# STUDY ON PHYTOCHEMICAL CONSTITUENTS, NUTRIENTS AND ISOLATION OF FATTY ACIDS FROM THE LEAF OF *CLINACANTHUS NUTANS* L. (SNAKE-GRASS)

Ei Ei Chaw[1], Kay Khine Nyunt[2] , Saw Hla Myint[3]

## Abstract

Plants are the main source of natural products that are used in medicine. *Clinacanthus nutans* (Arcathanceae), was collected from Yangon University Campus, Yangon Region and identified by the botanist, Department of Botany, University of Yangon. According to the preliminary phytochemical tests, it was found that glycosides, flavonoids, phenolic compounds, saponins, tannins and terpenoids were present in the dried leaves powders. Qualitative elemental analysis by EDXRF revealed that the leaves of *Clinacanthus nutans* contained K (2.204 %) and Ca (2.25 %) as major elements and Fe, S, Mn, Zn, Cu and Rb as trace elements. The nutritional values determined by AOAC method indicate the percentage of moisture (13.95), ash (19.27), fat (2.01), fibre (28.45), protein (18.12) and carbohydrate (18.2) in the leaves of *Clinacanthus nutans*. From the ethyl acetate fraction of 95 % ethanol extract of the leaf of *Clinacanthus nutans*, carboxylic acids were isolated after three successive column chromatographic methods, namely vacuum liquid chromatography (VLC), flash chromatography, using medium pressure, and micro-column chromatography using pasteur pipette giving a single spot on TLC. Carboxylic acids were characterized as long chain fatty acids by TLC staining and FTIR. Three fatty acids were further identified by GC-MS in C, namely linoleic acid (or) (*Z, Z*)-9,12-octadecadienoic acid (**C-1**), 11,14-octadecadienoic acid (**C-2**) and 11,14-eicosadienoic acid (**C-3**).

**Keywords**: *Clinacanthus nutans*, nutritional values, long chain fatty acids, linoleic acid

## Introduction

Plants are used as medicine at least to the middle period, since 60 000 years ago. The genus *Clinacanthus nutans* (family Acathaceae) consists of two species. *Clinacanthus nutans* is a small shrub about one meter tall native to tropical Asia and it is used in traditional system of medicine.

### Botanical Aspect of *Clinacanthus nutans*

Family : Acanthaceae
Genus : *Clinacanthus*
Species : *nutans*
Scientific name : *Clinacanthus nutans*
Common name : Snake- grass

**Figure 1** *Clinacanthus nutans* (snake-grass) plant and leaf

[1] Lecturer, Department of Chemistry, Dagon University
[2] Dr, Lecturer, Department of Chemistry, University of Yangon
[3] Dr, Professor and Head (Retired), Department of Chemistry, University of Yangon

A mixture of nine cerebrosides and a monoacyl monogalatosylglycerol are present from the ethyl acetate soluble fraction of the ethanol extract of the leaves (Tuntiwachwuttikul, 2004). The structures of the cerebrosides were characterized as 1-*O*-b-D-glucosides of phytosphingosines, which comprised a common long-chain base, (2*S*, 3*S*, 4*R*, 8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol with nine 2-hydroxy fatty acids of varying chain lengths (C16, C18, C20-26) linked to the amino group. Fatty acids are widely occurring in natural fats and dietary oils and they play an important role as nutritious substances and metabolites in living organisms.

The leaves are also consumed as raw vegetable or mixed with other juices (apple, sugarcane or green tea) or served as fresh drink or refreshing beverage in Thailand. The decoction of the dried leaves is applied on the affected part to treat herpes infection and envenomation (Kapoor, 2001).

## Materials and Methods

### Sample Preparation

The collected fresh leaves sample was washed with distilled water and air dried at room temperature for one week and the dry leaves were ground into powder and then stored in the air tight container. The plant sample was identified at Department of Botany, Yangon University.

### Preliminary Phytochemical Screening

In order to classify the secondary metabolites present in the leaf sample, preliminary phytochemical tests were carried out according to the appropriate reported methods.

### Determination of Nutrients

The determination of percentage of the nutrients in the powdered sample was carried out according to the Chemical Analysis of Food Method. Protein content was determined by using Macro-Kjeldahl's method. Fat content was determined by the Soxhlet Extraction method. Fiber content was determined by Fiber-cap method.

### Extraction and Isolation of Compounds

Ethyl acetate soluble fraction of a 95% ethanolic extract was prepared and submitted to three successive separations by vacuum liquid chromatography, flash chromatography and micro-column chromatography using Pasteur pipette to finally obtain carboxylic acids mixture C, giving a single spot on TLC.

### Vacuum Liquid Chromatography

The vacuum liquid chromatography (VLC) employed in the present work is a modification of the Dry Column Vacuum Chromatography (DCVC) (Pedersen & Rosenbohm, 2001). In DCVC, Silica Gel 60 (15–40 μm) is used whereas in VLC of the present work Silica Gel 60 (63–200 μm) was used. Another difference is the use of a normal glass column instead of a sintered glass funnel, and also a usual wet packing of the column instead of a dry packing. Fractions of 70 mL each of PE: EA 9:2, 9:4, 9:6, 9:8, EA only, EA:MeOH 9:1, 1:1 and MeOH only were collected, and 10 fractions were obtained. Each of the fractions was concentrated by evaporating the solvent and monitored by TLC. The ethyl acetate sub fraction from the VLC was rechromatographed by flash chromatography (Still *et al.*, 1978). The solvent system chloroform: methanol (9:1) which gave good separation and also moved the major component to $R_f = 0.35$ on analytical TLC was chosen. Further purification on a micro-column (Millar, 2012) yielded C.

### Rapid Methylation of Isolated Compound C

The isolated compounds by micro-column chromatography was identified as acid by TLC with reagent bromocresol green (yellow spots against blue background), and C was converted to its methyl ester for GC-MS analysis by a rapid methylation method (Ichihara and Fukubayashi, 2010).

## Results and Discussion

The results of preliminary photochemical tests of *Clinacanthus nutans* showed that the leaves contain alkaloids, flavonoids, glycosides, organic acids, tannins, phenolic compounds and terpenoids were present in Table 1.

**Table 1 Results of Phytochemical constituents on the Leaves of *Clinacanthus nutans***

| No. | Phytochemical tests | Extracts | Test reagents | Observation | Remark |
|---|---|---|---|---|---|
| 1 | Alkaloids | 1% HCl | Mayer's reagent<br>Dragendroff's reagent<br>Wagner's reagent<br>Sodium picrate | White ppt<br>Orange ppt<br>Reddish brown ppt<br>Yellow ppt | +<br>+<br>+<br>+ |
| 2 | α-amino acids | $H_2O$ | Ninhydrin | Purple spot | + |
| 3 | Carbohydrate | $H_2O$ | 10% α-naphtnol + Conc: $H_2SO_4$ | Red ring | + |
| 4 | Cyanogenic glycosides | $H_2O$ | Conc: $H_2SO_4$ and Sodium picrate | No color change | - |
| 5 | Flavonoids | EtOH | Conc: HCl and Mg turning | Pink color | + |
| 6 | Glycosides | EtOH | 10% Lead acetate | White ppt | + |
| 7 | Organic acid | $H_2O$ | Bromocresol green solution | Yellow colour | + |
| 8 | Phenolic compound | EtOH | $K_3Fe(CN)_6$ + 1% $FeCl_3$ | Brown ppt | + |
| 9 | Reducing sugars | $H_2SO_4$ | Benedict's solution | No brick red ppt | - |
| 10 | Saponins | $H_2O$ | Distilled water | Frothing | + |
| 11 | Starch | $H_2O$ | $I_2$ solution | Blue black | + |
| 12 | Steroids | PE | Acetic-anhydride & conc: $H_2SO_4$ | Red colour | + |
| 13 | Tannins | EtOH | 1% gelatin | Brown | + |
| 14 | Terpenoids | $CHCl_3$ | Acetic anhydride | Pink | + |

(+) = Presence, (-) = Absence, (ppt) = Precipitate

### Analysis of Nutrients in the Leaves of *Clinacanthus nutans*

According to the results, the ash, carbohydrate, fat, fibre, moisture and proteins were found to be 19.27 %, 18.20 %, 2.01 %, 28.45 %, 13.95 % and 18.12 %, respectively, (Table 2).

**Table 2 Nutritional Values for the Leaves of *Clinacanthus nutans***

| No. | Parameter | Contents (%) |
|---|---|---|
| 1 | Ash | 19.27 |
| 2 | Carbohydrate | 18.20 |
| 3 | Fat | 2.01 |
| 4 | Fiber | 13.95 |
| 5 | Moisture | 18.12 |
| 6 | Protein | 28.45 |
| 7 | Energy values (kcal/100g) | 163.37 |

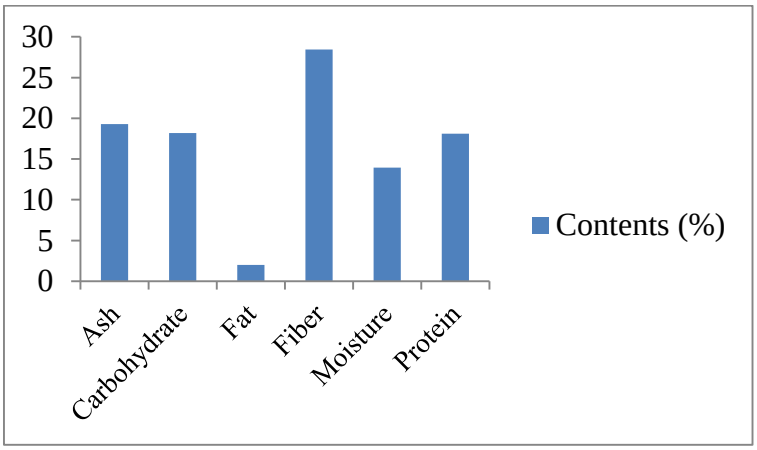


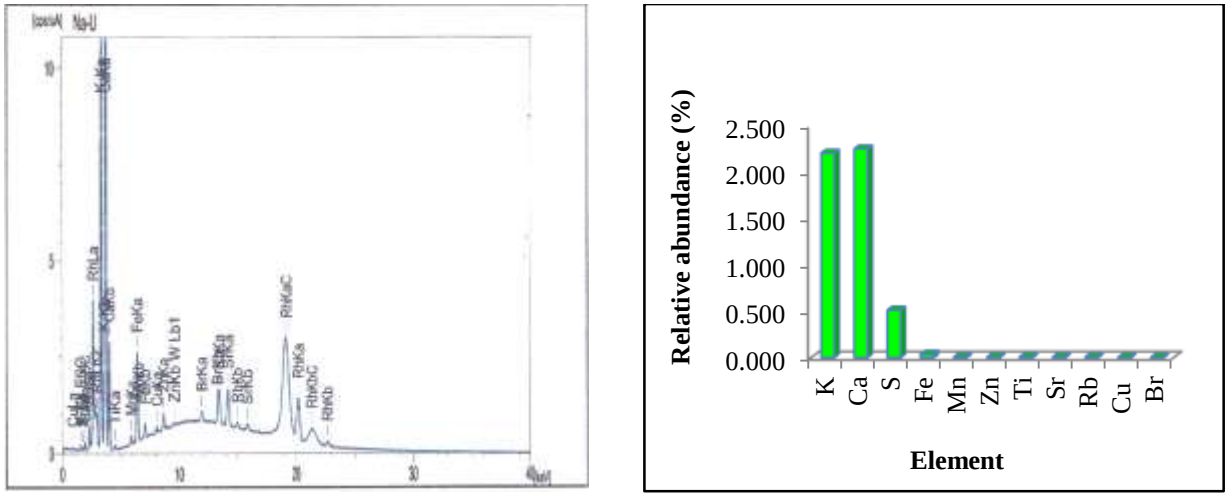

**Figure 2** Bar graph showing nutrients in the leaves of *Clinacanthus nutans*

**Qualitative Elemental Analysis by EDXRF**

In this study relative abundance of elements present in the leaf of *Clinacanthus nutans* was determined by EDXRF. Qualitative elemental analysis by EDXRF spectrometry revealed that the leaves of *Clinacanthus. nutans* was found to contain K (2.204%) and Ca (2.251%,) as major elements and Fe, S, Mn, Zn, Cu and Rb as trace elements, (Figure 3 and Table 3).

**Table 3 Relative Abundance of Elements in Leaves of *Clinacanthus nutans* by EDXRF**

| No. | Elements | Relative abundance (%) |
|---|---|---|
| 1 | K | 2.204 |
| 2 | Ca | 2.251 |
| 3 | P | - |
| 4 | S | 0.513 |
| 5 | Fe | 0.037 |
| 6 | Mn | 0.005 |
| 7 | Zn | 0.003 |
| 8 | Ti | 0.006 |
| 9 | Sr | 0.004 |
| 10 | Rb | 0.004 |
| 11 | Cu | 0.001 |
| 12 | Br | 0.001 |




(a) (b)

**Figure 3** EDXRF spectrum (a) and bar graph showing the elements (b) of *Clinacanthus nutan* leaf

## Isolation of Carboxylic Acids

The ethyl acetate soluble fraction of ethanol extract was subjected to vacuum liquid chromatography (VLC) by applying suction elution using a vacuum pump, collecting 70 mL fractions. Common column silica gel (63 – 200 µm) was used. Due to the rapid flow rate under suction, the running time was shortened considerably. Next the selected eluted fraction from the column was further separated by flash chromatography (Still *et al.*, 1978) on a finer silica gel (40–63 µm) in which positive pressure elution was employed using compressor. Purer fraction containing compound was obtained in a short time due to the increased flow rate used. The small fraction was further purified on a micro-column (Millar, 2012) using a pasteur pipette filled with common column silica gel (63 – 200 µm) and a rubber bulb for pressure elution, collecting 1 mL fractions. Finally, fraction containing C was obtainedwhich gives single spot on TLC was obtained. If the finer silica gel (15–40 µm) used in dry column vacuum chromatography (DCVC) (Pedersen and Rosenbohm, 2001) were available, the purification could have been done by a single column.

## Analysis of the Carboxylic Acids Composition of C by GC-MS

The observed IR bands may be explained as follows: the broad O-H stretching band with maximum at 3365cm$^{-1}$, characteristic of a carboxyl group, with the carbonyl C=O stretching band at 1720 cm$^{-1}$ indicates a carboxylic group. The $CH_3$ asymmetric and symmetric stretching bands at 2957 and 2858 cm$^{-1}$, the $CH_3$ asymmetric and symmetric bending bands at 1461 and 1380 cm$^{-1}$ indicate presence of methyl groups. Furthermore, the methylene rocking band at 731 cm$^{-1}$ suggests a long-chain fatty acid rather than a pentacyclic triterpenic acid (Table 4). Thus from the TLC reagent tests and the FT IR spectrum (Figure 4), **C** is a long-chain fatty acid. The presence of linoleic acid (palmitic acid) in the leaf of *Clinacanthus nutans* has also been reported on page 5 of a review article on *Clinacanthus nutans* (Khoo *et al.*, 2018).

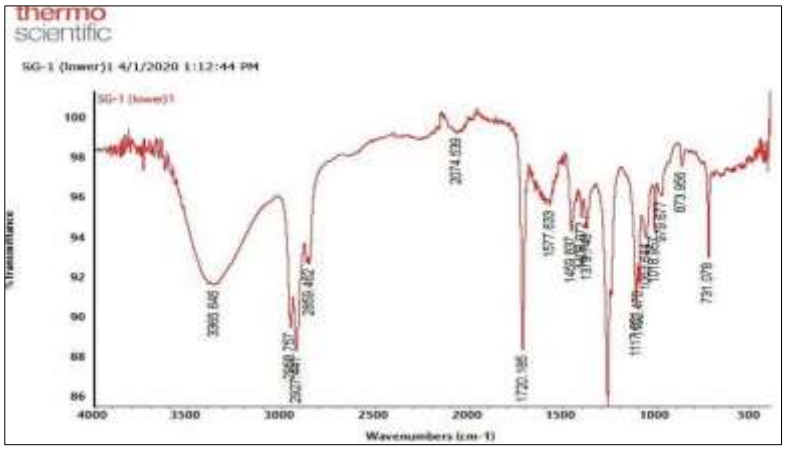


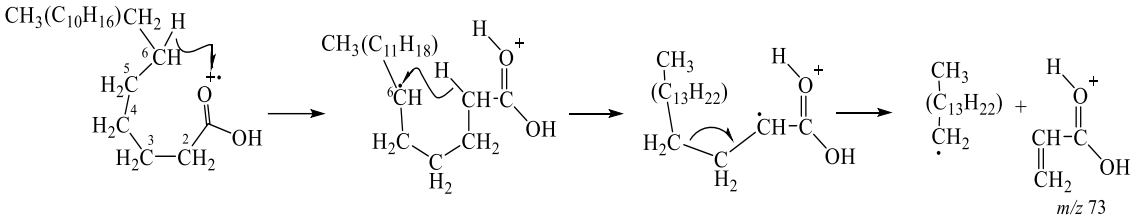

**Figure 4** FT IR spectrum of the isolated compound

**Table 4 FT IR Band Assignment of Isolated Compound C**

| **Wavenumber ($cm^{-1}$)** | **Vibrational Mode** | **Assignments** |
|---|---|---|
| 3500-2500 (very broad) | ν O-H | OH of carboxyl |
| 2957 | $\nu_{as}$ $CH_3$ | $CH_3$ |
| 2927 | $\nu_{as}$ $CH_2$ | $CH_2$ |
| 2858 | $\nu_{sy}$ $CH_3$ | $CH_3$ |
| 1720 | ν C=O | carboxyl |
| 1461 | δ $CH_2$, δas $CH_3$ | $CH_3$, $CH_2$ |
| 1380 | $\delta_{sy}$ $CH_3$ | $CH_3$ |
| 1266, 1117, 1063, 1018 | ν C-O | C-O |
| 731 | □ $CH_2$ | $(CH_2)_n$ with $n \geq 4$ |

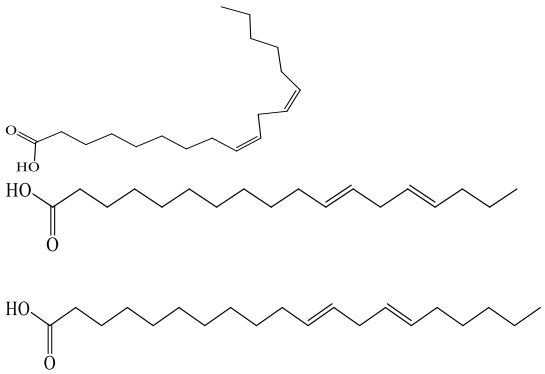
C can be a mixture of fatty acids since it is usually impossible to isolate individual acids on a silica gel column or by TLC. Therefore the carboxylic acid composition and their structures in C was analysed by GC-MS after preparation of the methyl ester. In the present method used for the preparation of fatty acid methyl esters (Ichihara and Fukubayashi, 2010), the methanolysis reaction is improved by enhancement of the solubility of hydrophobic acids and esters, if any, by adding toluene, and the use of heating in the sealed tube condition 100 °C shortened significantly the reaction time. The desired groups of methyl esters is the reaction mixture after further workup procedure were separated from other products by micro-column and then by PTLC.

Three fatty acids have been identified by GC-MS in isolated compound. From the selected peaks in the TIC chromatogram, Figure 5, linoleic acid (or) (*Z, Z*)-9,12-octadecadienoic acid (**C-1**) (RT 16.861 min) (Figure 6) was observed as free fatty acid (FFA) in the GC-MS. The second is 11,14-octadecadienoic acid (**C-2**) as its methyl ester (RT 19.511 min), Figure 7 and 11,14-eicosadienoic acid (**C-3**) as its methyl ester (RT 21.835 min) Figure 8. All the mass spectra show the low mass ion series corresponding to the formula $C_nH_{2n-3}$, namely 53, 67, 81, 95, 109,123, 137, 151 etc., suggesting a long chain hydrocarbon containing two double bonds, which is present in all the three compounds. A diagnostic peak at m/z 73 for a long chain fatty acid can be observed in the mass spectrum of **C-1**.




In the mass spectra of the fatty acid methyl esters of **C-2** and **C-3**, *m/z* 263 and 291 peaks for [M-31] corresponding to [M-$OCH_3$] by loss of methoxyl radical are very small. Refined search in the total ion chromatogram (TIC) may give better mass spectra for these esters.

**C-1**
Linoleic acid (or)
(*Z*,*Z*)-9,12-octadecadienoic acid



**C-2**
11,14-octadecadienoic acid

**C-3**
11,14-eicosadienoic acid

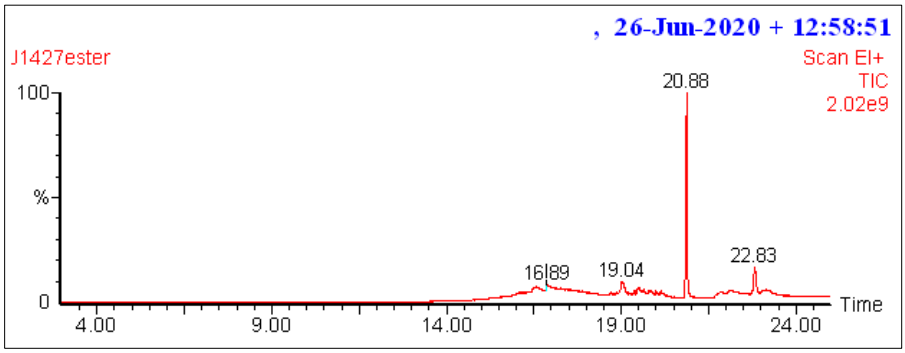



**Figure 5** Total ion chromatogram (TIC) of isolated compound

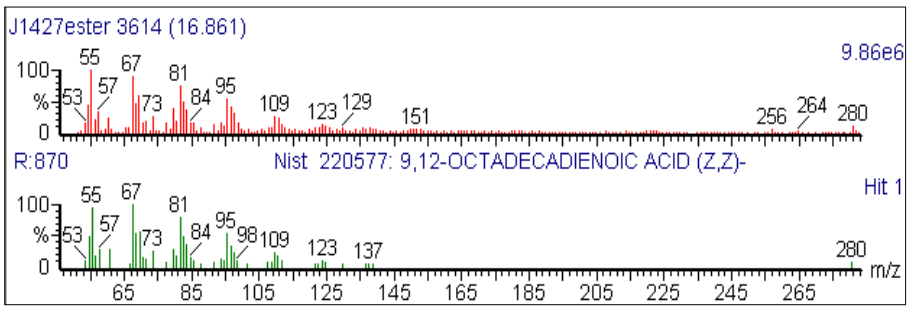



**Figure 6** Comparison of the EI mass spectra of C-1 (upper) and 9,12- octadecadienoic acid

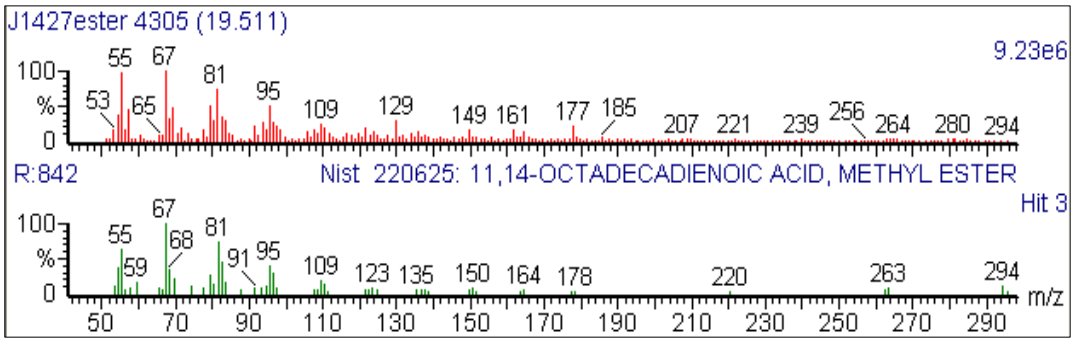



**Figure 7** Comparison of the EI mass spectra of C-2 methyl ester (upper) and methyl 11,14-octadecadienoate (lower)

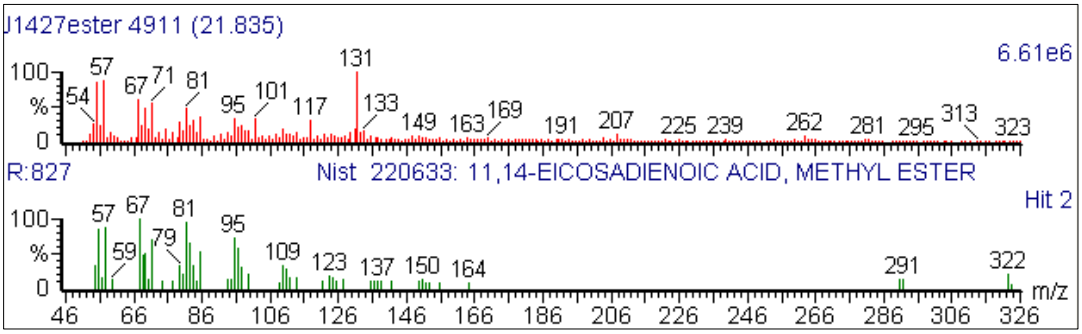



**Figure 8** Comparison of the EI mass spectra of C-3 methyl ester (upper) and methyl 11,14-eicosadienoate (lower)

## Conclusion

From the leaves of *Clinacanthus nutans*, carboxylic acids **C** were isolated after passing through different column chromatographic techniques. **C** giving a single spot on TLC was deduced as long-chain fatty acids by the FT IR and physicochemical characteristics. Three fatty acids have been further identified by GC-MS in **C**, namely linoleic acid (or) (*Z,Z*)-9,12-octadecadienoic acid (**C-1**), 11,14-octadecadienoic acid (**C-2**) and 11,14-eicosadienoic acid (**C-3**).

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