analysis for toluene extract of *Octagon Good Knight* mosquito coil was found to have the major constituents as 3-pentanol-2,3-dimethyl, benzyl benzoate, allethrin, acridine, cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester, stearic acid, anthraquinone-2-methyl and cholestadiene.

The compound cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester is reported to be toxic in inhalation, ingestion, or skin contact with material may cause severe injury or death. The contact with molten substance may cause severe burns to skin and eyes. Whereas when heated, vapors may form explosive mixtures with air indoors, outdoors, and sewers explosion hazards. Linoleic Acid hydroperoxide is formed during free radical attack on long-chain unsaturated fatty acids with linoleic acid (octadecanoic acid) which is an important source of biomembrane damage and is implicated in the onset of atherosclerosis, hepatic diseases, and food rancidity. 3,5-cholestadien-7-one (Oxysterol) can induce the cytotoxic effect and the apoptosis on gallbladder epithelial cells. Acridine is a known human carcinogen that causes frame shift mutations in incorporating into the DNA, and doing so creating an additional base on the opposite strand. If that mutation occurs in a coding sequence, it almost always leads to inactivation of the protein it encoded. Benzyl Benzoate is more risky to elderly patients who are more likely to have age-related xerosis of the skin, which may make their skin more susceptible to the drying effects of benzyl benzoate, and irritation may be worse in this age group. 2-Methyl anthraquinone is explosive in nature, harmful by inhalation, contact with skin and if swallowed. It can cause severe burns, irritating to the eyes, respiratory system and skin. Limited evidence of a carcinogenic effect has been found. It may cause heritable genetic damage in humans and is toxic to aquatic organisms and possible risk of impaired fertility.[32,33]

In the similar work conducted in smoke of mosquito coils in Tamilnadu India, Ahamedabad India, USA and Taiwan.[5,16,17,27] allethrin, other pyrethroid compounds and similar moieties of compounds were found after GC-MS analysis. In the study conducted in Taiwan, aliphatic aldehyde and allethrin were found in smoke of mosquito coil.[6,27]

CHAPTER VI

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The chemical compounds present in the mosquito repellant coil (viz. *Surya* mosquito coil and *Octagon Good Knight* mosquito coil) were identified by GC-MS technique. Major chemical constituents were allethrin, palmitic acid, carpic acid, acridine, cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester, stearic acid, decanoic acid, octadecanoic acid, hexadecanoic acid, n-heptadecanol-1, 3-pentanol- 2,3-dimethyl acid, benzyl benzoate, anthraquinone-2-methyl and cholestadiene. The mosquito coils were found to have chemicals such as sterol (cholestadine), acidic compounds which were not found on the package label. The carbonyl compound analysis by dinitro-salisylic acid (DNS) method was done for the samples obtained after burning the mosquito coil in which *Octagon Good Knight* mosquito coil and *Surya* mosquito coil were found to have carbonyl compound concentration of 4.44 mg/mL and 4.78 mg/mL respectively.

6.2. Recommendation

1. Toxicity test *in vivo* and *in vitro*: To evaluate the hazardous chemicals in mosquito repellants relevant bioassays should be done for complete toxicity of the chemicals present in the coils.
2. Government should develop the policy of the regulation and distribution of pesticides like mosquito repellants which directly effect human health. Hence a protocol should be developed to address this problem.
3. Government should launch the awareness programs regarding the knowledge, attitude and practices of such harmful items.

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Appendix I: Questionnaire

National Products Research Laboratory (NPRL)

NAST

Questionnaire

Name:……………………………………….

Age:…………………………………………..

Marital status: a). Married b). Single

Gender: a). Male b). Female

Telephone Number:………………….

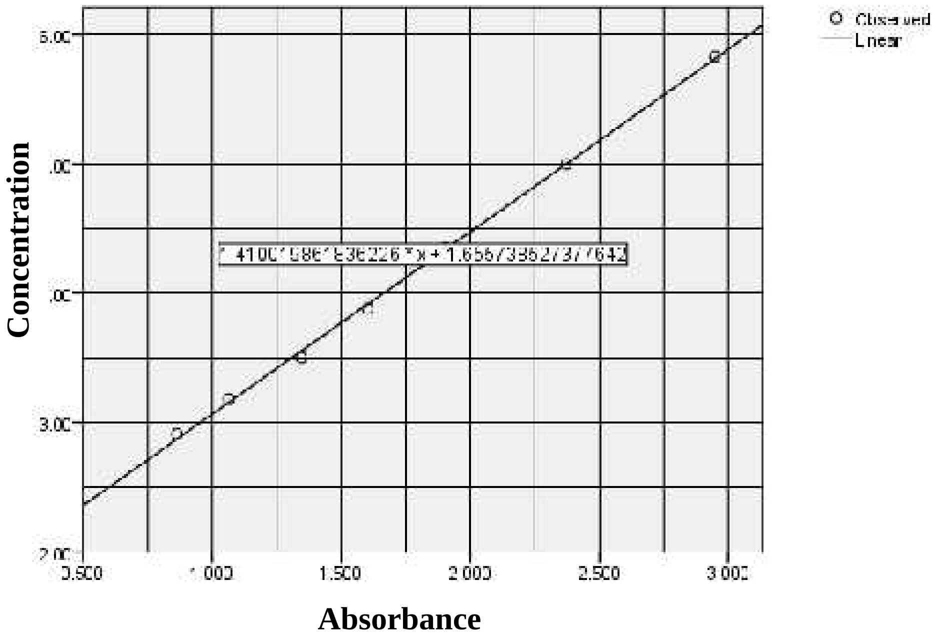
Address:…………………………………….

1. Do you use mosquito repellant?
   a. Yes
   b. No
2. What do you use as the mosquito repellant?
   a. Chemicals
   b. Net
   c. Others………….(specify)
3. If you use chemicals as the mosquito repellant. What do you use?
   a. Liquid
   b. Mats
   c. Coils
   d. Cream
   e. Other…………..(specify)
4. For Liquid user, what you use as mosquito repellant?
   a. Mortin
   b. Good Knight
   c. Others…………….(Specify)
5. For Coil user, what you use as mosquito repellant?
   a. Octagon Good Knight
   b. Surya
   c. Fumikilla
   d. Others…………….(Specify)
6. For Mats user, what you use as mosquito repellant?

a. Super Mat
b. King Mat
c. Others……………..(Specify)

7. For Cream user, what you use as mosquito repellant?
   a. Good Knight Cream
   b. Others………………(Specify)

Appendix – II: Standard Calibration curve analysis of carbonyl compound present in samples obtained from burning mosquito coils



R Square value= 0.999

Equation of line Y = 1.41x + 1.66

Where x= Absorbance value

S. N	Formaldehyde Concentration (mg/mL)	Absorbance at 550nm
1	5.83	2.947
2	5.0	2.374
3	4.35	1.903
4	3.88	1.602
5	3.5	1.345
6	3.18	1.065
7	2.91	0.863

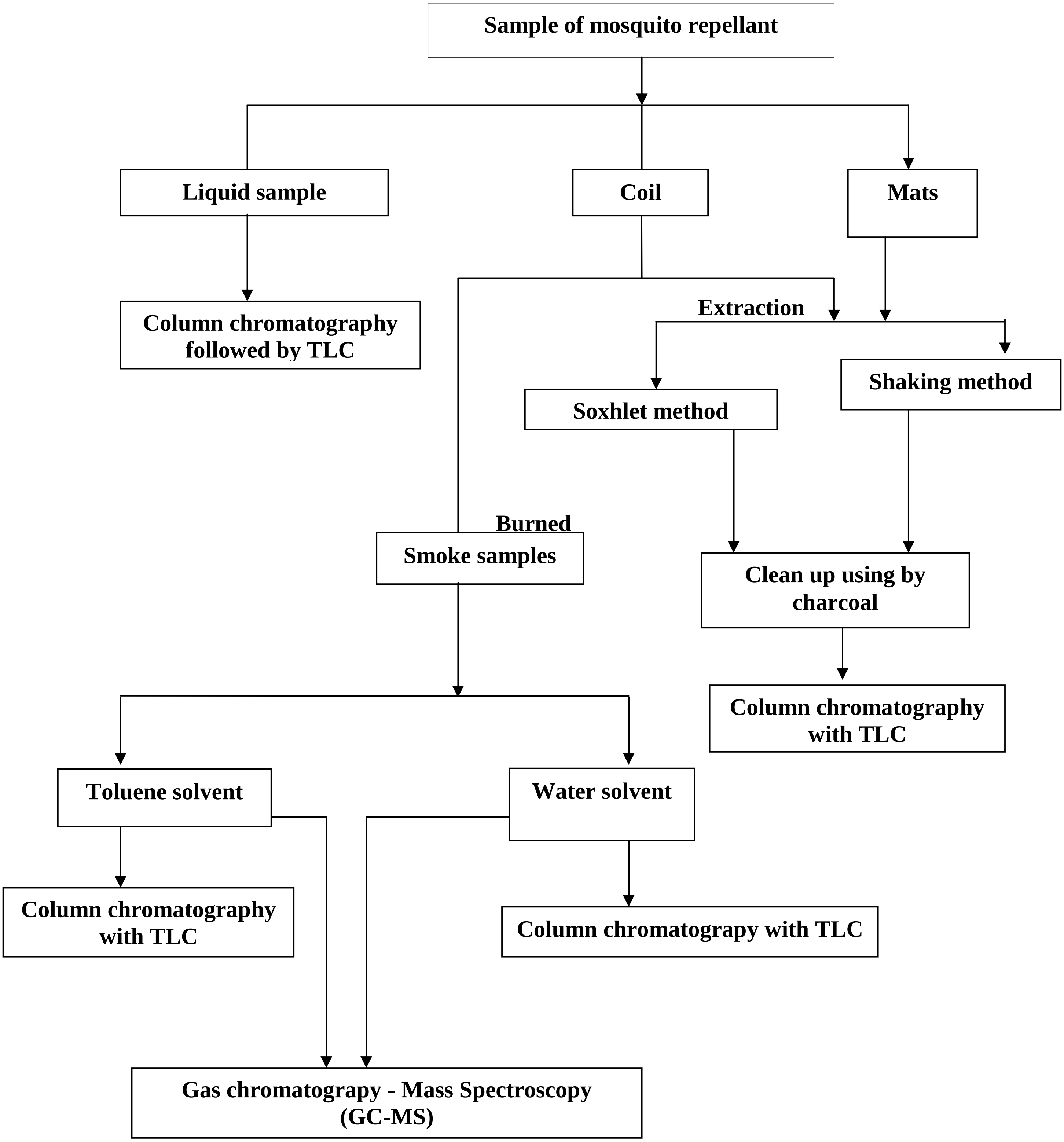
So Absorbance value of *Octagon Good Knight* sample is 1.971 and *Surya* mosquito coil sample is 2.216.

Then by equation,

Octagon Good Knight mosquito coil = 4.44mg/mL

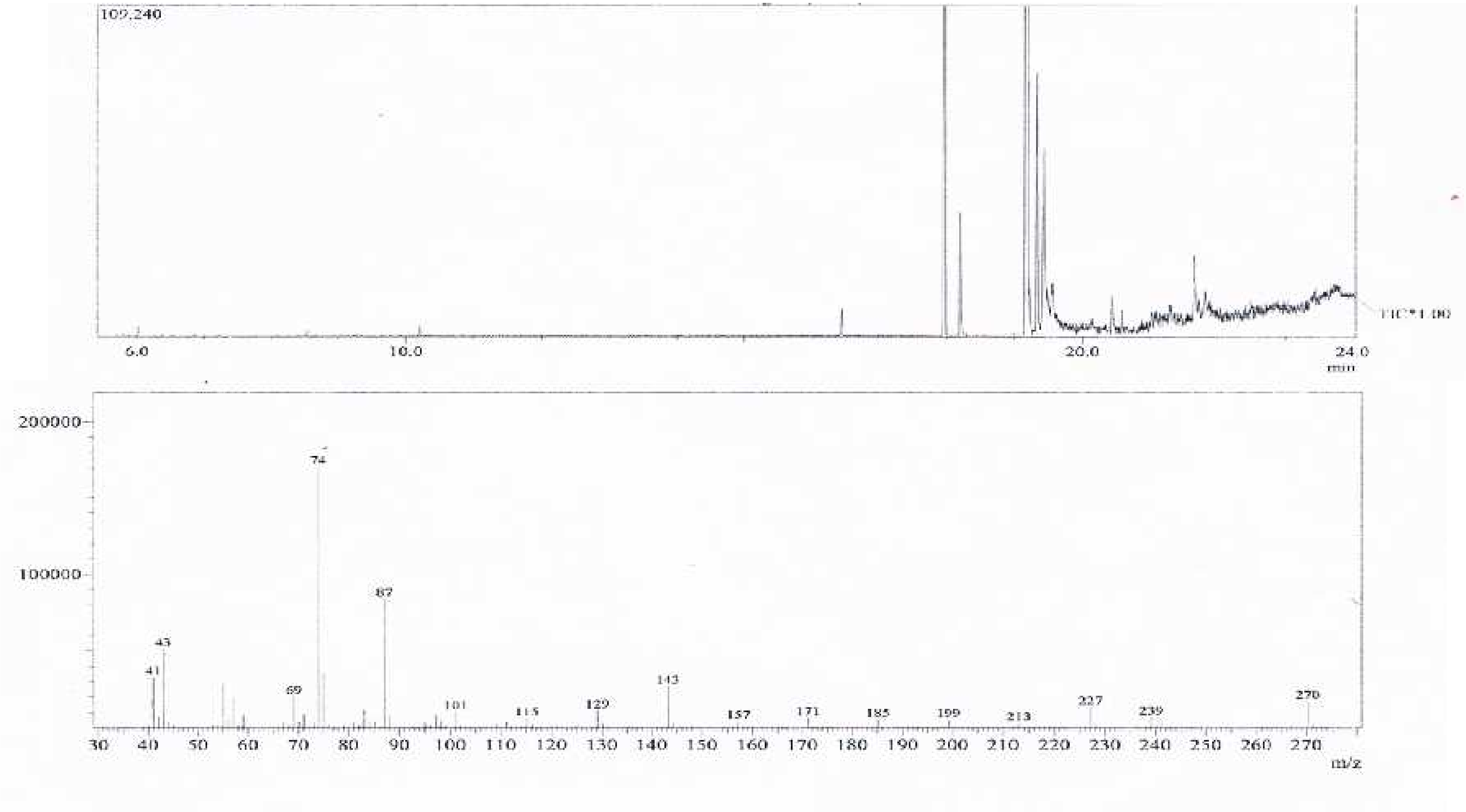
Surya mosquito coil = 4.78 mg/mL

Appendix-III: Flow chart methodology of extraction of active ingredients of mosquito repellants

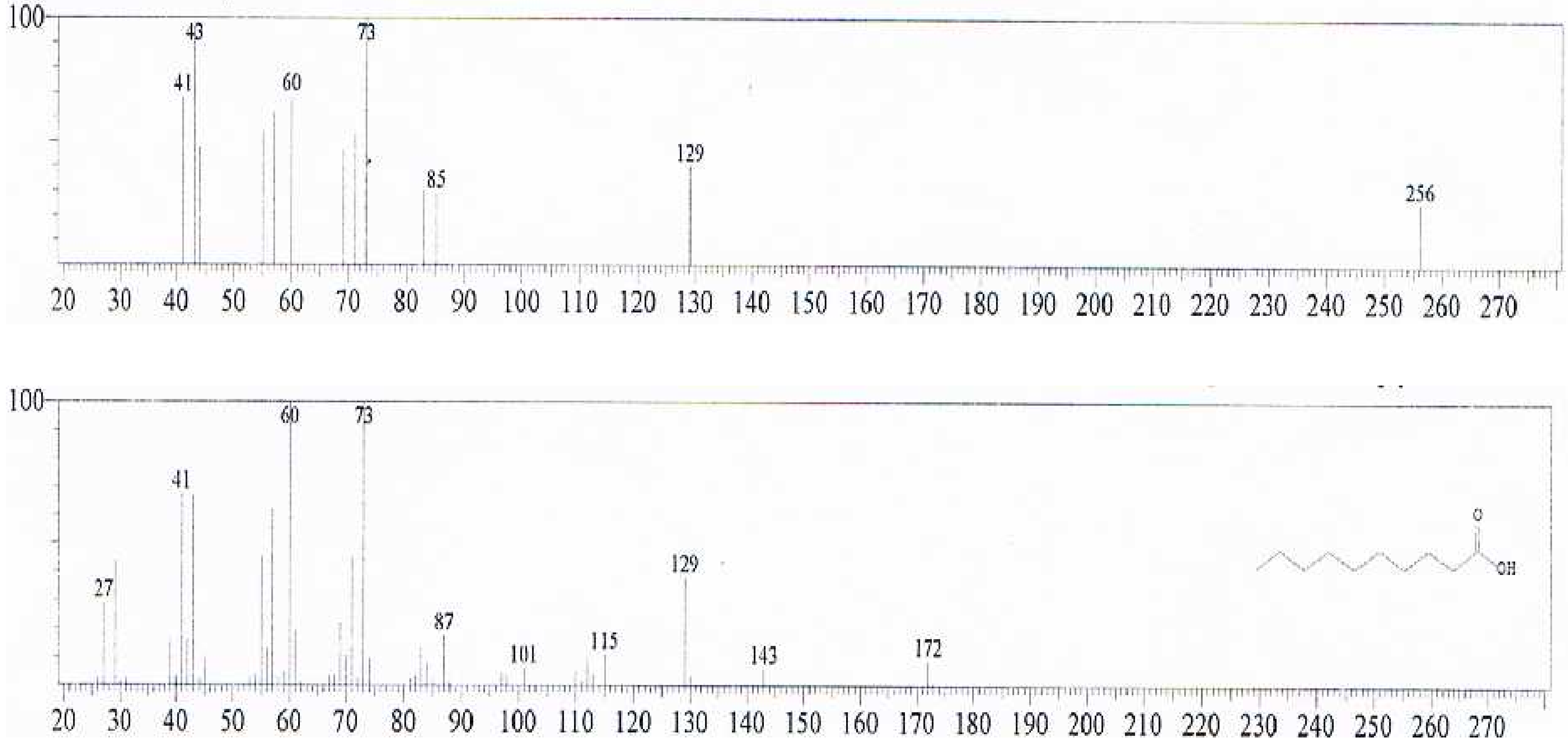


Appendix IV- GC-MS Spectra

Spectra 1: GC-MS analysis of sample in water solvent obtained after burning *Surya* mosquito coil

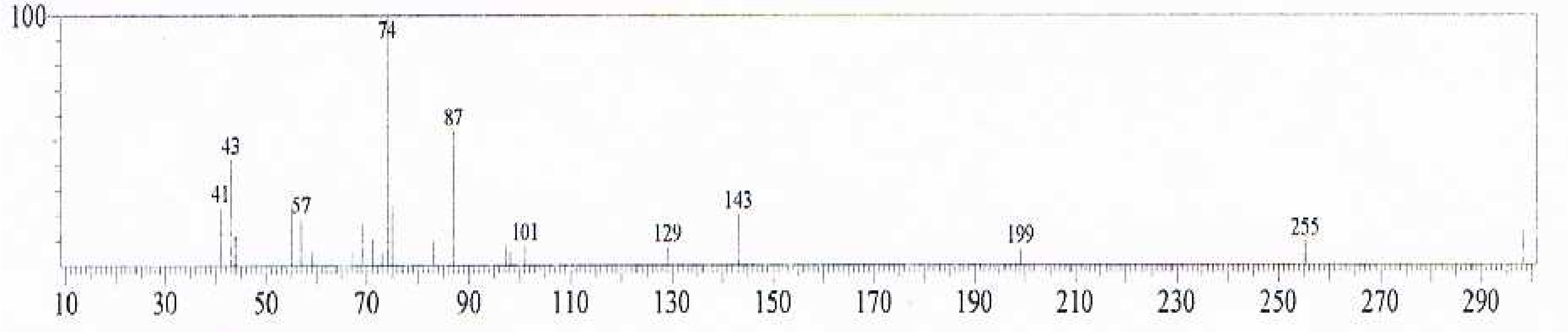


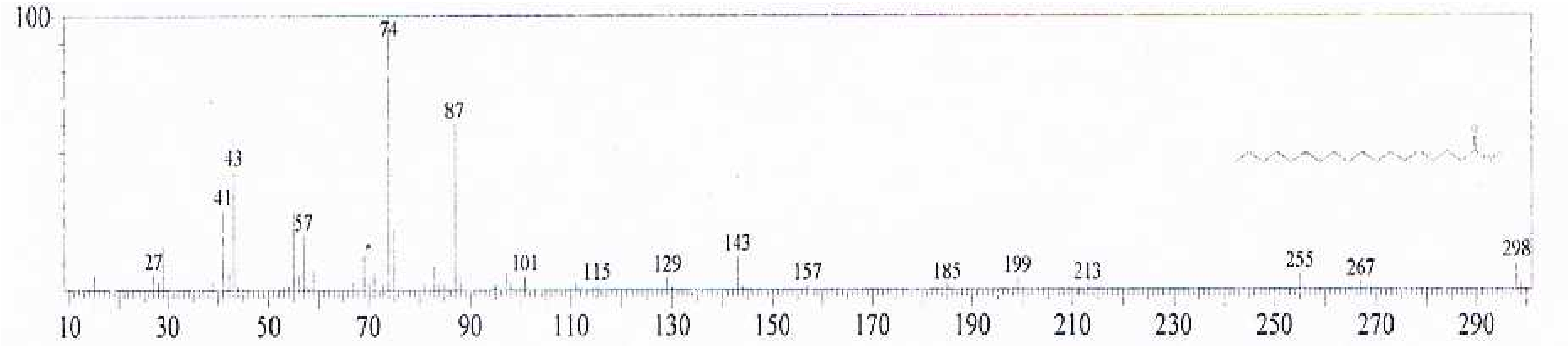
Spectra 2. GC-MS analysis of decanoic acid in water solvent obtained after burning *Surya* mosquito coil



(NIST Library search)

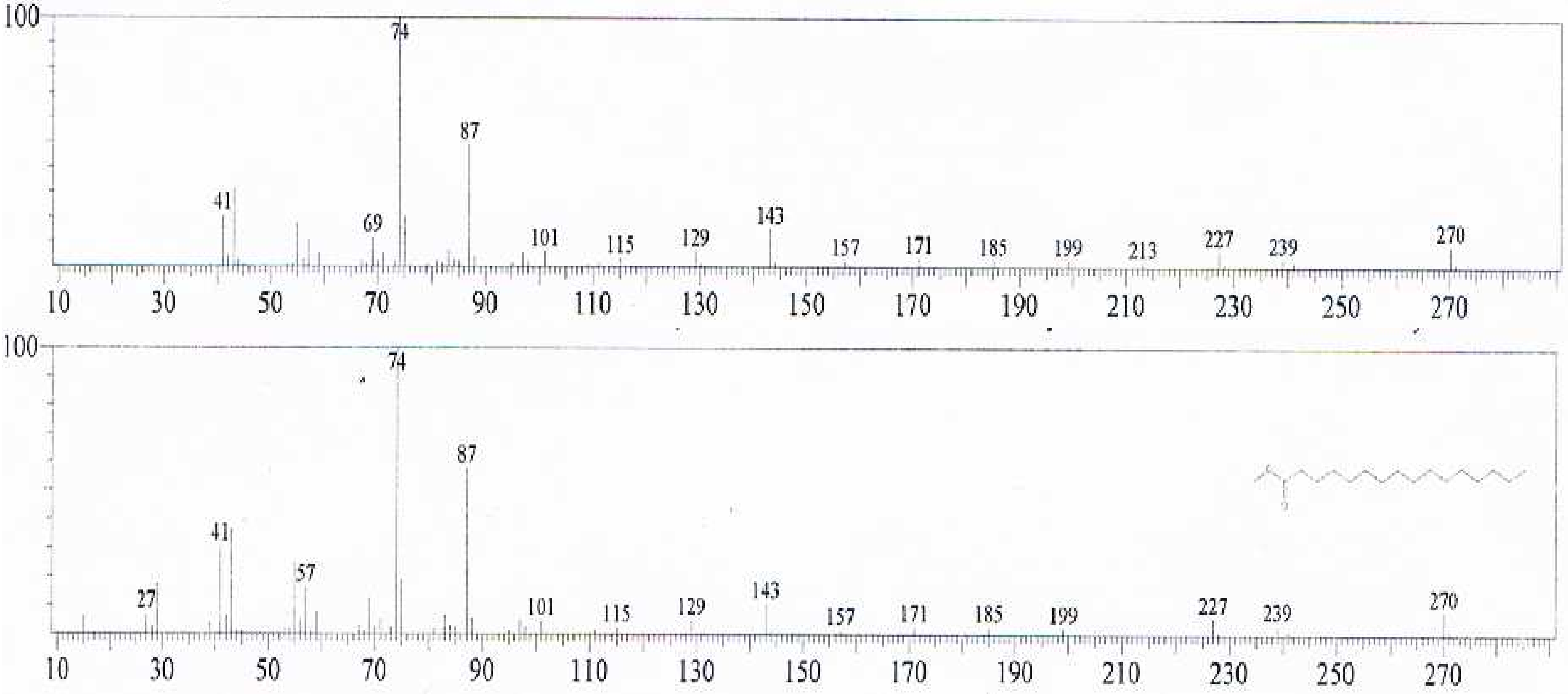
Spectra 3. GC-MS analysis of octadecanoic acid in water solvent obtained after burning *Surya* mosquito coil





(NIST Library search)

Spectra 4. GC-MS analysis of hexadecanoic acid in water solvent obtained after burning *Surya* mosquito coil



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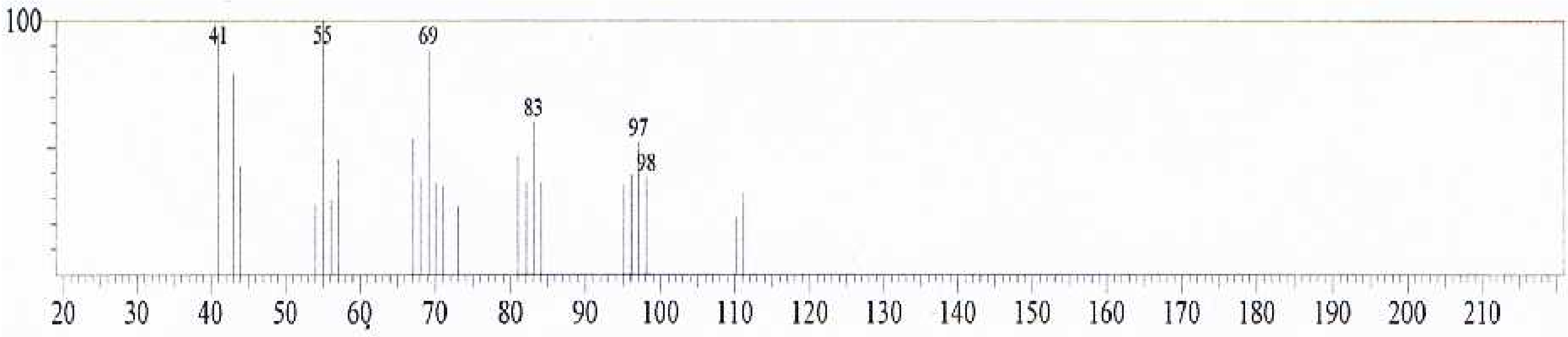
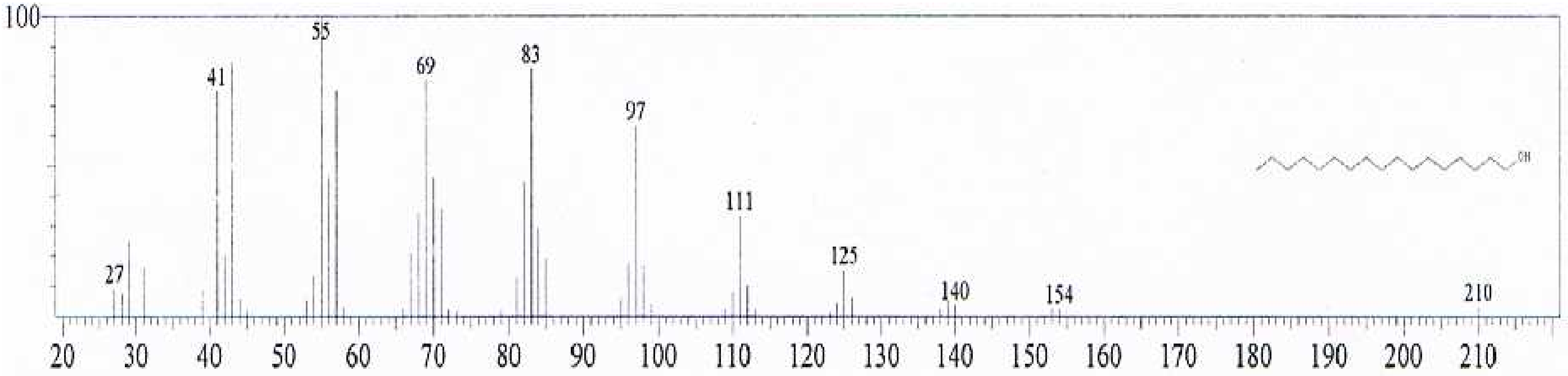
Spectra 5. GC-MS analysis of n-heptadecanol-1 in water solvent obtained after burning *Surya* mosquito coil





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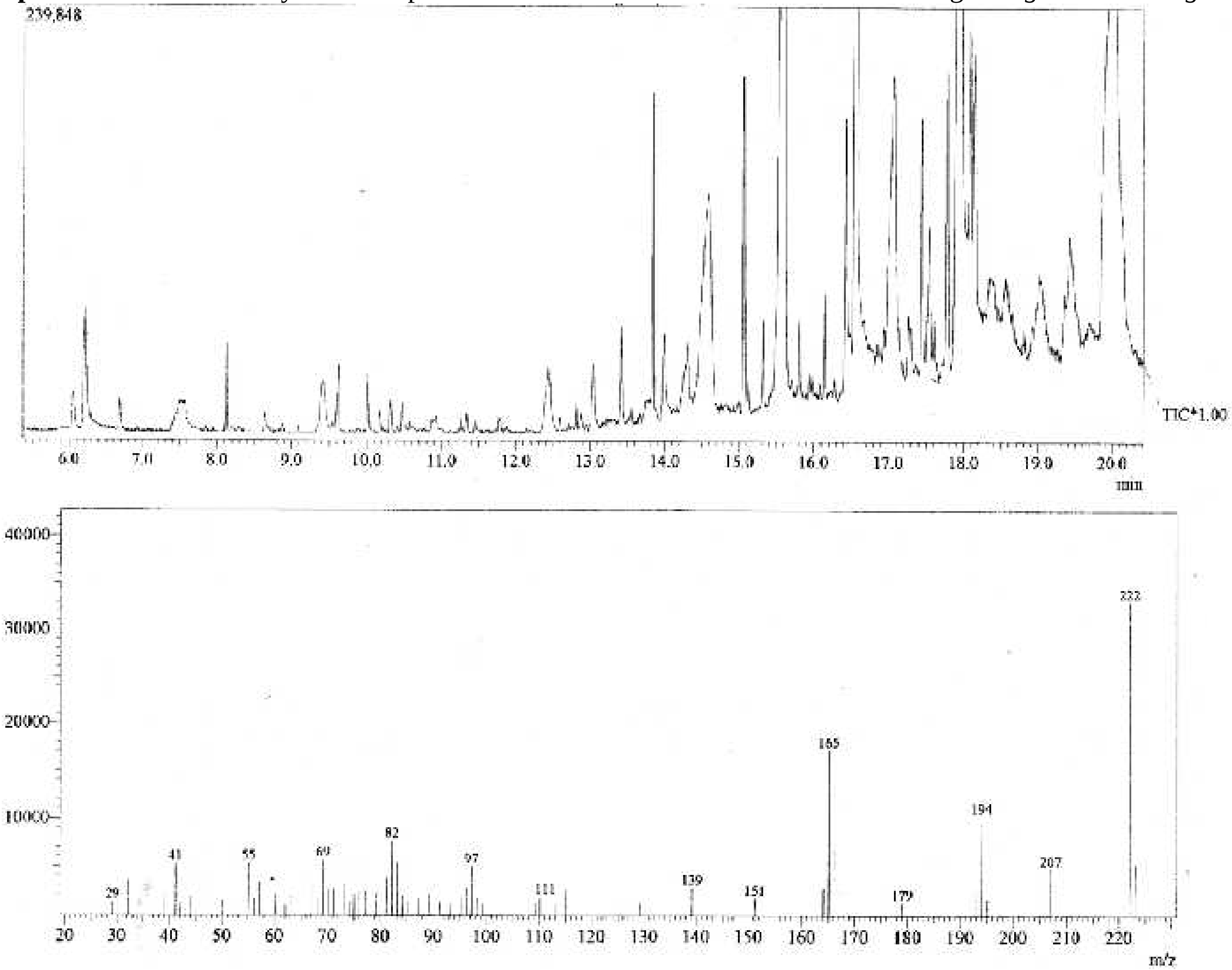
Spectra 6. GC-MS analysis of sample acid in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil



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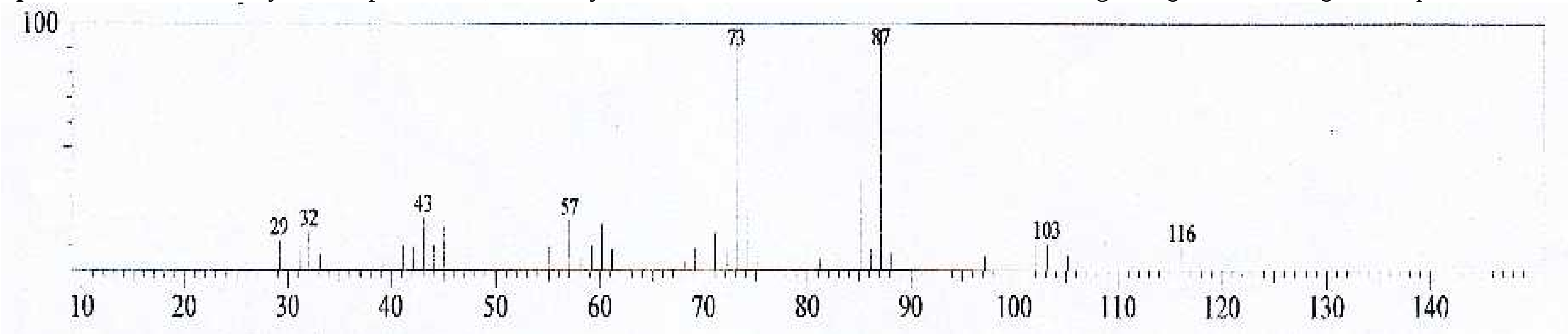
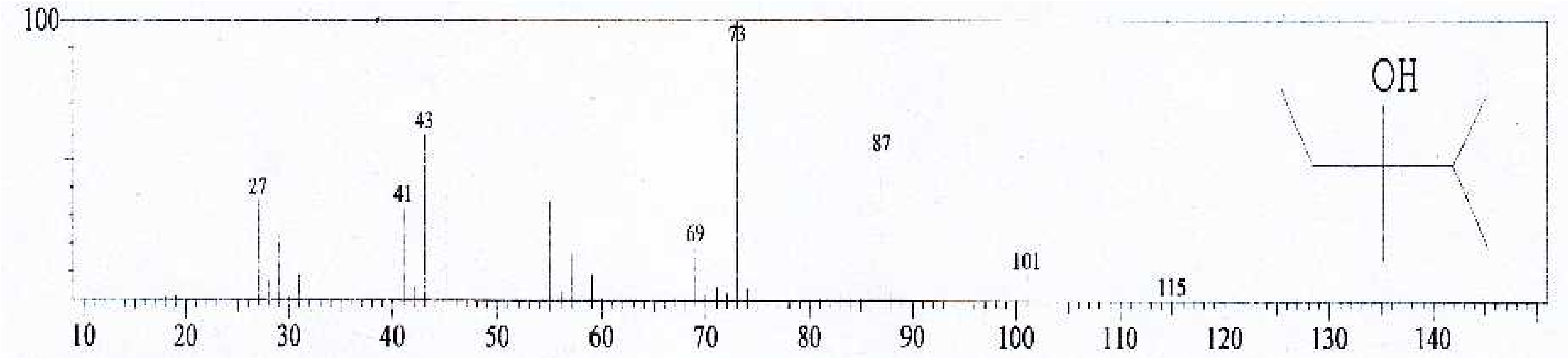
Spectra 7. GC-MS analysis of 3-pentanol-2,3-dimethyl acid in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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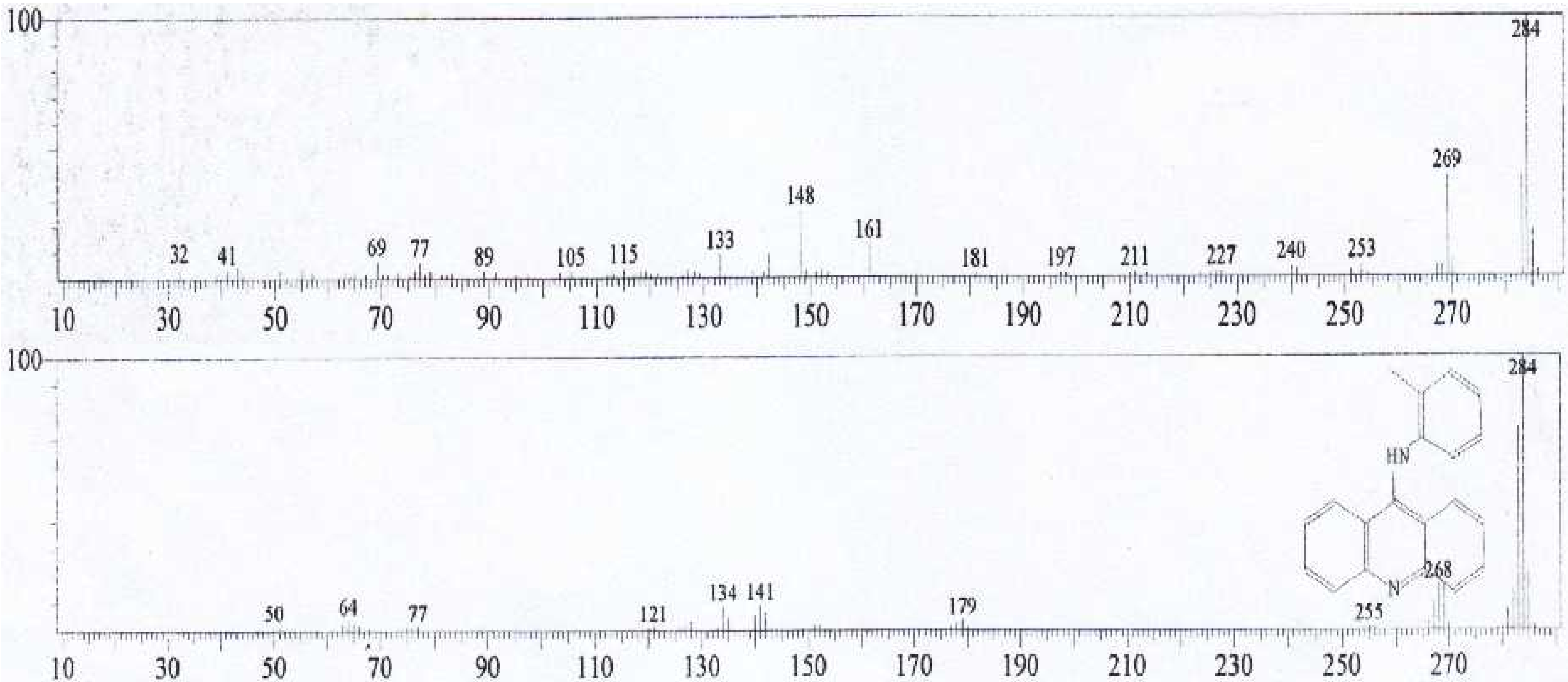
Spectra 8. GC-MS analysis of acridine in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil



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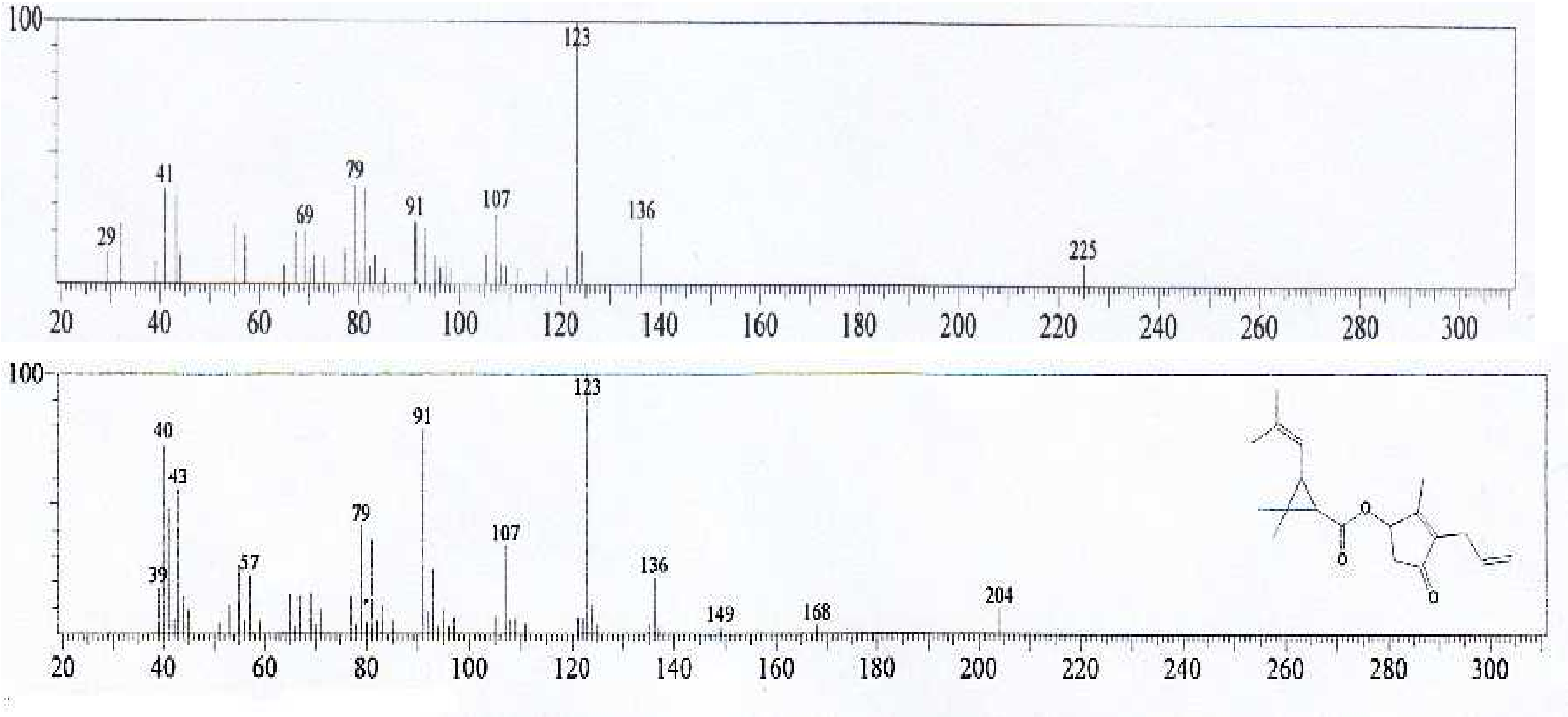
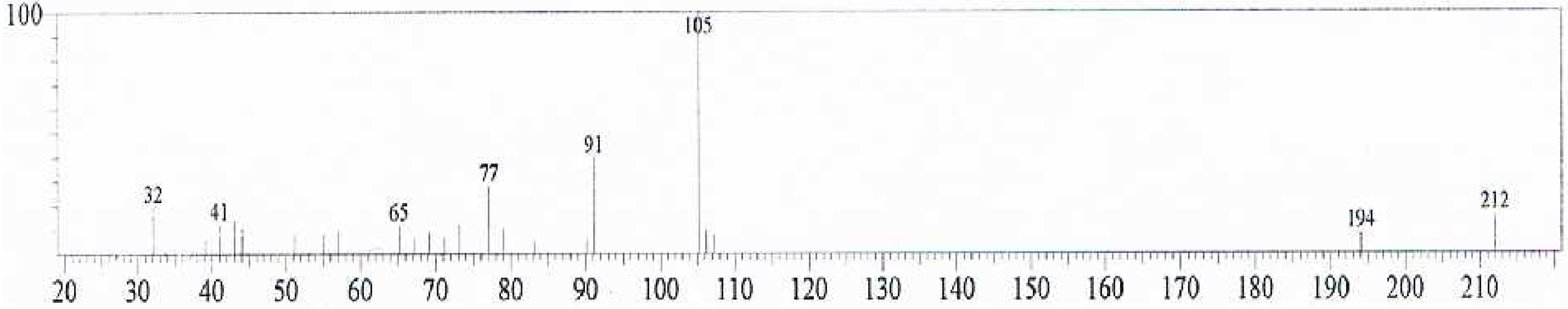
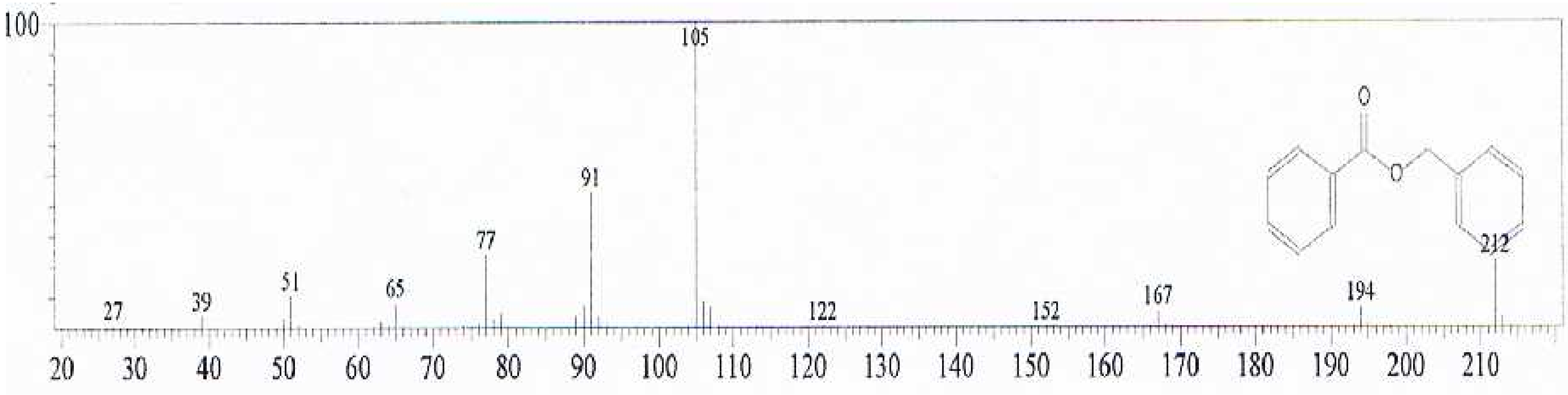
Spectra 9. GC-MS analysis of allethrin in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil



(NIST Library search)

Spectra 10. GC-MS analysis of benzyl benzoate in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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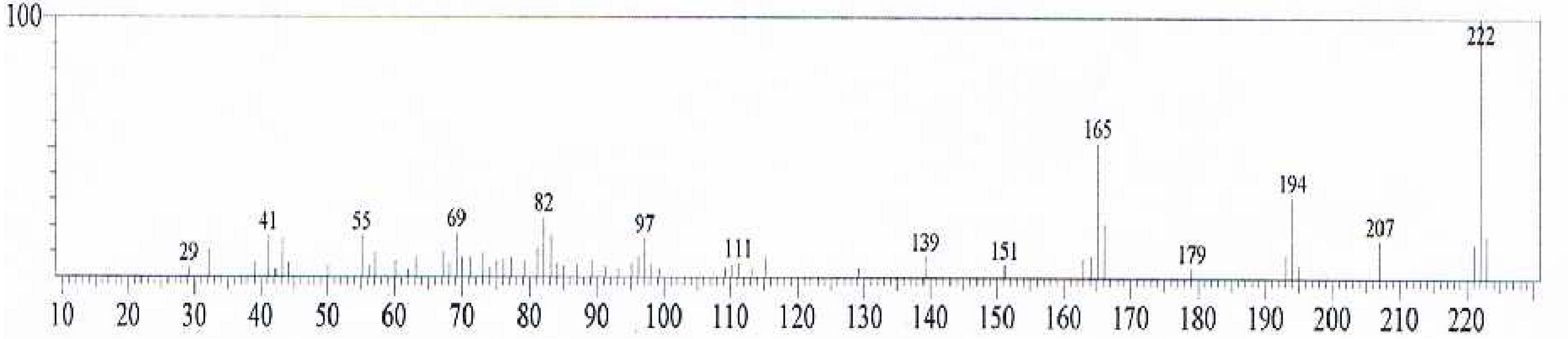
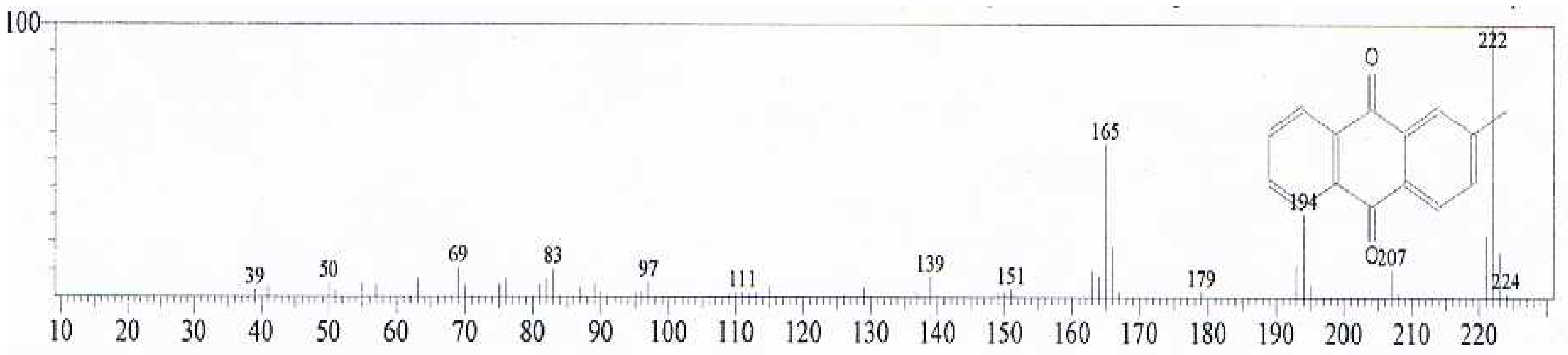
Spectra 11. GC-MS analysis of anthraquinone 2-methyl in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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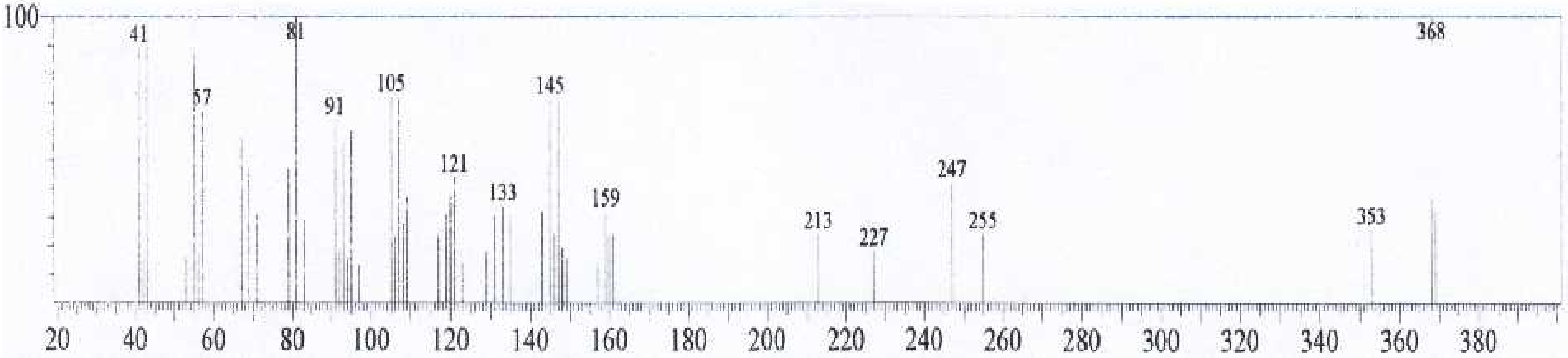
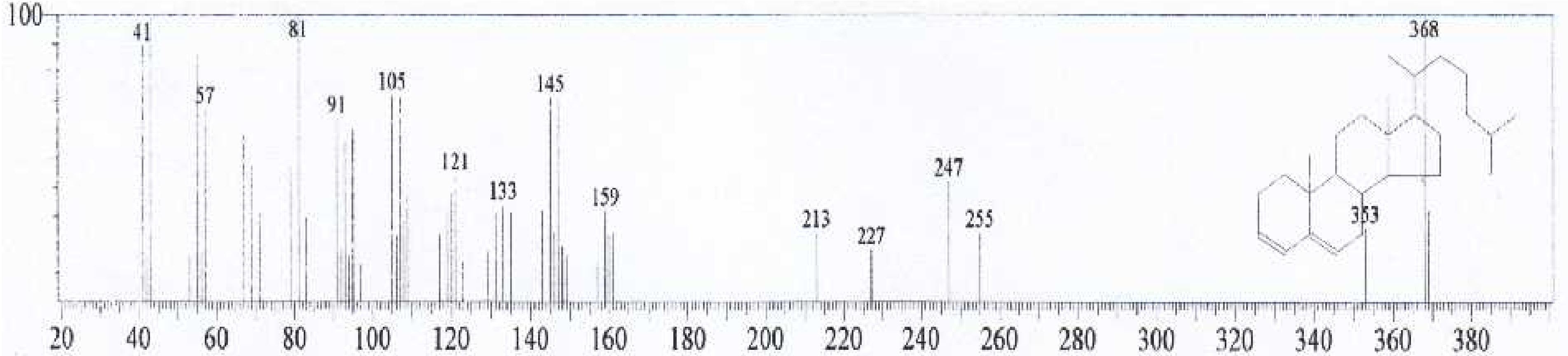
Spectra 12: . GC-MS analysis of cholestadiene in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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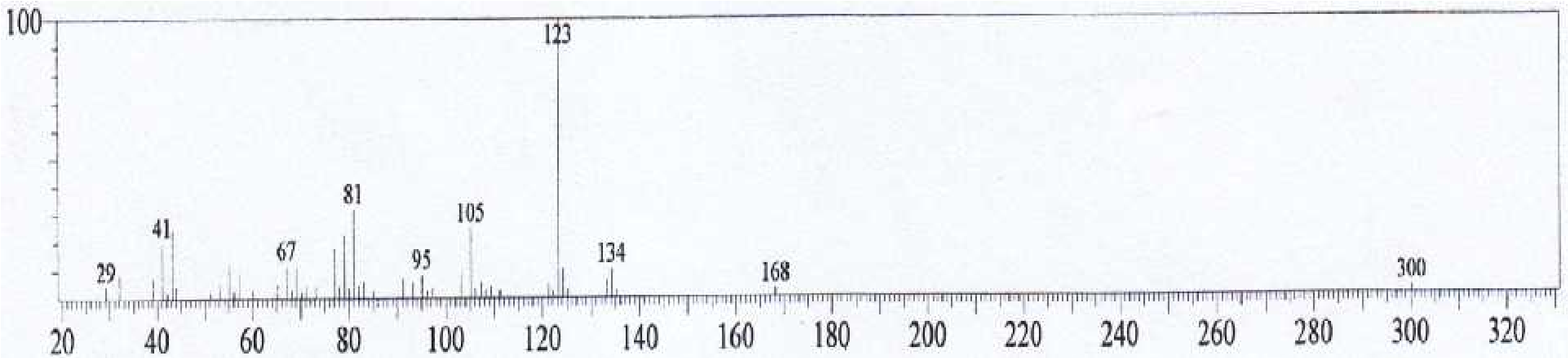
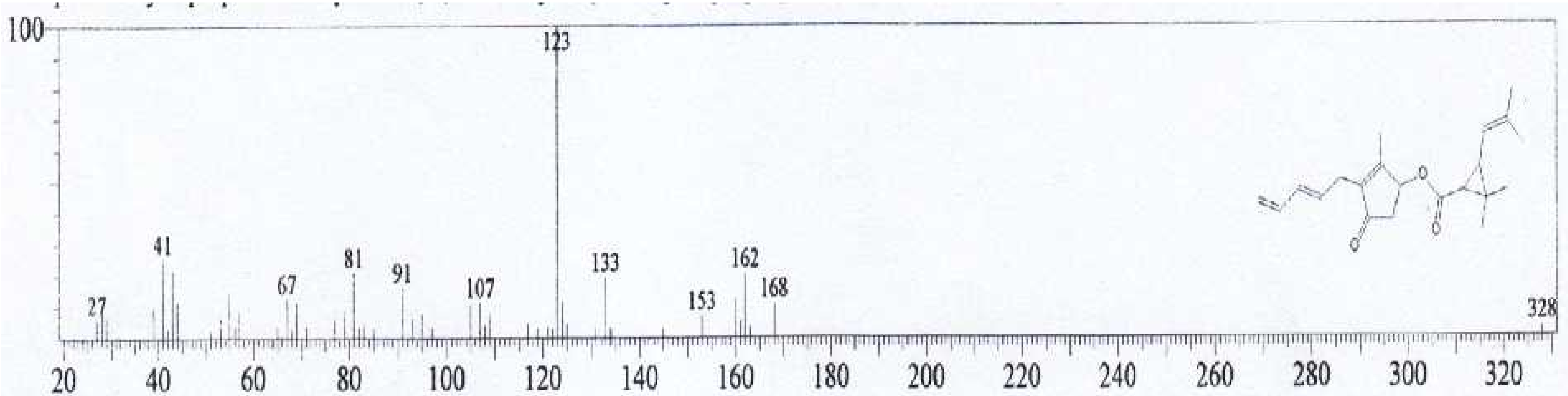
Spectra 13. GC-MS analysis of cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-,2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl ester in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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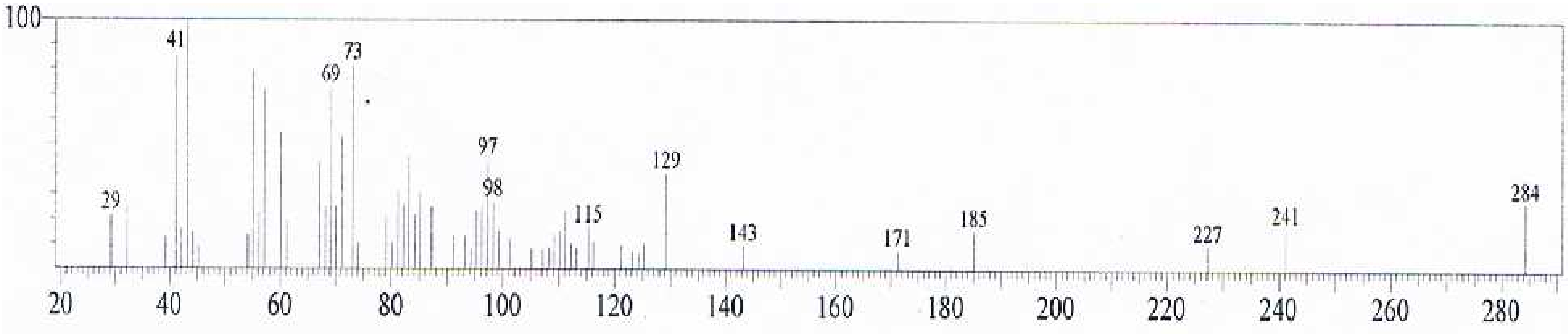
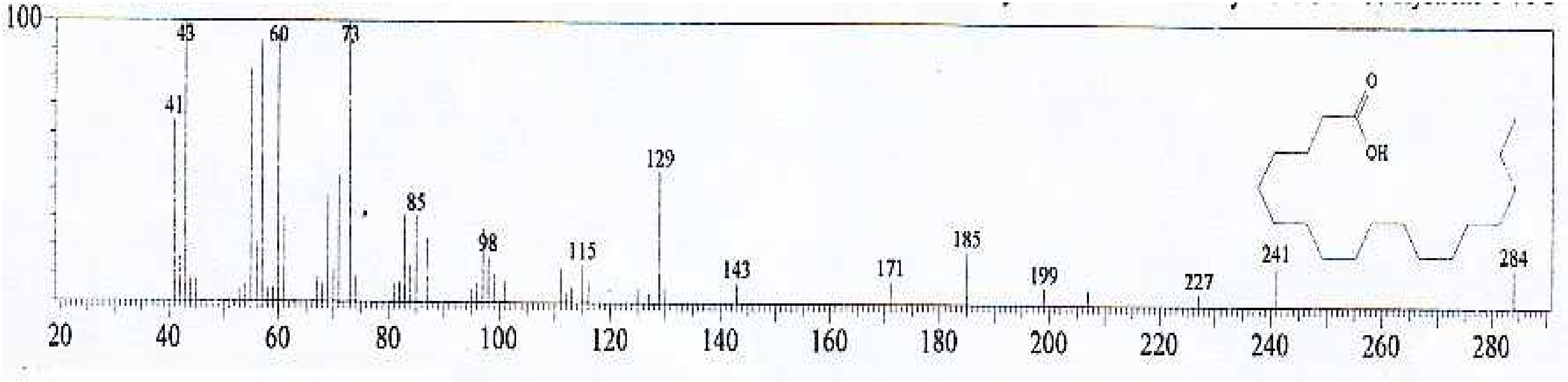
Spectra 14. GC-MS analysis of stearic acid in toulene solvent obtained after burning *Octagon Good Knight* mosquito coil





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