GC-MS Analysis and Antioxidant Activity of Leaves of *Aegle marmelos* L. Correa (Ok-shit)

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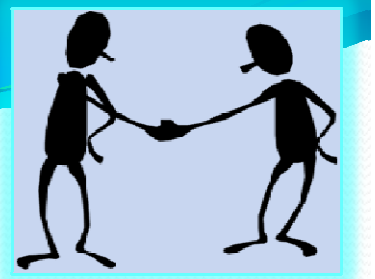
Introduction



- Medicinal plants has been increasing all over the world due to minimal toxicity, cost effective, pharmacologically active
- Provide easy remedy for many human ailments when compared to synthetic drug (1)
- WHO estimated 75% of population in developing countries applied traditional medicine for primary health care (2)
- Phytochemicals are bioactive chemicals of plant origin, and known as secondary metabolites and provide health benefits to human illness (3)
- Gas chromatography mass spectrometry (GC-MS) provides advantage of both chromatography as a separation method and mass spectrometry as an identification method (4)

Introduction (Contd.)

- **Fourier Transform Infrared Spectrophotometer (FTIR) is the most powerful tool for identifying the functional groups present in compounds [5]**
- **Many researchers have been studied for more cost effective and active phytochemical which possess antioxidant activity**
- **Thin layer chromatography, TLC- bioautogarphy and *In- vitro* DPPH radical scavenging methods are useful for finding of new antioxidants from plant extract [6]**

Introduction (Contd.)

- *Aegle marmelos* L. Correa, popularly known as **Bael tree**, **Ok-shit** in Myanmar, belongs to family **Rutaceae**
- distributed - India, Sri Lanka, **Myanmar** and Thailand [7]
- *Aegle marmelos* - edible & possess many medicinal properties
- Crude extracts of Ok-shit - **antioxidant, antidiabetic, anticancer, anti-hyperlipidaemic, anti-inflammatory, antimicrobial activities** on various animal models [1]
- **Young fruit** - Traditional Medicine Formulation No 41 (**Wunmeetauk Hsay**) [8]
- **Antihyperglycemic activity** by Soe Sandar Phyo *et al.*, 2015 **lipid lowering effect** on Wistar Albino rats by Aye Aye Mya, 2016, **antibacterial activities** by Lei Lei Win *etal.*, 2018 [9,10,11]
- **Anti-diarrhoeal activity** of unripe fruit of ဥသျှစ် was studied by Khin Tar Yar Myint *et al.*, 2017 [12]

Scientific evaluation

- **bioactive constituents**
- **antioxidant activity**

Ok-shit leave has not been published yet, in Myanmar

General objective

To evaluate chemical compositions, antioxidant activity, total phenolic content and acute toxicity study of leaves of *Aegle marmelos* L. Correa (Ok-shit)

Specific objectives

- **To find out phytochemical constituents of Ok-shit leaves**
- **To investigate bioactive constituents from Ok-shit leaves**
- **To assign the functional groups of ethanolic extract of Ok-shit leaves**
- **To measure antioxidant activity of Ok-shit leaves**
- **To determine phenolic content of Ok-shit leaves**
- **To evaluate LD_{50} of extract by acute toxicity test**

Materials

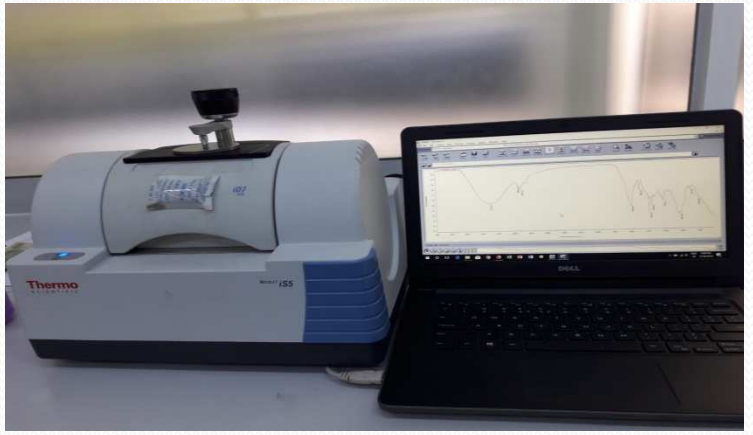
Analytical grade reagents

- Ethanol, Methanol, n- Hexane, Ethyl acetate (Merck)
- L -Ascorbic acid (BDH, England)
- 1, 1- diphenyl-2-picryl-hydrazyl (DPPH) (Merck)
- Gallic acid
- Sodium carbonate
- Folin- Ciocalteu reagent (Merck)
- Thin layer chromatography (TLC) silica gel plate (F245)
- ICR (Institutional Cancer Research) mice

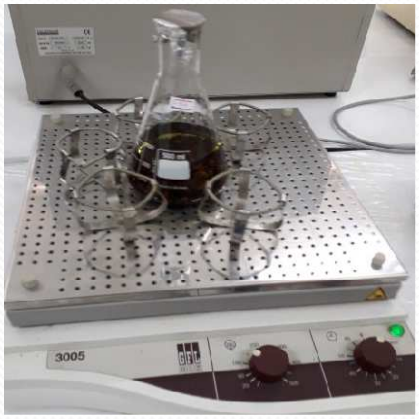
Materials (Contd.)

Gas Chromatography - Mass Spectrophotometer (GC-MS), QP-2020

Fourier Transform Infrared Spectrophotometer FTIR, Nicolet is5 (Thermo Scientific)



GFL Orbital Shaker (Germany)



UV-Lamp (Lambda-40) Perkin-Elmer

Rotary Evaporator

UV- Visible Spectrophotometer (UV 1601 PC)

Methods

Plant authenticity

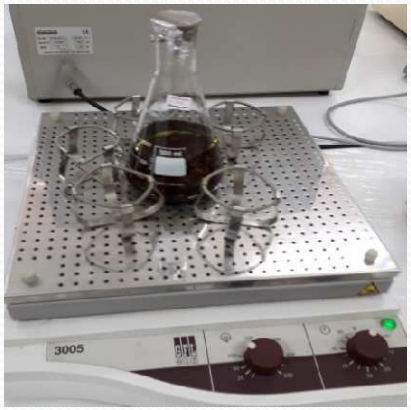
- collected fresh samples with their inflorescences were studied and identified for specific botanical name by competent taxonomist[13]

Sample Collection

- Fresh leaves samples
- Pyin Oo Lwin township, Mandalay Region, in May 2017

Methods (Contd.)

Preparation of extracts[14]



50 g of Ok-shit with 500 ml 95% EtOH

Macerate

orbital shaker, three days, filter

GC-MS analysis; 1 mL Filtrate -diluted with solvent 1:3 ratio

Remaining liquid extract

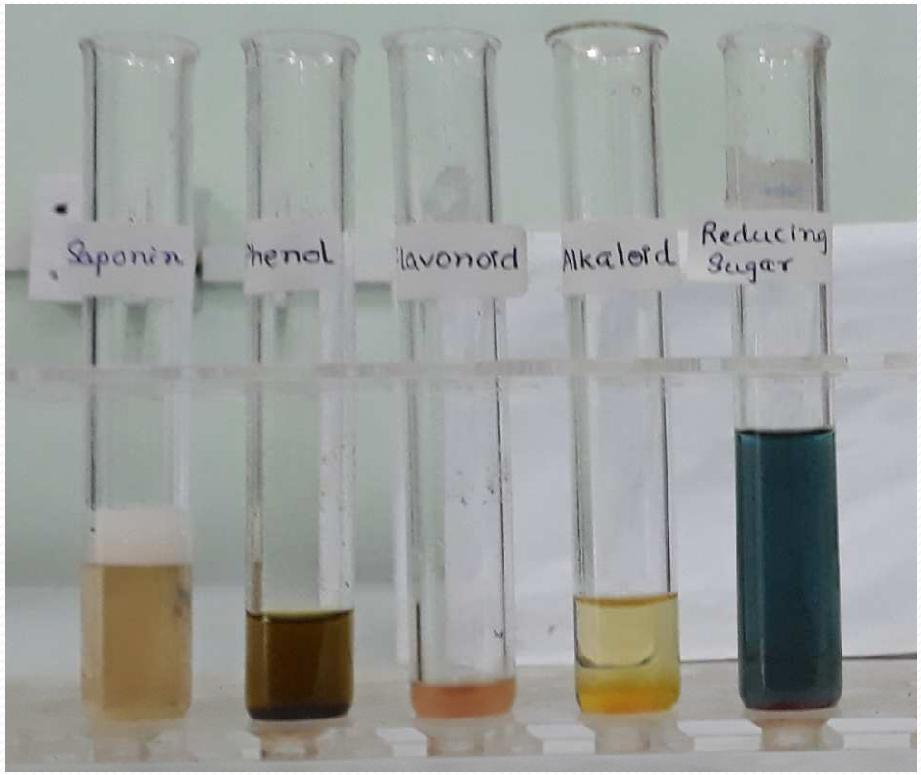
dried in air

crude extract

Methods (Contd.)

Phytochemical tests for types of compounds

Raaman (2006) Phytochemical Methods[15]

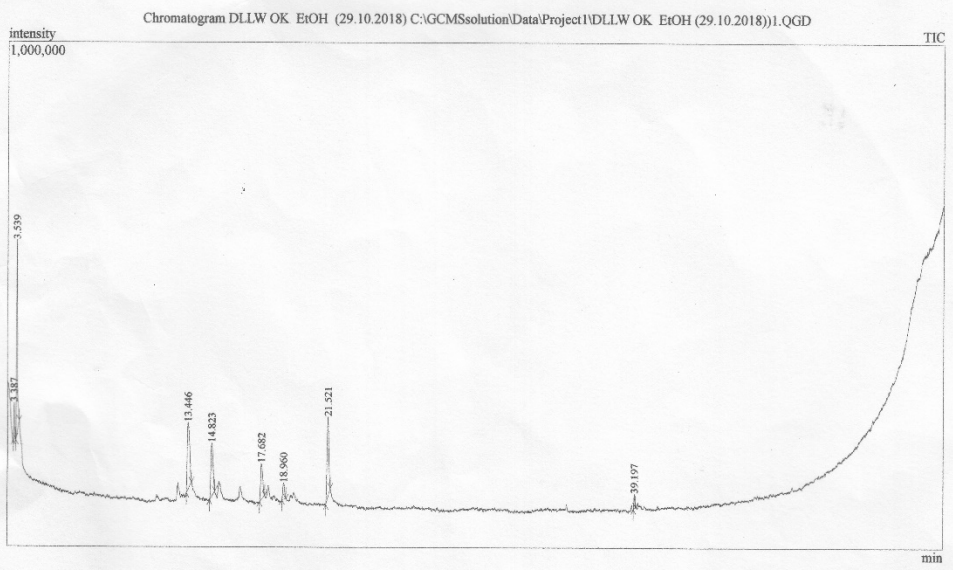


Methods (Contd.)

Gas Chromatography- Mass Spectrometry Analysis[6]

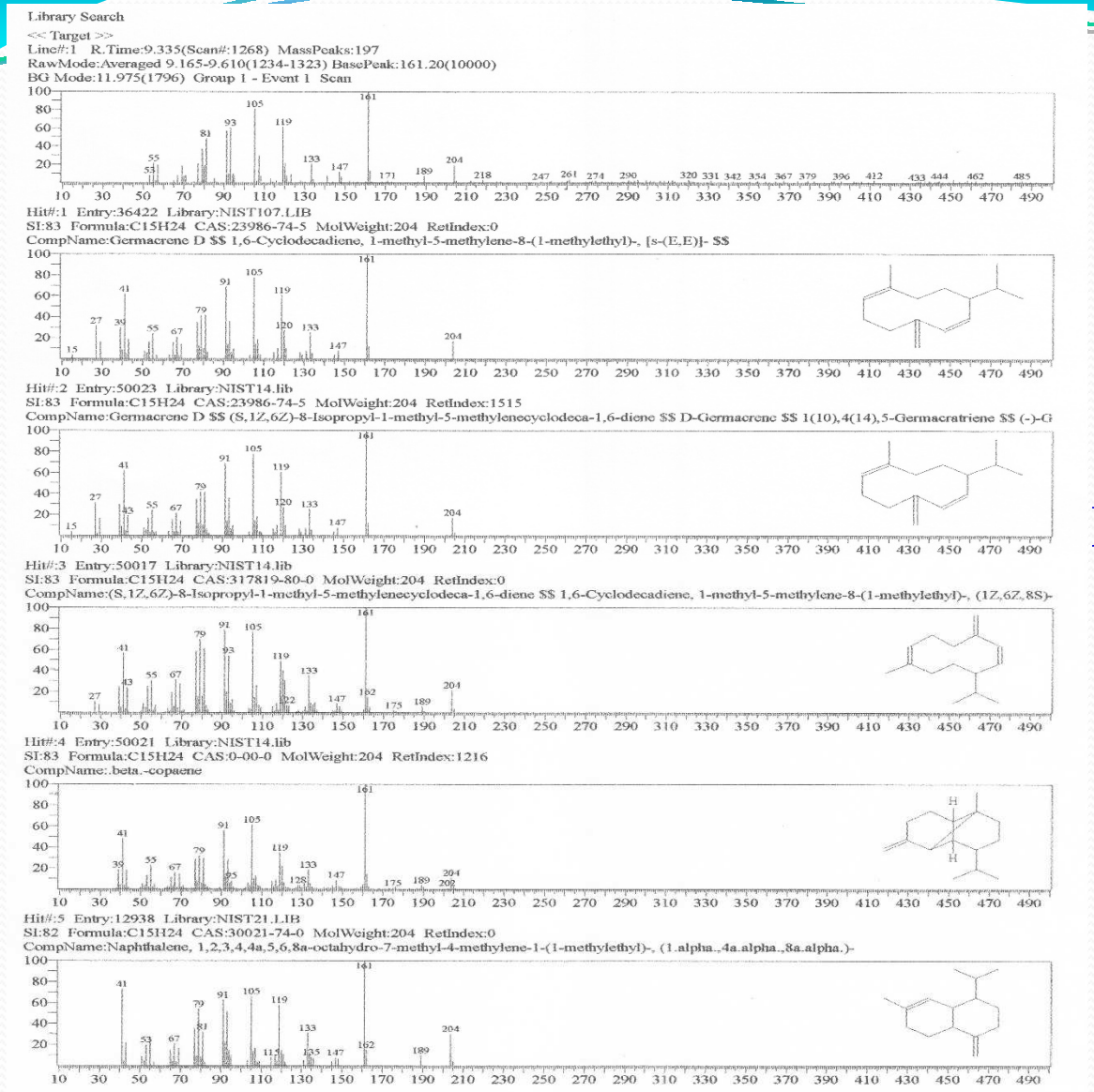
- methyl silicone - coated fused -silica capillary column DB-5 MS (0.25 mm i.d. x 30 m, 0.25 μm film thickness) autosampler and autoinjector (AOC-20 i +s)
- injection temperature 250 °C
- interface temperature 230 °C
- ion source temperature 230 °C
- electron impact (EI, 70 eV)
- oven temperature- 100 °C, increase 2 °C min^{-1} to 150 °C ramped up at 5 °C min^{-1} to 310 °C
- Helium gas flow rate of 1.57 ml min^{-1}
- mass range 40–650 *m/z*

Methods (Contd.)



- **gas chromatogram shows relative concentrations of various compounds getting eluted as a function of retention time (RT)**
- **heights of peak indicate relative concentrations of components present in sample**
- **large compound fragments into small compounds giving rise to appearance of peaks at different mass by charge (m/z) ratios**

Methods (Contd.)



mass spectrometer

- nature
- structure of compounds eluted at different times

Bioactive components

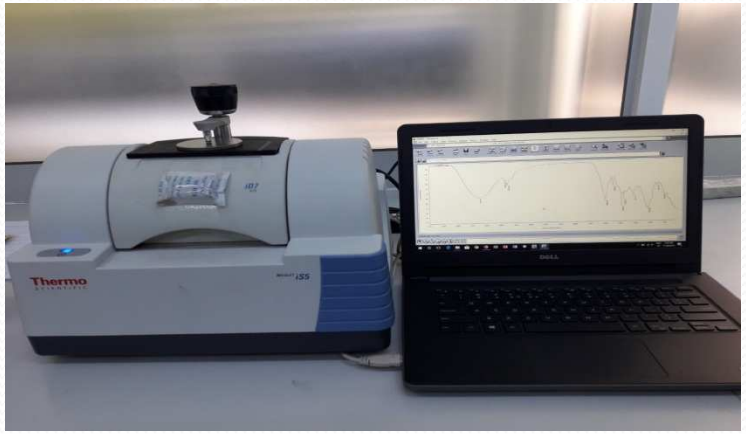
- unknown components
- known compound
- similarity index (SI)
- molecular formula (MF)
- molecular weight (MW)
- molecular structure

National Institute Standard and Technology (NIST) Libraries

Methods (Contd.)

FTIR Spectroscopic Analysis

- **Nicolet is5 FTIR spectrophotometer,**
- **Detection characteristic of peaks & their functional groups (Attenuated Total Reflectance) accessory**
- **IR scan - 4000-650 cm^{-1} (mid infrared range) [15,16]**
- **Delta Science Company Limited**



Methods (Contd.)

Thin Layer Chromatography

- TLC silica gel plate
  in solvent pre saturated glass chamber
- n-hexane: ethylacetate (1:1)
- under UV light (365 nm and 254 nm

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

TLC-bioautography analysis

- 0.25% DPPH in methanol sprayed - dried TLC plate
- yellow spots against purple background
- noted the R_f value[6]

Methods (Contd.)

Measurement of *In- vitro* Antioxidant Activity[17,18]

Preparation of freshly prepared DPPH solution (60 µM)

DPPH (2.36 mg) + 95 % ethanol (100 ml)
(60 µM)(Purple)

Final solution in volumetric flask

Methods (Contd.)

Preparation of different concentrations of ascorbic acid and Ok-shit extracts

10 mg/10 ml, 95% ethanol

50% ethanol

(a) (b)

Serial dilution

0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16

µg/100µL

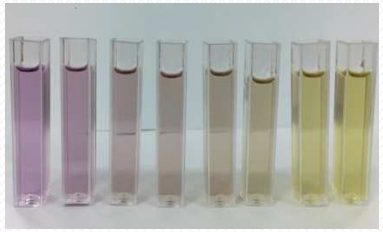
a = ascorbic acid , b = Ok-shit extract

Methods (Contd.)

The scavenging reaction between DPPH and test sample of an antioxidant

30 mins

RT



517 nm

Test sample 1ml

DPPH 2 ml

Mixing

$$\% \text{ Inhibition} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

$A_{control}$ = the absorbance of the control solvent (DPPH)

A_{test} = the absorbance in the presence of the tested

{ sample expressed in terms of 50% inhibition concentration (IC_{50})}

Methods (Contd.)

Determination of total Phenols[19]

Preparation of Gallic Acid Standard Curve

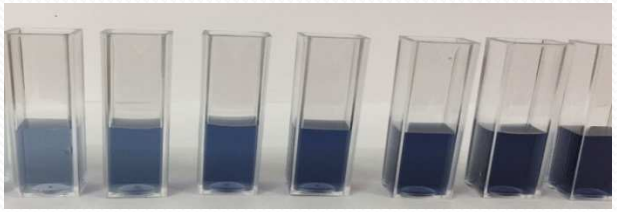
D W

10mg/10mL
95% Ethanol

0.1
mg/mL

Serial dilution

5
10
15
20
25
30
35
40



100µL
F C

300µL, 20 %
Na_2CO_3

40 °C, 30 mins

765 nm

µg/1600µL Gallic acid

Methods (Contd.)

Determination of total Phenols

Preparation ethanolic extract of Ok-shit

10mg/10ml
95% ethanol

30µL
Extract

1570µL
D W

40 °C

30 mins

765 nm

1600µL
Extract

100µL
F C

300µL, 20 %
Na_2CO_3

Mixing

Methods (Contd.)

Acute toxicity test of ethanolic extract Ok-shit leaves

- limit test at the dose of 5000 mg/kg, OECD 425 guideline
- fasted food for 3-4 hours
- dose was calculated upon fasted body weight
- single dose administration, food may be withheld 1-2 hours[20]
- observed for 24 hours, changes in behavioral responses
- 14 days recorded with individual work sheet
- LD_{50} was calculated by AOT 425 Stat program (US Environmental Protection Agency) [21]

Methods (Contd.)

Statistical Analysis

- Microsoft Excel v. 2007
- Antioxidant activity- Linear regression equation
  " Y= a X + b"
- Total phenolic content- Gallic acid standard curve

Results and Discussions

Botanical Identification

collected fresh samples were identified
Department of Botany, University of Mandalay

Aegle marmelos **L. Correa**

Extracts of Ok-shit leaves

- **Yield percent- 95% ethanolic extracts - 33.09%**

Results and Discussions (Contd.)

Table 2. Phytochemical constituents of Ok-shit leaves

No.	Phytochemical	Reagents	Observation	Result
1.	Alkaloids	Dragendorff's solution	Orange ppt	+
2.	α amino acid	Ninhydrin	Pink color	+
3.	Carbohydrate	α –naphthol, Con: H_2SO_4 solution	Pink ring color	+
4.	Flavonoids	Con: HCl, Mg turning	Reddish brown	+
5.	Glycosides	10 % lead acetate solution	Yellow ppt	+
6.	Phenols	10% Fe Cl_3 solution	Blue	+
7.	Protein	10 % Na OH, 10 % $CuSO_4$ solution	Red or Violet	+
8.	Reducing sugar	Benedict's solution	Brick red ppt	+
9.	Saponins	H_2O, Shaken 15 minutes	2 cm foam	+
10.	Starch	Iodine solution	Blue	–
11.	Steroids	acetic anhydride, Con:H_2SO_4 solution	Greenish blue Solution	–
12.	Tannins	1% Fe Cl_3, Dil: H_2SO_4 solution	Yellowish brown	+
13.	Tri-terpene	$CHCl_3$, acetic anhydride, Con: H_2SO_4 solution	Reddish brown coloration	+

(+) = Detected (-) = Not detected

Results and Discussions (Contd.)

In phytochemical analysis

- **Ok-shit leaves -alkaloids, carbohydrates, flavonoids, glycosides, phenols, protein, reducing sugar, saponins, tannins and tri-terpene**

- **alkaloids, flavonoids, phenols & tannins –antioxidant activity**[11]

Results and Discussions (Contd.)

Gas Chromatography - Mass Spectrometry Analysis

- Eight compounds were identified
- The retention time and peak area percent of various compounds were presented in figure 1 and table 2

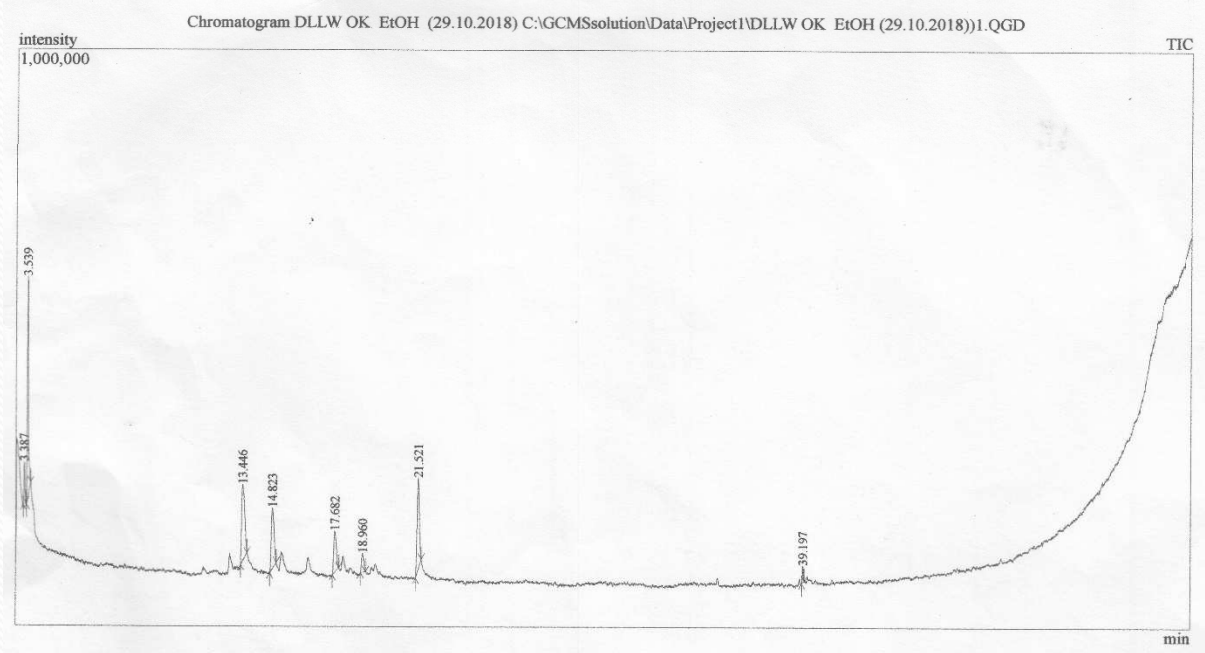


Fig 1. GC-MS chromatogram of ethanolic extract of Ok-shit leaves

Results and Discussions (Contd.)

Table 2. Bioactive compounds from ethanolic extract of Ok-shit leaves

Sr No	RT (min)	SI	Name	MF	MW	Peak Area %	Library	Compound nature	Activity[22,23,24,25]
1	3.39	94	α- pinene	$C_{10}H_{16}$	136	4.01	NIST 107 LIB	Mono-terpene	antioxidant, anticancer, Antiseptic, antimicrbial anti-inflammatory
2	3.54	96	β-ocimene	$C_{10}H_{16}$	136	24.79	NIST 14 lib	Mono-terpene	Antifungal
3	13.45	95	1-ethenyl-1-methyl-2,4-bis (1-ethylethenyl) -[1S-(1-alpha., 2 beta.,4 beta)], Cyclohexane	$C_{15}H_{24}$	204	21.69	NIST 21 LIB	Cyclo alkane	Antibacterial
4	14.82	96	caryophyllene	$C_{15}H_{24}$	204	15.5	NIST 14 lib	Bicyclic Sesqui-terpene	antioxidant, antitumor, anxiolytic, antibacterial, analgesic, neuroprotective anti-inflammatory
5	17.68	92	β-copaene	$C_{15}H_{24}$	204	9.67	NIST 14 lib	Triclic Sesqui-terpene	Antioxidant
6	18.96	92	8-Isopropenyl-1,5-imethyl-1,5-cyclodecadiene	$C_{15}H_{24}$	204	3.87	NIST 14 lib	Terpene	anticancer, anti-inflammatory
7	21.52	81	germacrene	$C_{15}H_{24}$	204	18.93	NIST 14 lib	Sesqui-terpene	antioxidant, antimicrobial, insecticidal
8	19.36	92	9-octadecenoic acid (Z) - methyl ester	$C_{19}H_{36}O_2$	296	1.53	NIST 14 lib	Fatty acid ester	antioxidant, antifungal, antimicrobial, anticarcinogenic, hypocholesterolemic

Results and Discussions (Contd.)

According to GC-MS analysis

- **eight bioactive compounds - possess many medicinal properties α- pinene, caryophyllene, β-copaene, germacrene and 9-octadecenoic acid (Z) -methyl ester - antioxidant activity**
- **α-pinene, ocimene , caryophyllene, copaene & germacrene from current study agreed with following two studies**
- **Rathee D. *etal*.. 2017, - volatile oil of bael leaves presence of α-pinene, caryophyllene, α-ocimene, and α-copaene [6]**
- **Satyal P.*etal*., 2012, - essential oil of bael leaves contained limonene, (E)-β-ocimene, germacrene B, (E)- caryophyllene[26]**

Results and Discussions (Contd.)

FTIR Spectroscopic Analysis

The results of FTIR analysis were presented in Fig. 2 and Table 3

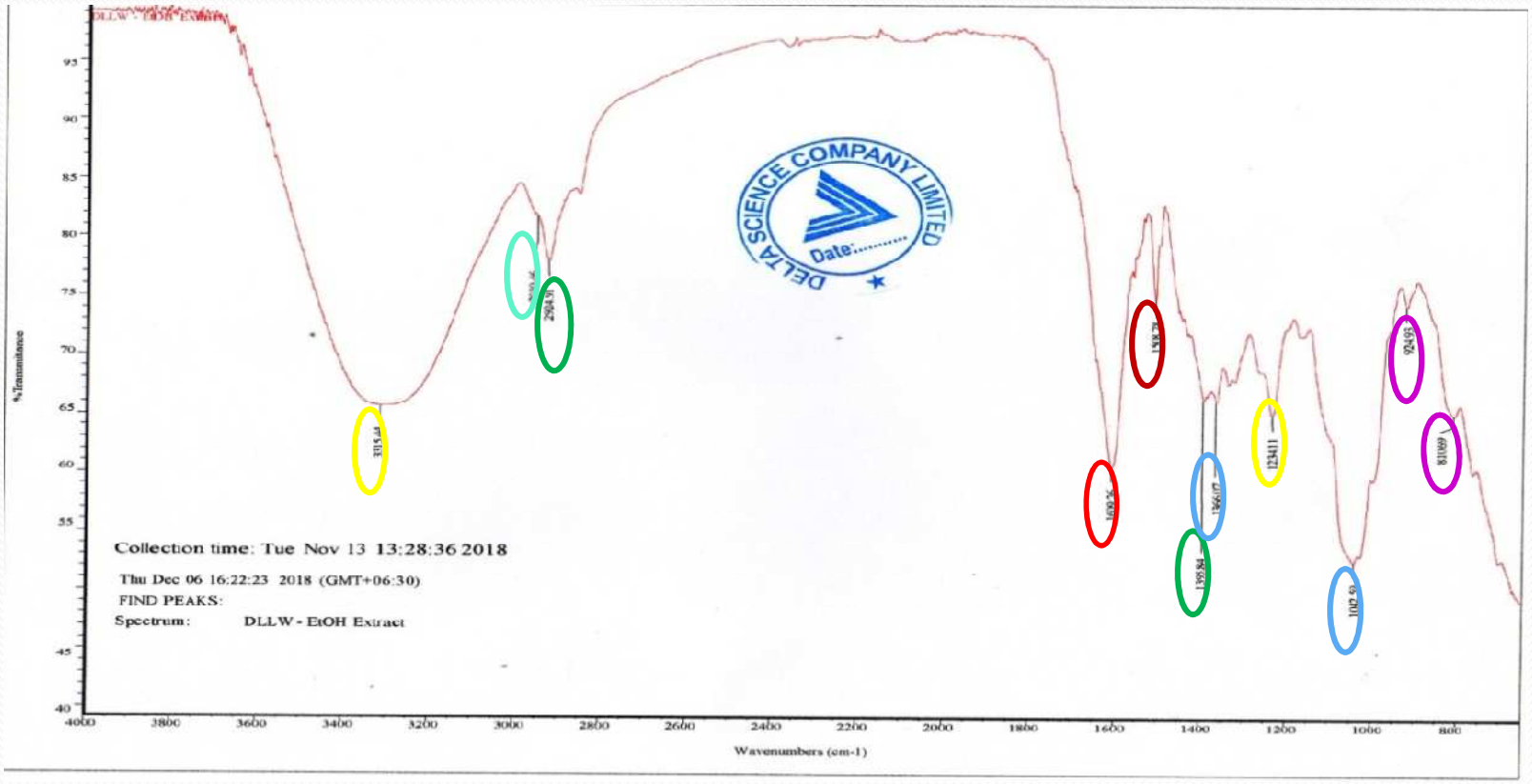


Figure (2) FT-IR Spectrum of ethanolic extract of Ok-shit leaves

Results and Discussions (Contd.)

Table 3. FT-IR Assignments of ethanolic extract of Ok-shit leaves

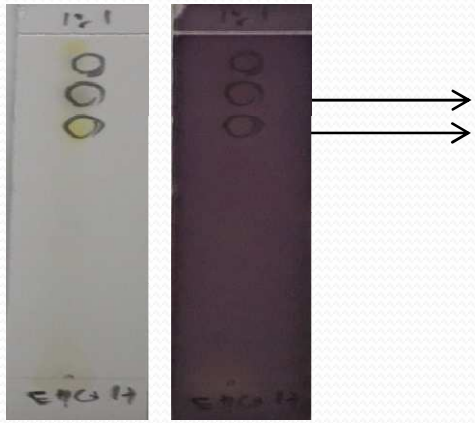
No	Wave number (cm^{-1})	Functional groups
1.	3315.44	O-H stretching vibration of hydroxyl group
2.	3010.46	C-H stretching vibration of sp^2 hydrocarbon
3.	2924.91	C-H stretching vibration of sp^3 hydrocarbons
4.	1609.26	C=O stretching vibration of carbonyl group
5.	1508.28	C=C stretching vibration
6.	1395.84	C-H bending of sp^3 C-H group
7.	1366.07	C-C-O stretching vibration of ester group
8.	1234.11	O-H bending vibration
9.	1042.59	C-O stretching vibration of ester group
10.	924.93	C-H bending vibration of trans or E alkenic goup
11.	810.69	C-H bending vibration of cis or Z alkenic goup

Results and Discussions (Contd.)

TLC-bioautography analysis

Antioxidant potential of compound on TLC plates

- **identified with DPPH reagent,**
- **the yellowish bands on the purple background**
- **R_f value - 0.75 and 0.85**



Yellow

antioxidant fraction

Fig 3: Thin Layer Chromatogram and Bioautograph for antioxidant fraction

- **TLC-bioautography analysis- distinct yellow region against DPPH**

Results and Discussions (Contd.)

Measurement of *in-vitro* antioxidant activity

- Antioxidants are significant prevention of human ageing
- % inhibition of ascorbic acid - 40 to 100% ($R^2 = 0.9933$)
- % inhibition of Ok-shit extract - 41.98 to 63.96% ($R^2 = 0.98$) at different concentrations ranging from 0.125- 16 µg/100µL

IC_{50} values

- ascorbic acid - 2.7µg/mL
- Ok-shit extract - 49.7µg/mL

Results and Discussions (Contd.)

Table 4. Mean percent inhibition and IC_{50} values of Ok-shit extract and ascorbic acid

Sr. No	Sample	Concentration (µg/100µL)/ % inhibition								IC_{50} (µg/mL)
		0.125	0.25	0.5	1	2	4	8	16	
1.	Ascorbic acid	40	50.27	64.82	90.16	100	100	100	100	2.7
2.	OK-shit Extract	41.98	42.75	43.66	45.03	47.48	49.61	54.96	63.96	49.7

Results (Contd.)

Determination of total phenols

- determined by Folin- Ciocalteu reagent
- Gallic acid standard curve equation

$y = 0.0516x + 0.0844$, $R^2 = 0.9963$

phenolic content of Ok-shit leaves - 47 mg GAEs/g

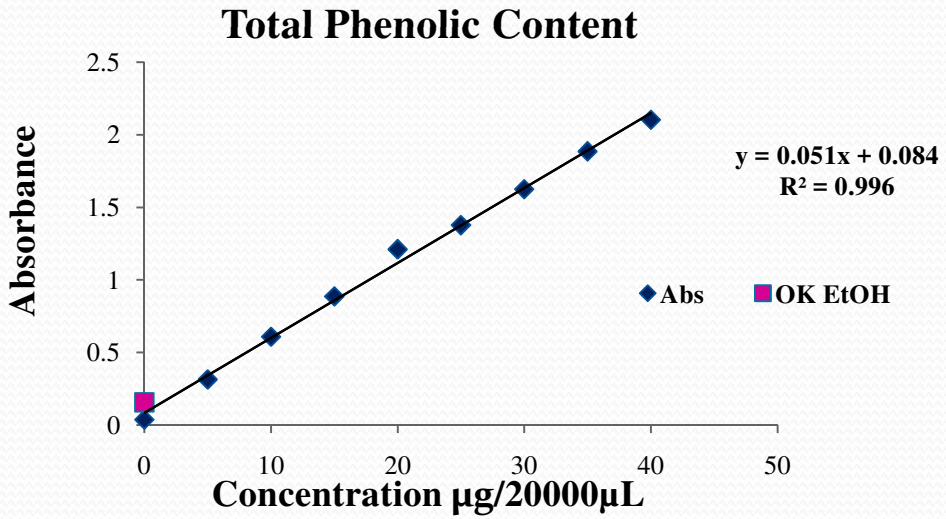


Fig 5: Standard curve of Gallic acid

Results and Discussions (Contd.)

- direct correlation - antioxidant activity & phenolic content
- our study results, ascorbic acid & Ok-shit extract IC_{50} values 2.7µg/mL & 49.7µg/mL, phenolic - 47 mg GAEs/g comparable to
- Raja *et al* (2017), - aqueous extract of bael leaf, IC_{50} = 125µg/mL and total phenolic content = 40.84mg/g [18]
- Wali *et al* (2015), - methanolic extract of bael leaf IC_{50} = 249.3 µg/mL and phenolic content = 16.5mg GAEs/g[25]
- Gupta *et al* (2014), - ascorbic acid and methanolic extract of unripe fruit of *Aegle marmelos* were IC_{50} value of 2.8µg/mL and 62.59µg/mL respectively[27]

Results and Discussions (Contd.)

Acute Toxicity

- OECD 425 guideline
- Hakim *et al*. (2003), ethanolic extract of Ok-shit leaves - non-toxic[28], limit test was used
- No toxic signs and lethality up to 14 days
- Skin and fur changes, eyes, mucous membrane, respiratory rate, motor activity and behavioral pattern were found to be normal
- Salivation, convulsion, cyanosis, tremors, & diarrhoea did not occur in all animals
- LD_{50} , above 5000 mg/kg
- Ok-shit leaves are acute safe for consumption

Results and Discussions (Contd.)

Qualitative and Quantitative variations of same species

- difference in geographical location
- climatic conditions
- time of harvest
- habitat of plant samples
- part used
- extraction method
- type of solvent and
- other environmental factors

Conclusion

- **scientifically proved that the presence of bioactive constituents, antioxidant activity, phenolic content and acute toxicity study of ethanolic extract of Ok-shit leaves**
- **antioxidant activity and phenolic content were related to bioactive constituents resulting from GC –MS chromatogram**
- **ethanolic extract of Ok-shit leaves exhibited antioxidant properties may be suggested new potential sources of antioxidant**
- **recommended as a highly antioxidant plant for traditional healers and acute safe for consumption**

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- **all of colleagues, who have willingly helped us throughout with their ability**

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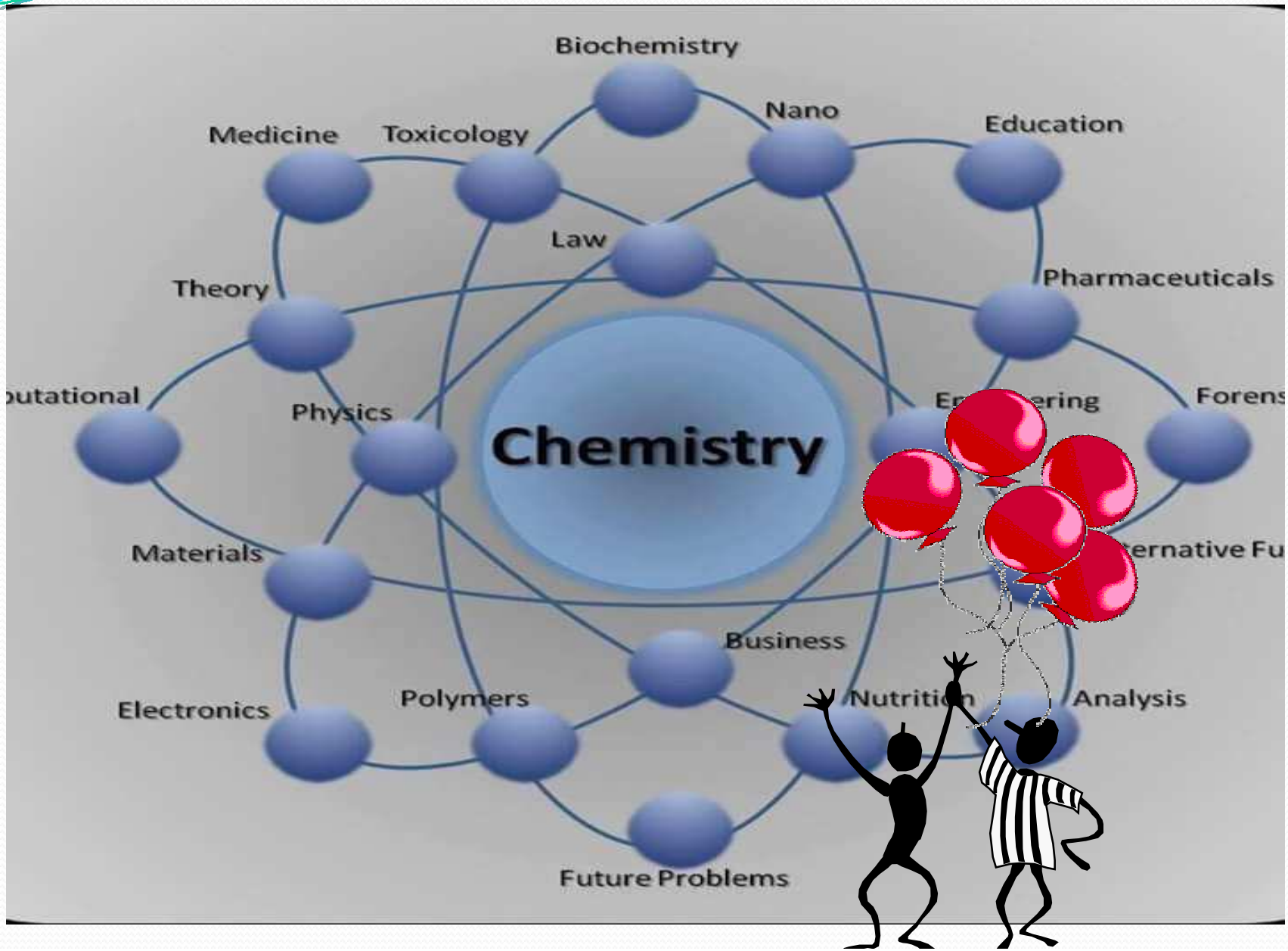
Thank you for your kind & patient attention



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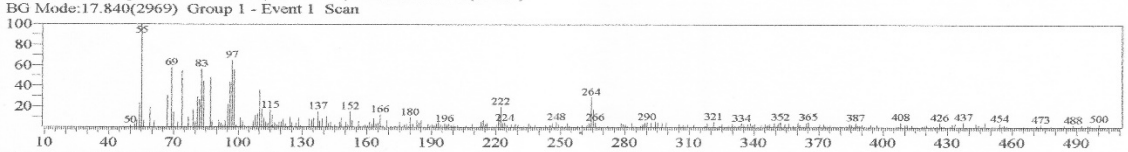

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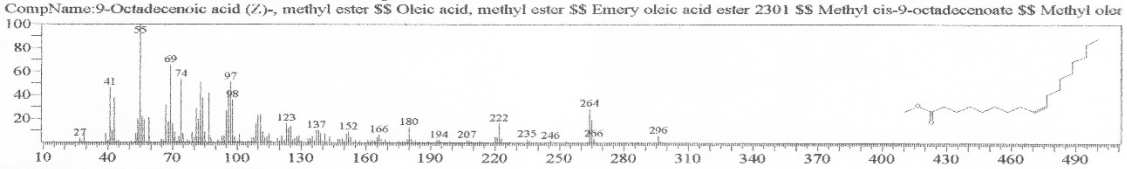

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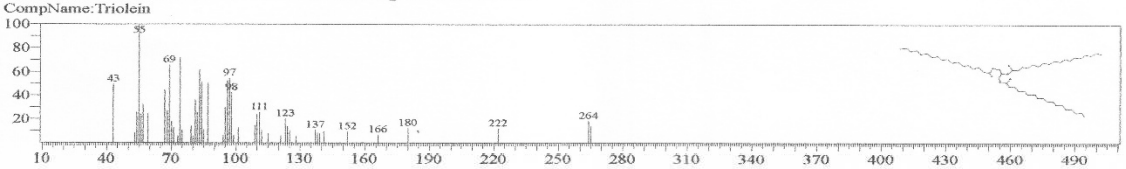

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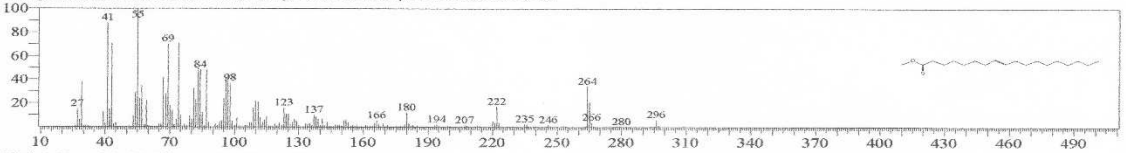

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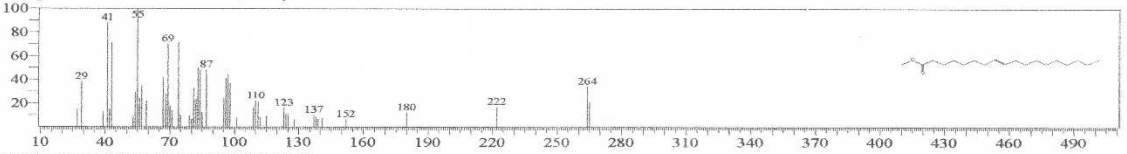

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