International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-2, Issue-10, October 2016
ISSN: 2395-3470
www.ijseas.com

# GC-MS Analysis and Antimicrobial Activity of Sudanese *Pithecellobium dulce* (Roxb.) Benth Fixed Oil

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## *Abstract:-*

The present study was aimed to quantify and identify the chemical constituents of *Pithecellobium dulce* **(Roxb.) Benth** seed oil and to evaluate its antimicrobial activity. Twenty one components were detected by GC-MS analysis. Major constituents are: 9,12-octadecadienoic acid(23.59%), 9-octadecenoic acid (22.65%),methyl 20-methylheneicosanoate(13.99%), hexadecanoic acid(12.88%),tetracosanoic acid(7.45%) and methyl stearate(5.78%) . Butylated hydroxytoluene, a potent antioxidant, was detected as a minor constituent(0.17%). The antibacterial activity of the oil was evaluated via cup plate agar diffusion assay against six standard human pathogens(Gram positive: *Staphylococcus aureus* and *Bacillus subtilis*; Gram negative : *Escherichia coli* and *Pseud*om*onasa aeruginos*a and the fungi *Candida albicans* and *Aspergillus niger*) . The oil showed different antimicrobial responses against test organisms. It gave partial activity against *Bacillus subtilis* and the fungi *Candida albicans* and *Aspergillus niger*. These results indicate that the oil is a candidate for further optimization

***Keywords***: *Pithecellobium dulce* , Fixed oil, GC-MS, Antimicrobial activity

## Introduction

*Pithecellobium* species are widely distributed through tropics,specially in Asia and America.They belong to the sub-family Mimosoideae in Legume family. *Pithecellobium dulce* **(Roxb.) Benth**.(Manila Tamarind) is a multipurpose tropical fruit tree used primarily for its fruits, which are eaten fresh or processed and seeds are processed for non-food uses (Lewis *et.al*.2005). *Pithecellobium dulce* is a small- to medium- sized semi-evergreen tree that grows up to 20m height. Crown is spreading but irregular. Trunk is short, about 1 m high, with crooked branches and somewhat shiny branch lets(Grandtner,2005). Manila tamarind originated from a large central American area, stretching from southern California to Colombia and Venezuela. It was successfully planted in small areas in the south Sahelian and north Sudanese zones. It is now widespread (planted and naturalized) in tropical regions (Grandtner,2005).

Manila Tamarind is reported to be a folk medicine for an array of human ailments including: leprosy, peptic ulcer, earache and toothache.It is also used by local healers as emollient .Stems are used to cure dysentery, while leaves are used for intestinal disorders. Seeds are claimed to treat ulcers(Chopra *et.al*,1992; Rajasab and Mahamad,2004; Sivakumar *et.al*.,2005).Seeds have been reported to contain steroids, saponins, lipids, phospholipids, glycosides, glycolipids and polysaccharides. Bark yielded 37% tannins of the catechol type. Quercitin, kaempferol, dulcitol and afezilin were reported from leaves(Lewis *et.al*.2005).Fatty acid analysis of seed extract yielded 9 saturated and 17 unsaturated fatty acids. *Pithecellobium dulce* is a versatile medicinal plant that attracted a worldwide prominence in recent years. All plant parts elaborates a vast array of biologically active phytochemicals and have been demonstrated to exhibit antidiabetic, free radical scavenging, spermicidal,anticonvulsant(Sharma and Mehta,2013),anti-inflammatory(Krishna *et.al*.,1970), arbortacient (Banerjee,2005), antimicrobial(Shanmugakumaran *et.al*.,2005), ,antivenom(Pithayanukul *et.al*.,2005) and protease inhibitory(Delgado *et.al*.,2004) properties .Some interesting secondary metabolites were isolated from this species and some were evaluated for bioactivity(Kulkarni et.al.,1992; Nigam *et.al*.,1997; Yoshikawa *et.al*.,1997; Saxena et.al.,1998; Niranjan et.al.,1998; Saxena VK, Singhal,1999) .

The plant is frequently used for bowel movements. The leaves, when applied as plasters is used for pain and venereal sores. Salted decoction of leaves is a treatment for indigestion, it is also used as abortifacient. The bark is used in dysentery, dermatitis and eye inflammation. In Mexico, decoction of leaves is used for earaches, leprosy and toothaches. In

Indian folk medicine, bark is used as astringent, in dysentery and as febrifuge. Also it is used for dermatitis and eye inflammations**.** Fruits are astringent and barks are claimed to treat ailments ranging from bronchitis, diarrhea, hemorrhages, sores, liver problems to spleen issues (Sugumaran *et.al*.,2008). Due to extensive use of antimicrobial agents in recent years, the antimicrobial resistance became a global issue. Some current antimicrobial agents are now inefficient in controlling diseases (Nascimento *et.al*.,2000; Hay *et.al*.,2005)[2.3] . To combat this problem measures for exploring novel, save and potent antimicrobial agents should urgently be taken. Plants which were used for centuries in traditional medicine proved to be a rich source for bioactive phytochemicals. Due to their diverse pharmacological properties,plant secondary metabolites (flavonoids,alkaloids…etc) have recently attracted considerable attention. Currently a lot of research is focusing on the bioconstituents of medicinal plants used in tradional medicine. Numerous reports appeared in literature describing the antimicrobial activity of secondary metabolites against a panel of human pathogens(Nascimento et.al.,2000; Hay et.al.,2005; Sibanda et.al.,2007; Indu *et.al*.,2006; Reddy *et.al*.,2007; Sudharameshwari and Radhika, Doughari and Manzara,2008) .Hence ,the present study was designed to identify and quantify the lipid constituents of *Pithecellobium dulce* seed oil and to evaluate the oil for its antimicrobial activity.

## Material and Methods

### Materials

### Plant material

Seeds of *Pithecellobium dulce* were collected from Khartoum state and authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum-Sudan.

### Instruments

A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μm, thickness)was used**.**

### Test organisms

*Pithecellobium dulce* oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in table(1).

**Table 1: Test organisms**

| Ser. No | Micro organism | Type |
|---|---|---|
| 1 | *Bacillus subtilis* | G+ve |
| 2 | *Staphylococcus aureus* | G+ve |
| 3 | *Pseudomonas aeroginosa* | G-ve |
| 4 | *Escherichia coli* | G-ve |
| 5 | *Aspergillusniger* | fungi |
| 6 | *Candida albicans* | fungi |

## Methods

### Extraction of oil from *Pithecellobium dulce* seeds

Powdered shade-dried seeds of *Pithecellobium dulce* (300g) were exhaustively extracted with n-hexane (Soxhlet).The solvent was removed under reduced pressure and the oil was kept in the fridge at 4$^{o}$C for further manipulation.

### Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in (100ml) methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml )of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and (7ml) of alcoholic sodium hydroxide were added followed by (7ml) of alcoholic sulphuric acid. The tube was stoppered and shaked vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added, then (2ml) of normal hexane were added and the tube was vigorously shaked for five minutes .The

Hexane layer was then separated.(5µl) of the hexane extract were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was directly injected in the GC-MS vial.

**GC-MS analysis**

*Pithecellobium dulce* seed oil was analysed by gas chromatography – mass spectrometry.A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 µm, thickness)was used. Helium (purity; 99.99 %) was used as carrier gas. Oven temperature program is given in Table 2, while other chromatographic conditions are depicted in Table 3.

Table 2: Oven temperature program

| Rate | Temperature($^{o}$C) | Hold Time (min.$^{-1}$) |
|---|---|---|
| - | 150.0 | 1.00 |
| 4.00 | 300.0 | 0.00 |

Table 3 : Chromatographic conditions

| | |
|---|---|
| Column oven temp. | 150.0$^{o}$ C |
| Injection temp. | 300.0$^{o}$ C |
| Injection mode | Split |
| Flow control mode | Linear velocity |
| Pressure | 139.3KPa |
| Total flow | 50.0ml/min |
| Column flow | 1.54ml/sec. |
| Linear velocity | 47.2cmlsec. |
| Purge flow | 3.0ml/sec. |
| Split ratio | -1.0 |

## Antimicrobial assay

**Preparation of bacterial suspensions**

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in (100 ml) of normal saline to produce a suspension containing about $10^8$-$10^9$colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism per ml of the stock suspension was determined by means of the surface viable counting technique.
Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

**Preparation of fungal suspensions**

Fungal cultures were maintained on dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

**Testing for antibacterial activity**

The cup-plate agar diffusion method was adopted, with some minor modifications, to assess the antibacterial activity of oil. (2ml) of the standardized bacterial stock suspension were mixed with (200ml) of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4). Each one of the halves was designed for a sample. Separate Petri dishes were designed for standard antibacterial chemotherapeutic, (ampicillin and gentamycin).
The agar discs were removed, alternate cups were filled with (0.1 ml) sample using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.
The diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

## Results and discussion

**GC-MS analysis of *Pithecellobium dulce* fixed oil**

*Pithecellobium dulce* seeds oil was analyzed by GC-MS and the characterization of the constituents was initially accomplished by comparison with the MS library (NIST) and also confirmed by interpretation of the recorded fragmentation pattern.

The GC-MS analysis revealed the presence of 21 components (Table 4).The typical total ion chromatograms(TIC) of hexane extract are shown in Fig.1.

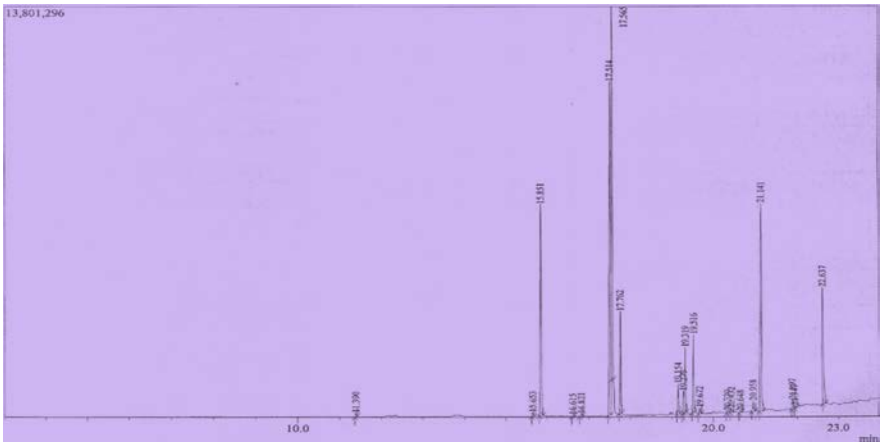


Fig.1: Total ion chromatograms

Table 4 :The typical total ion chromatograms(TIC)

| Peak# | R.Time | Area | Area% | Name |
|---|---|---|---|---|
| 1 | 11.390 | 173449 | 0.17 | Butylated Hydroxytoluene 9- |

| | | | | |
|---|---|---|---|---|
| 2 | 15.653 | 184499 | 0.18 | Hexadecenoic acid, methyl ester, *(z)* - |
| 3 | 15.851 | 12947477 | 12.88 | Hexadecanoic acid, methyl ester |
| 4 | 16.615 | 76299 | 0.08 | cis-lO-Heptadecenoic acid, methyl ester |
| 5 | 16.821 | 136513 | 0.14 | Heptadecanoic acid, methyl ester |
| 6 | 17.514 | 23724870 | 23.59 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ( |
| 7 | 17.565 | 22780049 | 22.65 | 9-Octadecenoic acid, methyl ester, (E)- |
| 8 | 17.762 | 5816472 | 5.78 | Methyl stearate |
| 9 | 19.154 | 1676226 | 1.67 | Tridecanedial |
| 10 | 19.279 | 1355682 | 1.35 | Oxiraneoctanoic acid, 3-octyl-,methyl ester |
| 11 | 19.319 | 3585956 | 3.57 | 11-Eicosenoic acid, methyl ester |
| 12 | 19.516 | 4354065 | 4.33 | Methyl 18-methylnonadecanoate |
| 13 | 19.672 | 390840 | 0.39 | Methyl 15-hydroxy-9,12-octadecadienoate |
| 14 | 20.339 | 91979 | 0.09 | Heneicosanoic acid, methyl ester |
| 15 | 20.432 | 248071 | 0.25 | Phenol, 2,2•-methylenebis[6-(1,1-dimethyll |
| 16 | 20.648 | 96254 | 0.10 | Octadecanoic acid, 2,3-dihydroxypropyl e! |
| 17 | 20.958 | 636972 | 0.63 | cis-lO-Nonadecenoic acid, methyl ester |
| 18 | 21.141 | 14071742 | 13.99 | Methyl 20-methyl-heneicosanoate |
| 19 | 21.897 | 594787 | 0.59 | Tricosanoic acid, methyl ester |
| 20 | 21.947 | 124058 | 0.12 | Oxiraneoctanoic acid, 3-octyl-, methyl est( |
| 21 | 22.637 | 7490660 | 7.45 | Tetracosanoic acid, methyl ester |
| | | 100556920 | 100.00 | |

Fatty acids constituted the major bulk of the oil and two antioxidants :butylated hydroxytoluene and 2,2`-Methylene-bis-[6-(1,1-dimethylethyl)-4-methyl]phenol were detected as minor constituents;0.17% and 0.25% respectively.Some important constituents are discussed below:

**9,12-Octadecadienoic acid methyl ester(23.59%)**

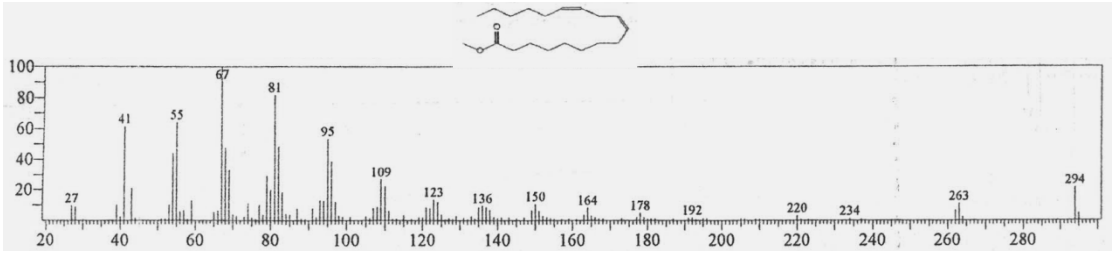


Fig. 3: Mass spectrum of 9,12-octadecadienoic acid methyl ester

The EI mass spectrum of 9,12-octadecadienoic acid methyl ester is shown in Fig. 3.The peak at m/z 294, which appeared at R.T. 17.514 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z263 corresponds to loss of a methoxyl function.

**9-Octadecenoic acid methyl ester(22.65%)**

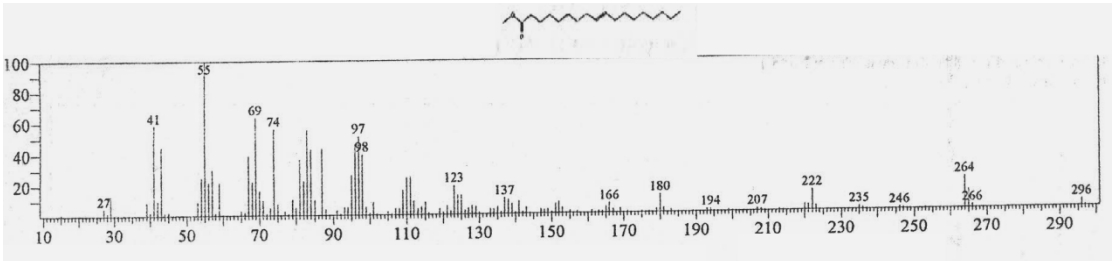


Fig. 4: Mass spectrum of 9-octadecenoic acid methyl ester

The EI mass spectrum of 9-octadecenoic acid methyl ester is shown in Fig.4.The peak at m/z 296, which appeared at R.T. 17.565 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z265 corresponds to loss of a methoxyl function.

**Hexadecanoic acid methyl ester(12.88%)**

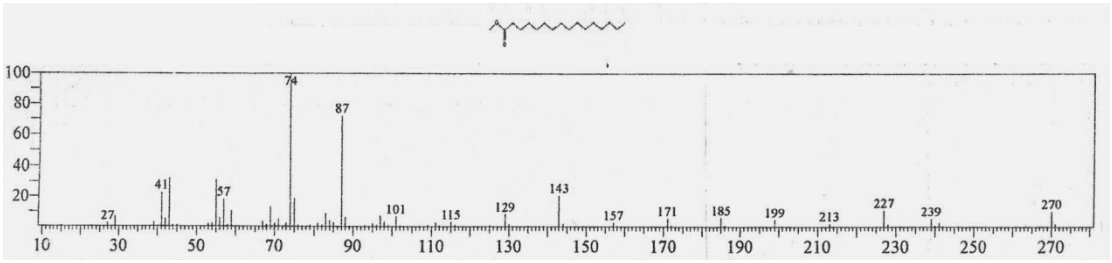


Fig. 2: Mass spectrum of hexadecanoic acid methyl ester

The EI mass spectrum of hexadecanoic acid methyl ester is shown in Fig. 2.The peak at m/z 270, which appeared at R.T. 15.851 in total ion chromatogram, corresponds to $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z239 corresponds to loss of a methoxyl function.

**Methyl stearate(5.78%)**

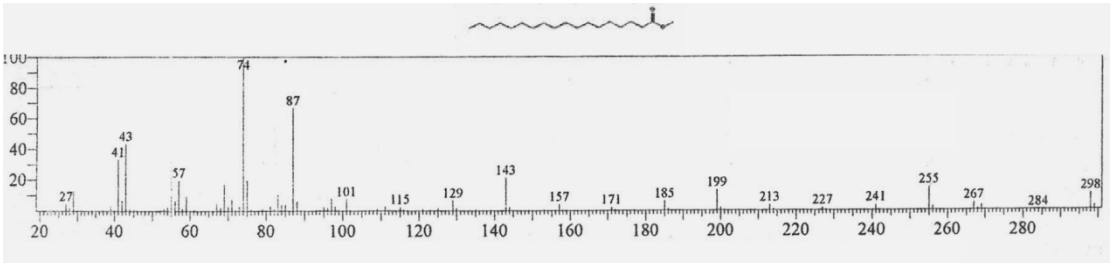


Fig. 5: Mass spectrum of methyl stearate

The EI mass spectrum of methyl stearate is shown in Fig. 5.The peak at m/z 298, which appeared at R.T. 17.762 in total ion chromatogram, corresponds to $M^+[C_{19}H_{38}O_2]^+$ .The peak at m/z267 corresponds to loss of a methoxyl function

**Cis-11-Eicosenoic acid methyl ester(3.57%)**

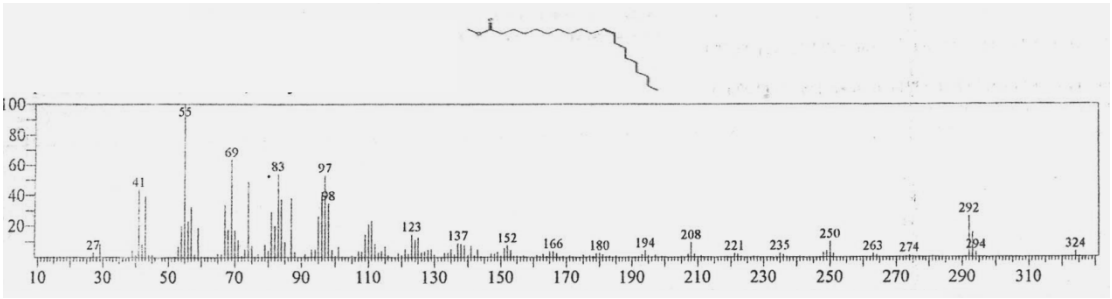


**Fig**. 6: Mass spectrum of Cis-11-Eicosenoic acid methyl ester

The EI mass spectrum of Cis-11-eicosenoic acid methyl ester is shown in Fig. 6.The peak at m/z324, which appeared at R.T. 19.319 in total ion chromatogram, corresponds to $M^+[C_{21}H_{40}O_2]^+$ .The peak at m/z293 corresponds to loss of a methoxyl function.

**Butylated hydroxytoluene(0.17%)**

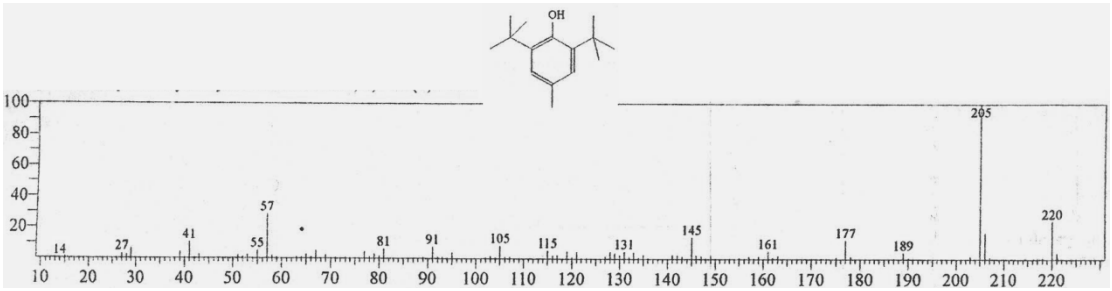


Fig. 7: Mass spectrum of butylated hydroxytoluene

The EI mass spectrum of butylated hydroxytoluene is shown in Fig. 7.The peak at m/z 220, which appeared at R.T. 11.390 in total ion chromatogram, corresponds to $M^+[C_{15}H_{24}O]^+$ . The peak at 205 is due loss of a methyl function.

**2,2`-Methylene-bis-[6-(1,1-dimethylethyl)-4-methyl]phenol(0.25%)**

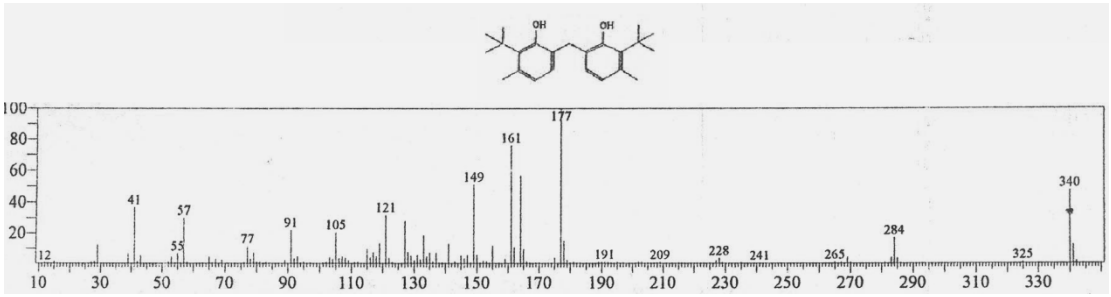


Fig. 8: Mass spectrum of 2,2`-Methylene-bis-[6-(1,1-dimethylethyl)-4-methyl]phenol

The EI mass spectrum of 2,2`-methylene-bis-[6-(1,1-dimethylethyl)-4-methyl]phenol is shown in Fig. 8.The peak at m/z340, which appeared at R.T.20.432 in total ion chromatogram, corresponds to $M^+[C_{15}H_{24}O]^+$ .The peak at m/z325 corresponds to loss of $CH_3$ .

**Antimicrobial activity**

The oil was evaluated for antimicrobial potency against standard organisms. The average of the diameters of the growth inhibition zones are shown in Table (5) .The results were interpreted in terms of the commonly used terms (<9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .Tables (6) and (7) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 5 : Antibacterial activity of *Pithecellobium dulce* oil :M.D.I.Z (mm)

| Drug | Conc.(mg/ml) | Ec | Ps | Sa | Bs | Ca | An |
|---|---|---|---|---|---|---|---|
| oil | 100 | - | - | 8 | 11 | 11 | 10 |

Table 6 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

| Drug | Conc. mg/ml | Bs. | Sa. | Ec. | Ps. |
|---|---|---|---|---|---|
| Ampicillin | 40 | 15 | 30 | - | - |
| | 20 | 14 | 25 | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | 10 | 11 | 15 | - | - |
| Gentamycin | 40 | 25 | 19 | 22 | 21 |
| | 20 | 22 | 18 | 18 | 15 |
| | 10 | 17 | 14 | 15 | 12 |

Table 7 : Antifungal activity of standard chemotherapeutic agents against standard fungi

| Drug | Conc. mg/ml | An. | Ca. |
|---|---|---|---|
| Clotrimazole | 30 | 22 | 38 |
| | 15 | 17 | 31 |
| | 7.5 | 16 | 29 |

Sa.: *Staphylococcus aureus*
Ec.: *Escherichia coli*
Pa.: *Pseudomonas aeruginosa*
An.: *Aspergillus niger*
Ca.: *Candida albicans*
*Bs.: Bacillus subtilis*
M.D.I.Z: Mean diameter or growth inhibition zone (mm)..

The oil showed partial activity against *Bacillus subtilis* and the fungi *Candida albicans* and *Aspergillus niger*. These results indicate that the oil is a candidate for further optimization.

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