# Chemical Composition Study and Biological Activities of Oil of *Casuarina Equisetifolia* Leaves

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**Abstract:** The fixed oil extract from *Casuarina equisetifolia* leaves was tested for antifungal and antioxidant activities. The antifungal activity was determined against some phytopathogenic fungi by food poison technique. The results indicated that the percentage inhibition of mycelia growth increased with increasing concentrations of fixed oil for all tested strains. It was clear that *Rizoctonia solani*, *Macrophomina phaseolina* and P*hytophthera cactorum* showed strong sensitivity to extract and the EC50 values were 3228.12, 3945.74 and 4351.63ppm, respectively. Meanwhile, it displayed moderate antifungal activity against *Sclerotium rolfsii*, *Aspergillus niger* and *Pestalotia longisetula*. In addition to, the fixed oil exhibited high DPPH radical scavenging activity which recorded 78.5%. While, the chemical composition of the extracted fixed oil was determined by GC. The results illustrated that twenty five saponification and twenty seven unsaponification matter were identified in the oil. The major saponification compounds were lignoceric (10.8%) and cis-10-heptadecanoic acids (10.5%). The main unsopnification matter was trans-1, 2-dimethyl cyclopentane which recorded 51.3%. These results showed that, the antifungal and antioxidant activities of fixed oil extracted from *Casuarina equisetifolia* leaves could be due to the higher concentration of lignoceric and cis-10-heptadecanoic acids.

**Keywords:** *Casuarina equisetifolia*, antifungal , antioxidant, GC, lignoceric and cis-10-heptadecanoic acids.

## 1.Introduction:

The fungi are major disease causing agents on plants and can lose up to 90% of agricultural yield. Various systemic fungicides have been used to control the plant diseases, but due to indiscriminate use of synthetic fungicides, various important pathogens have been developed resistance to many of the currently available fungicides (**Gangawane, 1990**). Beside, this fungicide also pollutes soil and water.

Sometimes the fungicide adversely affects the non target organisms. Hence it is necessary to search new antifungal compounds as an alternatives, safe, ecofriendly, cheap to synthetic fungicides from plants, since they produce different secondary metabolites which perform defensive role in plants and protect the plants from their invaders. Plant extracts and essential oils has been investigated throughout the world for their antifungal activity against wide range of fungi (**Ezzat, 2001; Abd-El-Khair and Hafez, 2006; Gupta *et al*., 2008)** *Casuarina equisetifolia* L. is predominantly a monoecious species which belongs to the family Casuarinacease. The plant has the capability of growing in a wide range of soil conditions particularly on coastal and limestone soils near the shore. It tolerates light to heavy textured soils **(Ramanathan *et al*., 2012).**

Medicinal plants are natural resources yielding valuable products which are often used in the treatment of various ailments (**Dulger, *et al*., 2004**). Recently, many attempts have been made to investigate the indigenous drugs against infections diseases in order to help safer antimicrobial drugs can be developed.

*Casuarina equisetifolia* L. is a plant that is used in folk medicine for the treatment in diarrhea, cough, ulcers, toothache and diabetes (**Swamy et al., 2013**). The present study aims to screening Casuarina *equisetifolia* leaves oil for its antifungal and antioxidants activities. In addition to, study chemical composition of this oil (saponification and unsaponification) by using GC equipment.

## 2. Materials and methods:

### 2.1. Plant collection and preparation:

Leaves of *Casuarina equisetifolia* were collected from Central Agriculture Pesticides Laboratory (CAPL) farm. Identities of plants species were authenticated by referring standard literature. The plant leaves were brought to the laboratory washed in running tap water to remove debris and dust particles and then rinsed in distilled water for 5min. Then leaves were air dried under shade and powdered using electrical blender.

### 2.2. Fungal strains:

Cultures of plant pathogenic fungi were provided by Fungicide, Bactericide and Nematicide Department, CAPL . Each fungus were maintained on potato dextrose agar (PDA) and stored at 5° C for further studies.

### 2.3. Antifungal assay:

Antifungal activity of plant was determined by food-poisoned technique (**Mohanty *et al*., 2012**). Standard extracts at 3000, 4500, 6000, 7500 and 9000 µg/ml were mixed with 50ml of sterilized PDA medium and transferred equally into three Petri dishes. The media was allowed to solidify. Then seven day old fungal culture disk of 6-mm diameter was taken and inoculated to the center of Petri dishes containing plant extracts**.** Instead of PDA medium without plant extract served as control. All dishes were incubated at 27±2°C and radial

growth of colony was measured when the mycelia of control had almost filled the Petri dishes. Each test was performed in triplicate.

## 2.4. Extraction of fixed oil:

A dried and powdered Casuarina *equisetifolia* leaves (50g) were macerated three times for a 3-day-period in n-hexane (120ml) and filtered. The combined filtrate was evaporated under reduced pressure to dryness (oil matter). The fixed oil was kept at 4°C in the dark bottle.

The percentage yield was calculated as follows:

$$\%\text{Yield} = \frac{\text{Weight of extract recovered} \times 100}{\text{Weight of dry powder}}$$

## 2.5. Antioxidant activity:

### 2.5.1. The 2-diphenyl-1-picrythydrazyl (DPPH) radical scavenging assay:

The antioxidant activity of the fixed oil was assessed by their ability to scavenging DPPH stable radicals as reported earlier (**Mimica-Dukic *et al.*, 2003**) . The fixed oil (250µg/ml) was mixed with DPPH solution (1ml; 90µM) and then with methanol 95% to a final volume of 4ml. Synthetic antioxidant butylated hydroxyl toluene (BHT) was used as control. After 1h incubation period at room temperature, the absorbance was recorded at 515nm. Percent radical scavenging concentration was calculated using the following formula:-

$$\%\text{ radical scavenging} = \frac{\text{A (blank)- A(sample)} \times 100}{\text{A(blank)}}$$

Where:-

A (blank) = Absorbance of the control

A (sample) = Absorbance of the test sample

### 2.5.2. Inhibition of linoleic acid peroxidation:

The antioxidant activity of *Casuarina equisetifolia* leaf oil was evaluated in terms of percent inhibition of peroxidation in linoleic acid system (**Iqbal and Mi, 2005**). The fixed oil (5mg) was mixed with 0.31ml linoleic acid solution, 10ml ethanol 99.8%, 10ml sodium phosphate buffer (0.2M, pH=7) and diluted to 25ml with distilled water. The solution was incubated at 40C for 1h and extent of oxidation was investigated using colorimetric method. Then, at 0.2ml sample solution, 10ml ethanol75%, 0.2ml ammonium thiocyanate 30% and 0.2ml ferrous chloride solution (20mM in 3.5% Hcl w/v) were added consecutively. After stirring for 3min, the absorbance of mixture was calculated at 500nm. A control was also performed only with linoleic acid. The synthetic antioxidant such as BHT was used as positive control.

% inhibition of linoleic acid oxidation was investigated with the following equation :-

$$\%\text{ inhibition} = 100 - \frac{\text{(abs. increase of sample at 1h)} \times 100}{\text{(abs. increase of control at 1h)}}$$

## 2.6. Identification of fatty acids by using GLC:

For analysis fatty acids of the extracted oil were esterified with 2M NaOH in MeOH at room temperature as described by **A.O.A.C (1990).** Methyl esters of fatty acids were separated by using GLC apparatus (Agilent technologies, 6890N) (Network GC system, U.S.A). With the following conditions:-

Column: Quartz capillary column HP-5ms(5% phenyl methyl siloxane) (Agilent , United states) with a size 30.0m ×0.25mm×0.25nm, temperature gradient from 100 to 325°C, heating rate 5°C/min.

Mobile phase (carrier gas)- nitrogen, evaporator temperature 220°C with the consumption of the carrier gas- 30mi/min(nitrogen) , air- 300ml/min, hydrogen -30ml/min, the inflow rate -1.1ml/minand the flow division-1:27.

Oven temperature was programmed from 70°C, held for 5min and raised at 4°C/min to a final temperature of 240°C and held for 15min.

## 2.7. Identification of unsaponification matter by using GLC:

Preparation unsaponfication matter from extracted oil by adding 0.5g oil to 10ml alcoholic sodium hydroxide 4%, placed on a heater for 1hour with black flow condenser. The mixture was cooled at room temperature and removed to 50-ml flask. Then 20ml of diethyl ether were added, mixed and the upper ether layer was removed and weighted (unsaponification matter for injection) according to **A.O.A.C (1990).** unsaponfication matter were separated by using GLC apparatus (**Agilent technologies, 6890N) ( Network GC system, U.S.A)**. With adopting the same

$$\% \text{ unsaponification matter} = \frac{\text{Unsaponification matter} \times 100}{\text{Weight of fixed oil}}$$

## 3.Results and Discussion:

### 3.1.The yield of fixed oil:

The percentage yield of hexane extract (fixed oil) from the dry powder of *Casuarina equisetifolia* leaves was 5.5%, as shown in Table(1). The fixed oil extracted from *Peroskia abrotanoides* leaves was 3.6% **(Ashraf *et al*., 2014)**, while, **Rai *et al*., 2014** showed that the percentage yield of hexane extract from *Acacia nilotica* stem bark was 1.7%.

### 3.2. Antifungal activity:

The antifungal activity of hexane extract ( *Casuarina equisetifolia* ) *in vitro* against 8 species. (Table 2) summarizing the fungal growth inhibition which was calculated due to treatment against control using the following formula:-

**Table 1**. The percentage yield of fixed oil from *Casuarina equisetifolia* leaves.

| Plant part | solvent | Wt. of plant (g) | Wt. of extract (g) | Percentage yield (%) | Colour of extract |
|---|---|---|---|---|---|
| leaves | hexane | 50.0 | 2.75 | 5.5 | brown |

$$\% \text{ inhibition} = \frac{C\text{-}T \times 100}{C}$$ **(Satya *et al*., 2014)**

Where C is the average of three replicates of hyphal extension (mm) of control and T is the average of three replicates of hyphal extension (mm) of plates treated with tested material ( Casuarina oil ) . EC50 values were determined by the linear regression (LPD line computer program) of the probit of the tested fungs percentage inhibition vs. Logs the concentrations ( ppm) of the prepared casuarina oil . The Ec50 notation used to indicate the effective concentrations (ppm) that causes 50% growth inhibition. In essence, the lower the value of EC50 is the higher the efficacy of prepared oil in the test under consideration. The antifungal activity of Casuarina oil was prepared at the concentration ranged from 3000to 9000 ppm . The results showed that the percentage inhibition of mycelial growth increased with increasing concentration of prepared casuarina extract for all tested strains in a dose – manner . These results agreed with (**Nehad and Abd ulrahaman, 2012)** who reported that the ethanol extract of *C. equisetifolia* exhibited remarkable antifungal activities against the tested fungi in the order of sensitivity as *A.flaves >A.niger>A.fumagitus >C. albicans* . The half inhibitory concentration of antifungal activity was expressed as EC50 . It was clear *that R. solani , M. phaseolina,* and *P . cactorum* showed strong sensitivity to fixed oil . The EC50 values were 3228.12, 3945.74 and 4351.36ppm, respectively. Whereas it displayed moderate high antifungal activity against *Phoma sp., P. longisetula, Colletotrichum sp* and *S. rolfsii.* The EC50 values were 6409.17, 6442.25, 5614.84 and 5682.02, respectively. The less effective one which recorded EC50 (8860.27ppm)in case of *A. niger.*

The previous studies showed that leaves and fruit extracts of *Casuarina equisetifolia* have antibacterial and antifungal activity. The plant based products have been effectively proven for their utilization as source for antimicrobial compounds as described by **Swamy *et al.*, 2013,**while, **Mathur *et al.*, (2011)** who demonstrated that hexane extract of *Andrographis paniculate* have antifungal against *Aspergillus niger* and *R. solani* but no antifungal activity against *Candida allicans*. **Yenjit *et al.*, (2010)** lauric acid have antifungal activity against *Colletorichum gloesporiodes* and *Rhizoctonia solani* **(Yenjit *et al.*, 2010)**while, linoleic and linlenic acids have antifungal activity against *Rhizoctonia solani* ( **Walters *et al.*, 2004)**.

Antifungal mechanisms of free fatty acids may disrupt the cell membrane, especially in cells with low sterol content. They may inhibit myrisorylation of proteins and subsequent targeting of these proteins to the cell membrane. They may inhibit B-oxidation, triacylglycerol synthesis and sphingolipid synthesis, also they may inhibit topoisomerase activity **(Carolina *et al.*, 2011)**.

**3.3.Antioxidant activity:**

Antioxidants are an important part of the defense system of the human body and help to cope antioxidant analysis of plants (**Zia-Ui-Haq *et al.*, 2012**). DPPH is increasingly used quickly assessing the ability of antioxidants to transfer the labile H atoms to radicals**.** This hydrogen donation ability leads to formation of stable complex of free radicals, resulting in termination of damages caused by these radicals **(Zia-Ui-Haq *et al.*, 2013)**.

**Table 2.** Antifungal activity of fixed oil extracted from *Casuarina equisetifolia* on some phytopathogenic fungi.

| Fungi | %Inhibition growth at the different concentrations(ppm) | | | | | EC50 |
|---|---|---|---|---|---|---|
| | 3000 | 4500 | 6000 | 7500 | 9000 | |
| *Sclerotium rolfsii* | 33.3 | 44.0 | 52.0 | 54.0 | 64.8 | 5682.09 |
| *Rhizoctonia solani* | 50.3 | 59.2 | 68.6 | 78.1 | 85.6 | 3228.12 |
| *Mucrophomina phasolina* | 44.4 | 51.1 | 60.8 | 68.1 | 78.6 | 3945.74 |
| *Aspergilus niger* | 18.6 | 27.8 | 37.8 | 42.6 | 51.9 | 8860.27 |
| *Colletotrichum sp.* | 37.0 | 50.0 | 58.6 | 71.4 | 84.4 | 4351.36 |
| *Phoma sp.* | 24.1 | 38.1 | 48.6 | 59.2 | 80.0 | 5614.84 |
| *Pestalotia longisetula* | 20.3 | 27.8 | 42.2 | 61.1 | 74.8 | 6409.17 |
| *Phytophthora cactorum* | 22.2 | 38.1 | 46.3 | 53.0 | 65.2 | 6442.25 |

**Table 3.** Antioxidant activity of *Casuarina equisetifolia* fixed oil.

| Tested sample | DPPH radical scavenging (µg/ml) | percent inhibition of linoleic acid peroxidation |
|---|---|---|
| Fixed oil | 78.5 | 65.5 |
| BHT (standard) | 87.8 | 92.0 |

The fixed oil extracted from *Casuarina equisetifolia* leaves was screened for their possible antioxidant activity by DPPH radical scavenging as shown in Table (3). The fixed oil exhibited high DPPH radical scavenging activity recording 78.5µg/ml, whereas, standard antioxidant compound BHT showed the highest DPPH radical scavenging activity (87.8µg/ml).

The percent inhibition of linoleic acid peroxidation was observed for fixed oil, being 65.5%. Whereas, synthetic BHT provided inhibition at the level of 92.0%, Table (3).

Hexane extract of *Sinapis alba* was higher antioxidant potential which recorded 65% **Sujatha et al., 2014**, while, **Ramamathan *et al.*, 2012** showed that ethanol extract of *Casuarina equisetifolia* leaves was a potent antioxidant at a concentration of 250mg/ml recording 85.8%.

Antioxidant effect of a plant extract and its fixed or essential oil is mainly due to various bioactive compounds like flavonoids, phenolic compounds, tannis and diterpenes **(Zia-Ui-Haq *et al.*, 2013)**. The previous studies illustrated that antioxidants activity of fixed oil could be due to the presence of cis-10-heptadecanoic acid and lignoceric acids.

**3.4.GC analysis of fixed oil.**

The percentage of fatty acids were 75% in the fixed oil extracted from plant. While, the unsponfication matter percentage of fixed oil was 25%, (Table 4).

**Table 4.** The percentage of saponification and unsaponification matter in fixed oil

| Wt. of fixed oil (g) | % saponification | % unsopnification |
|---|---|---|
| 0.5 | 75.0 | 25.0 |

Qualitative GC analysis of the fixed oil extracted from *Casuarina equisetifolia* was performed in order to identify different compounds in the oils, as shown in Tables(5 & 6) and Figure (1&2) .

The GC analysis identified 25 fatty acids( Table 5) and 27 un-saponification matter,( Table 6). The fixed oil of plant consisted of a mixture of different classes of compounds. The major fatty acids components found in fixed oil of plant were

Lignoceric (10.8%), Cis-10-Hertadecanoic acid (10.5%), Behenic (8.6%), Henicosanoic (6.3%) Erucic (6.1%) and Oleic (5.2%), followed by Capric (1.6%), Pelargonic (1.6%), caprylic (1.3%), Lauric (0.6%) and Myristic acid (0.6%), (Table 5).

**Table 5.** Relative percentage of saponification compounds in fixed oil extracted from *Casuarina equisetifolia* leaves

| Rt | Cn | saponification compounds | % |
|---|---|---|---|
| 5.4 | C6 | Caproic | 3.5 |
| 5.9 | C8 | Caprylic | 1.3 |
| 7.6 | C9 | Pelargonic | 1.6 |
| 8.4 | C10 | Capric | 1.6 |
| 9.1 | C11 | Undecanoic | 0.6 |
| 18.3 | C12 | Lauric | 0.6 |
| 22.0 | C14 | Myristic | 0.6 |
| 22.6 | C14:1 | Myristoleic | 3.4 |
| 25.1 | C15:1 | Cis-10-pentadecanoic | 3.6 |
| 29.1 | C16:1 | Palmitolic | 3.4 |
| 32.1 | C17 | Heptadecanoic | 4.3 |
| 32.4 | C17:1 | Cis-10-heptadecanoic | 10.5 |
| 33.5 | C18 | Stearic | 3.5 |
| 34.5 | C18:1 | Oleic | 5.2 |
| 35.3 | C18:2 | Linalelaidic | 1.2 |
| 36.1 | C18:3 | &: Linolenic | 2.1 |
| 40.4 | C20 | Arachidic | 3.4 |
| 41.3 | C20:3 | Cis-8,11,14-eicosaorienoic | 1.9 |
| 42.1 | C20:4 | Arachidonic | 1.7 |
| 43.4 | C21 | Henicosanoic | 6.3 |
| 35.4 | C22 | Behenic | 8.6 |
| 46.4 | C22:1 | Erucic | 6.1 |
| 50.3 | C24 | Lignoceric | 10.8 |
| 51.5 | C24:1 | Nervonic | 2.6 |

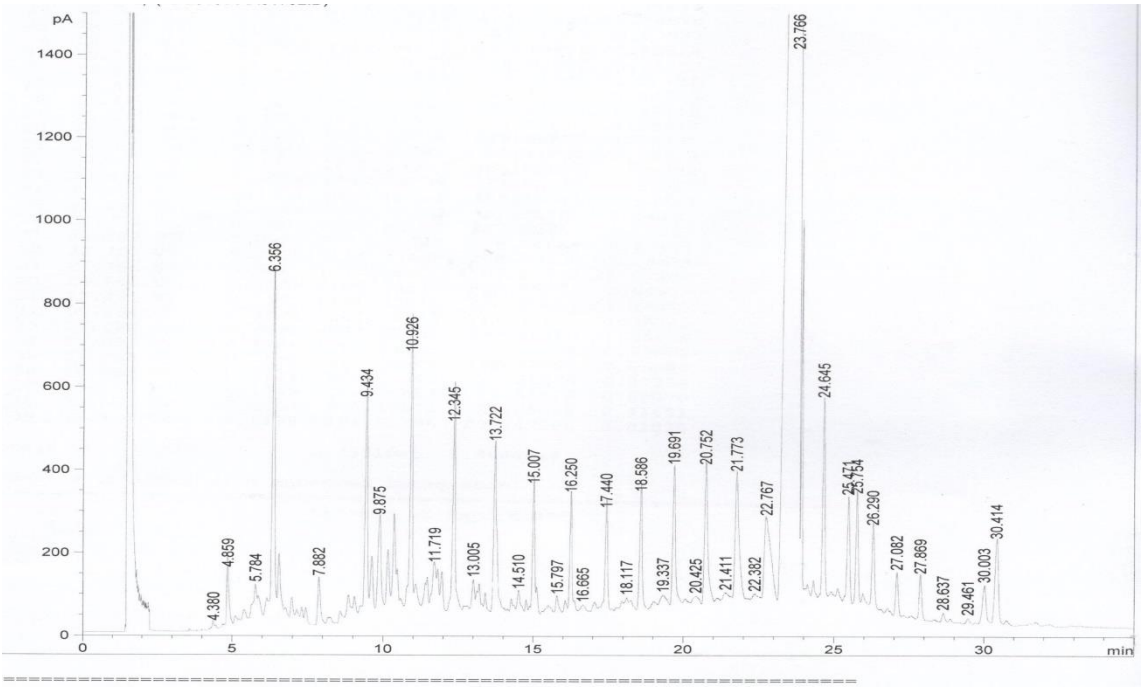


**Fig. 1.** GC analysis of saponification compounds in fixed oil

**Table 6.** Relative percentage of unsaponification matter in fixed oil extracted from *Casuarina equisetifolia* leaves

| Rt | Cn | unsaponification matter | % |
|---|---|---|---|
| 4.8 | C6 | 2,2-dimethylbutane | 0.4 |
| 5.7 | C8 | 2,3-didimethylbutane | 0.8 |
| 6.3 | C9 | 2-methylpentane | 4.5 |
| 7.8 | C10 | 3-methylpentane | 0.4 |
| 9.4 | C11 | n-hexane | 3.5 |
| 10.3 | C12 | 2,2-dimethylpentane | 2.2 |
| 10.9 | C13 | Methylcyclopentane | 2.8 |
| 11.7 | C15 | 2,2,3-trimethylbutane | 2.3 |
| 12.3 | C16 | Benzene | 2.2 |
| 13.7 | C17 | 3,3-dimethylpentane | 2.2 |
| 15.0 | C18 | Cyclo-hexane | 1.6 |
| 16.2 | C19 | 2-methylhexane | 1.4 |
| 17.4 | C20 | 2,3-dimethylpentane | 1.3 |
| 18.5 | C21 | 1,1-dimethylcyclopentane | 1.4 |
| 19.6 | C22 | 3-methylhexane | 2.2 |
| 20.7 | C23 | cis-1,3-dimethylcyclopentane | 1.9 |
| 21.7 | C24 | trans-1,3-dimethylcyclopentane | 2.5 |
| 22.7 | C25 | 3-ethylpentane | 2.4 |
| 23.7 | C26 | Trans-1,2-dimethylcyclopentane | 51.3 |
| 24.6 | C27 | n-heptane | 2.9 |
| 25.7 | C28 | 2,2-dimethylhexane | 1.5 |
| 26.2 | C29 | Ethylcyclopentane | 1.5 |
| 27.0 | C30 | 2,2,3-trimethylpentane | 1.8 |
| 28.6 | A | Cholesterol like compound | 1.4 |
| 29.4 | B | Stigmasterol | 1.2 |
| 30.4 | C | B-sitosterol | 1.0 |

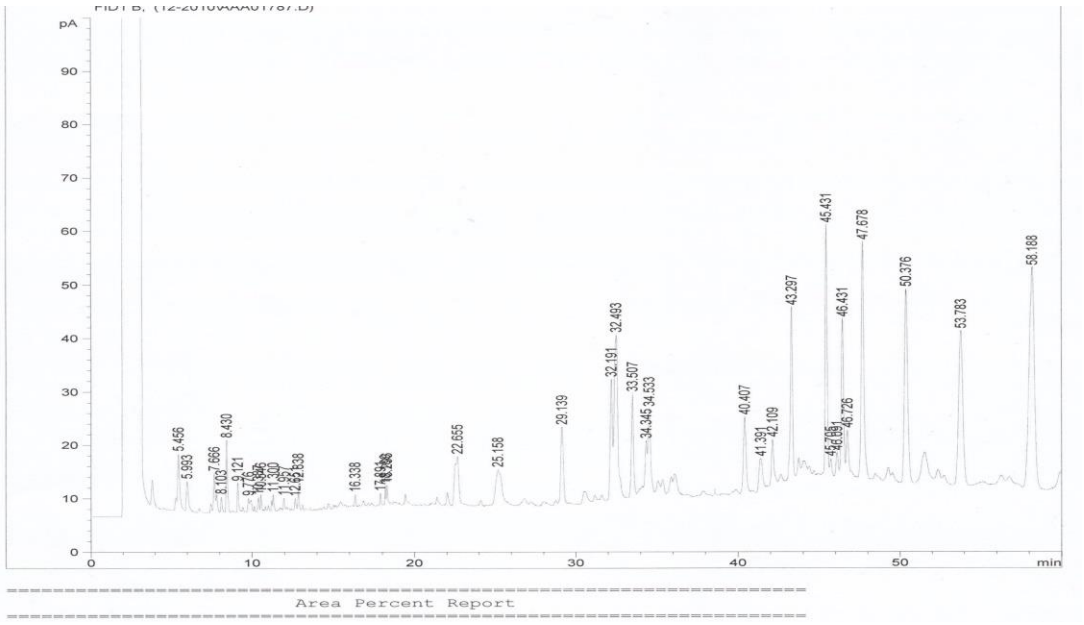


**Fig. 2.** GC analysis of unsaponification matter in fixed oil

On the other hand, the major un saponification matter component in fixed oil was trans 1,2-dimethylcyclopentane being 51.3% . The other concentrations identified were 2-methyl pentane (4.5%), methylcyclopentane (2.8%), 2.2.3trimethyl butane (2.3%) and 2.2-dimethyl pentane(2.2%), shown in Table 6.

Fatty acids are known to possess antibacterial, ant malarial and antifungal activity (**Carballeira,2008**). The development of resistance of microbes, including fungi and yeasts, towards antimicrobial agents already in use, necessitates the search for alternative antimicrobials, including fatty acids and their derivatives (e.g. ethylated and hydroxyl/fatty acids) (**Liu *et al.*, 2008**). Fatty acids refer to a class of natural compounds which are of special interests in their fungicidal values against plant pathogenic fungi **(Carolina *et al.*, 2011).**Numerous fatty acids have been demonstrated capable of effectively controlling pathogenic fungi such as *R. solani* **(Walters *et al.*, 2003),** *Phytophthra infestans* **(Avis and Belager, 2011),** and *Colletorichum gloesporiodes* **(Yenjit *et al.*, 2010)** that commonly occur worldwide.

The fungicidal fatty acids have been found to disrupt function of the fungal cytoplasmic membrane by inducing the release of intra cellular electrolytes and proteins due to increased membrane fluidity **(Carballeira, 2008).**

The membrane disorder induced by the elevated fluidity could thus modify membrane dynamics by affecting the activity of membrane-bound enzymes. This interaction between fungicidal fatty acids and cellular enzymes in sensitive fungi has been proved to be indirect and nonspecific **(Avis and Belaner,2001). Liu *et al.*, 2014** showed that the 2/5/3(w/w/w) mixed Caprylic-Pelargonic-Capric acids formulation has strong fungicidal activities against *R. solani*, *Colletorichum* sp., *Phytophthra infestans* and *Fusarium oxysporum*. This mixture can be exploited in controlling phytopathological fungi and in fungicide resistance managements.

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