Antioxidant and Antibacterial property of Ethanolic Extracts of *Trachyspermum ammi* L. Plant Seeds

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I. Introduction:

Natural products including essential oils are produced by the secondary metabolism in plants. Their constituents are used in human consumption as functional food, food additives, medicines, nutritional supplements and for the manufacture of cosmetics.

T. *ammi* L. is an ancient and well-known Ayurvedic spice that belongs to the family Apiaceae comprising 270 genera and species. [1, 2] Ajwain tends to grow in regions that are dry and barren. It is one of the ancient medicinal, aromatic, and spice plant which has been used to cure several stomach disorders along with respiratory distress. Medicinal properties of the plant include antioxidant, antispasmodic, antimicrobial, and antifungal.

T. ammi L. is local spice of Egypt but is now available in other parts of the worlds including South & West Asia, including Iran, India, Pakistan, and other countries. Gujarat and Rajasthan are regions within India that are well known for cultivating ajwain. [3] It is commonly known as Ajwain in Hindi, Bishop's weed in English, Yamini in Sanskrit, Lodhar Bengali in Punjabi, Ajma in Gujrati, Kath in Kashmiri, and Omam in Tamil. [4] Due to drug resistance and residual effect issue associated with antibiotics, these natural plant extracts have been screened for their potential uses as alternative medicines for the treatment of many infections.

It is an annual, aromatic, erect herb bearing white flowers and small brownish fruit with many leafy branches and small feather-like leaves. Seeds of Ajwain is commonly used in food dishes because of its distinct aromatic scent and flavour. Most of the research studies such as antibacterial, antifungal, and antiviral effects have been done on the seeds of this plant but very limited studies have been done on its leaves. The objective of the study was to find the antibacterial and antioxidant effects of leaf extracts of ajwain along with search of phyto-constituents of the plants using GC-MS analysis. Ajwain (*Trachyspermum ammi*) is a plant with medicinally useful chemicals that can be used for various therapeutic purposes.

T. ammi L. It is one of the ancient medicinal, aromatic and a spice plant which has been used to cure several stomach disorders along with respiratory distress. Medicinal properties of the plant include antioxidant, antispasmodic, antimicrobial and antifungal. Some researcher reported the active ingredients of the ajwain plant include six major chemical compounds including 49% thymol, 30.8% terpinene, 15.7% p-cymene, 2.1% b-pinene, 0.8% myrcene and 0.7% limonene. [5] In order to explore more regarding the properties of these herbs, the aim of this present study is to estimate the antimicrobial and antioxidant

properties of these natural feed additives which eventually allow the determination of its suitability as an alternative to antibiotic growth promoters in livestock and poultry production.

Medicinal properties of the plant include antioxidant, antispasmodic, antimicrobial, and antifungal reported the active ingredients of the ajwain plant include six major chemical compounds including 49% thymol, 30.8% γ-terpinene, 15.7% p-cymene, 2.1% b-pinene, 0.8% myrcene, and 0.7% limonene. [5] The main component is thymol (35– 60%), a strong germicide, antispasmodic, and fungicide agent. However, sometimes γ-terpinene and p-cymene exceed the thymol content. [6] Plant extracts containing phytochemicals, which have both antibacterial and antioxidant capabilities, must be used to control this issue because synthetic compounds can be hazardous in nature and may have side effects. Phytoconstituents of various Plants have role in plant-insect interactions. Some compounds extracted from *T. ammi* plant have insecticidal activity. Earlier studies suggest that the ethanolic seed extract of *T. ammi* shows antimalarial activity against different developmental stages of *Aedes aegypti* i.e. larva and pupa and considered an eco-friendly remedy. The mortality rate of larvae was three times faster than the pupa stage, hence we can observe *T. ammi* insecticidal properties as well. Ajwain is an exceptionally trustworthy plant source because it is abundant in components with potential bioactivity. [7] In recent years much attention has been given to non-chemical systems for seed treatment to protect them against many plant pathogens.[8] Since our country has favorable conditions for agriculture and allows the production of medicinal plants at relatively low cost, there is the possibility of using them in the fight against plant pathogens, along with other control methods that can prevent the indiscriminate use of antibiotics and pesticides. [9]

II. MATERIALS AND METHODS

1. Collection, Extraction, and Preparation of Plant Extract

In the present study, the *T. ammi* plant seeds were collected from local shops. The samples were authenticated by Dr. Rekha Maggirwar taxonomist from Department of Botany Shri Shivaji Science College Amravati District, Amravati Maharashtra. The Dry seeds of *T. ammi* were cleaned, dried and ground as a powder, and stored for experimentation.

A cold extraction process was used for the preparation of drug. About 20 g of powdered material was mixed in 100 ml ethanol in conical flasks; Flask was kept for 24 hours for maximum drug extraction from seed powder. After 24 hours both extracts was filtered through muslin cloth and centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was allowed to evaporate under vacuum conditions at 50 ± 2°C and stored at 4°C temperature for further experiments.

2. Qualitative phytochemical screening using Tube test:

Crude extracts were subjected to phytochemical tests for the presence of anthraquinones, saponins, tannins, steroids, terpenes, reducing sugars, flavonoids and alkaloids using standard procedures. [10]

3. Characterization of Plant Drug Extract:

a) DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay

The anti-oxidant activity of each of the plant extracts was determined using the colorimetric DPPH assay (Awley et al 2020) to determine the radical scavenging activity of the plant extracts. 0.1 mM solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol and Ascorbic acid (1 mg/ml) standard was prepared. [11]

The hydrogen donating capacity of test samples is quantified in terms of their ability to scavenge the relatively stable, organic free radical DPPH and by consequent reduction. The absorption of the deep violet DPPH solution is measured at 517 nm, after which absorption decreases due to decolorization to a yellow-white color, in the event of reduction. This decrease in absorption is stoichiometric according to the degree of reduction.

The free-radical scavenging activity was estimated by DPPH assay. The reaction mixture contained 100 µl of test extracts and 2.9 ml of methanolic solution of 0.1 mM DPPH radical. The mixture was then shaken vigorously and incubated at 37° C for 30 min. The absorbance was measured at 517 nm using ascorbic acid as positive control lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was calculated using the equation given below. The steps were used to study (%) DPPH scavenging activity of the extract along with standards different concentrations 0.2 to 1.0 mg/mL.

(%) DPPH scavenging effect = (Ac - As)/ (Ac) x100

Where,

Absorbance of control = Ac Absorbance of sample = As

B) Antibacterial activity of the Extract:

Two bacterial strains (1 Gram-positive and 1 Gram-negative) were selected for the study. Gram-positive bacterium *Staphylococcus aureus* (ATCC25923) , while Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC27853) was used for the study. The plates for culture were prepared using Mueller Hinton agar (M173) for bacteria cultures.

The viability tests for each isolate were carried out by resuscitating the organism in agar medium. The stock on nutrient agar medium (Hi Media, Mumbai, India) was incubated at 37 °C for 24 h (bacteria) following storage at 4 °C until required for sensitivity testing. [12]

Antibacterial activity testing using agar well diffusion technique

The antibacterial activity of the *T. ammi* seed extracts were determined by the agar well-diffusion method. [13] A pure isolate of each bacterium was first subcultured in nutrient broth at 37 ºC for 24 h. Standardized inoculum (100 µL, 106 CFU/mL; 0.5 Mac-Farland) of each test bacterium was spread with the help of a sterile spreader onto a sterile agar plate to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer (6.0 mm diameter) was used to bore the wells in the agar. Subsequently, 50 µL of each extract was introduced in wells of an agar plate. The plates were allowed to stand for 1 h for diffusion and then incubated at 37 ºC for 24 h. The inhibition zone diameter (IZD) was observed and measured in mm.

c) GCMS analysis of bioactive fraction:

The Ethanolic extract was diluted in HPLC grade ethanol, centrifuged at 4,000 r.p.m. and the supernatant was used for the GC-MS analysis to get the idea of the presence of several biochemical present in the extract. The sample was sent to BioRaj Lab Nagpur for GCMS analysis.

A gas chromatograph with a mass spectrometer (GC-MS/MS) instrument (Make-Bruker Scion, Model- TQ MS System) was used for the analysis of the plant extract sample by attempting the following conditions: Column DB-5MS Agilent (30m x 0.25mm1D), composed of 100% dimethyl polysiloxane). The sample was filtered with 0.45 microns and it was injected into the GC-MS instrument. For GC-MS detection, an electron ionization system with an ionization energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at constant flow rate 1.0 ml/min with a split ratio of 10:1. The oven temperature was operated according to the following oven temperature: 40°C held for 1 min, raising at the rate of 20°**C** min^{-1} up to 150°**C** then, raising at the rate of 3°**C** min^{-1}, hold for 0 min and raising at the rate of 20°**C** min^{-1} up to 300°C with 10 min held, injector temperature and volume 250°C and 2µL, respectively. The total GC running time was about 50 min. The MS operating conditions were ionization voltage 70 eV, source temperature of 250°C,inlet line temperature 280°C, mass scan (m/z)-30-500, solvent delay: 3.0 min, total MS running time 47 min. The mass spectra of compounds were identified by comparing the mass spectra obtained from their related chromatographic peaks with NIST mass spectral libraries.

III. RESULTS AND DISCUSSION

1. Collection, Extraction and Preparation of Plant Extract:

A seed sample of *T. ammi* was collected successfully from the local shop. Ethanolic extract prepared using the above-explained method was gelly-like, brownish-dark in color. Extracts and different dilutions were prepared successfully and stored for future use.

2. Characterization of Plant Drug Extract:

a) **Qualitative phytochemical screening:**

The different qualitative chemical tests were performed to establish a profile of the prepared plant extract for its chemical composition. Qualitative phytochemical analysis was done using the standard protocol. All the tests performed on extracts to detect various phytoconstituents present with the results are shown in Table 1.

The phytochemical analysis is of supreme importance in identifying new sources of therapeutically and industrially valuable compounds having medicinal plants that have been chemically investigated. In the present investigation, primary and secondary metabolites were qualitatively analyzed using *T. ammi* seed extract. The results are presented in Table 1.

Table 1: Preliminary phytochemical screening of *T. ammi* seed extract

S. no	Phytoconstituents	Name of the Test	Ethanolic extract of *T. ammi*
1.	Alkaloids	Mayer's test	–
		Dragondrofs test	-
2.	Carbohydrates:	Benedict's test	+
3.	Flavonoids	Ferric chloride test	+
4.	Glycosides	Borntrager's test	–
6.	Steroids	Liebermann–Burchard Test	+
7.	Tannins	Lead acetate test	+
8.	Starch	Iodine test	–
10.	Organic acids	Malic acid test	–
11.	Phenolic compounds	Lead acetate test	+
12.	Amino acids	Millons test	–
13.	Protein	Ninhydrin test	–

b) Antioxidant activity using DPPH free radical scavenging assay

The antioxidant activity of ethanolic extracts of *T. ammi* and ascorbic acid was evaluated in *in vitro* models using different concentrations ranging from 0.05 to 0.25 mg. The antioxidant reacts with the DPPH radical (purple color) and converts it into a colorless DPPH. The amount of DPPH reduced could be quantified by measuring the decrease in absorbance at 517 nm. The ethanolic extract significantly reduces DPPH radical in concentration concentration-dependent manner. With the increase in concentration of the extract the free radical scavenging activity increases. At the concentration of 0.25 mg Ethanolic extract and Ascorbic acid shows maximum % inhibition i.e. 54.51±2.98 and 96.55±2.56 respectively (Table 1). The ethanolic extract and Ascorbic acid show IC_{50} 0.176 and 0.07 mg respectively. The detailed results are shown in Table 2.

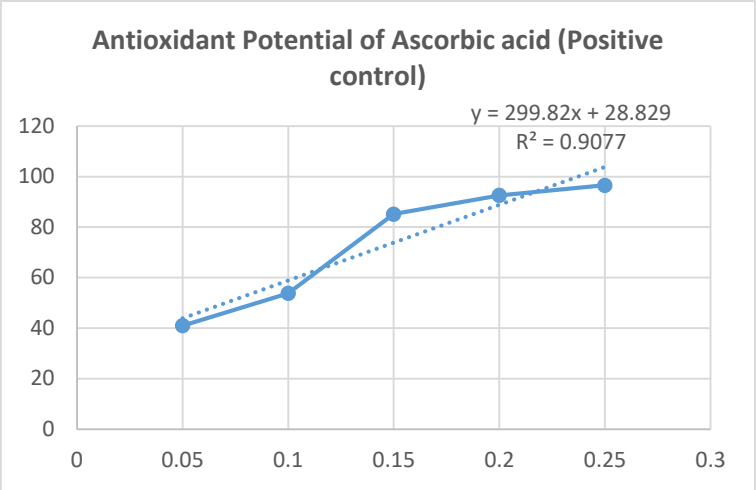
DPPH is commonly used for the evaluation of antioxidant activity because of its stability and simplicity during the assay. This assay gives reliable information related to the antioxidant ability of the compounds.

The medicinal ability of *T. ammi* has studied for arthritis, asthma, diabetes mellitus, colitis, and cancer in relation of their antioxidant and anti-inflammatory properties. The antioxidant and free radical scavenging property extracts of *T. ammi* are directly related with total phenolic and flavonoid presence. Extraction procedures suggest that the antioxidant activity depends on the polarity of the solvent. Earlier reports suggest that the essential oil of *B. dalzielii* was characterized by low antioxidant activity and that this was due to the extraction method adopted (e.g. low polarity of the solvent) that determined the absence of phenolics, especially flavonoids.

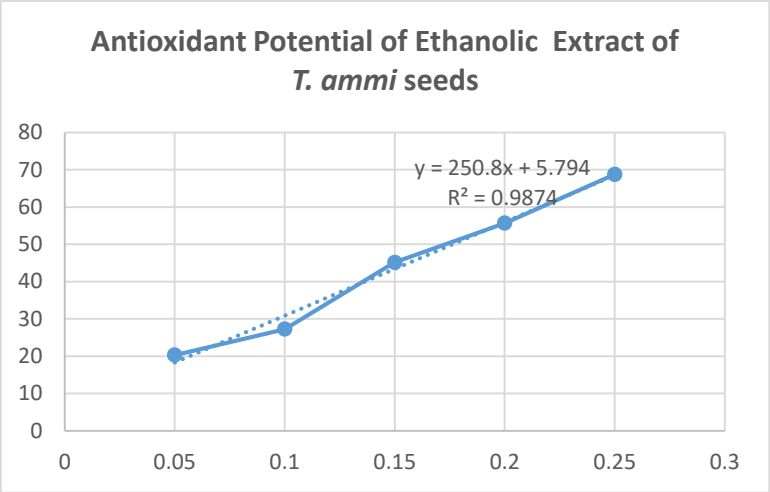
Table 2: DPPH free radical scavenging percent inhibition of *T. ammi* extracts

Conc. (in mg/mL)	% Inhibition by Ethanolic extract of *T. ammi*	% Inhibition by Ascorbic acid (Positive Control)
0.05	10.84±2.65	40.98±3.55
0.1	26.54±2.54	53.78±2.57
0.15	37.43±2.98	85.15±3.69
0.2	40.53±1.58	92.55±3.89
0.25	54.51±2.98	96.55±2.56
IC_{50}	0.176	0.07
y-equation	250.8x + 5.794	299.82x + 28.829
R^2	0.9874	0.9077

* All the data statistically analyzed with mean±SEM (n=3)



Graph 1: Antioxidant Potential of Ascorbic acid (Positive control)



Graph 2: Antioxidant Potential of Ethanolic Extract of T. ammi seeds

c) Antibacterial activity of Seed extract of *T. ammi* :

A total of two bacterial species were used to assess the antibacterial activity of seed extracts. The antimicrobial activities of the extracts were determined by the agar well-diffusion method against three Gram positive bacteria and two Gram negative bacteria (Table 3). The extracts were not active against both Gram positive whereas showing positive activity againts Gram negative bacteria. They were most effective against *Escherichia coli* (NCIM 5346) followed by *Pseudomonas aeruginosa* with inhibition zone diameter (IZD) of 35.0 mm and 30.0 mm respectively. In contrast to the negative control (DMSO), the ethanolic leaf extract exhibited active inhibition against all bacteria with the highest zones against *Bacillus subtilis* (NCIM 2920) and *Staphylococcus epidermidis* of 15.0 mm and 12.0 mm respectively) (Figure 2).

Some researchers investigated the antibacterial activity of *T. ammi* seeds ethanolic and acetonic extracts. They observed that *Pseudomonas* sp., *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* were found to be more susceptible to ethanolic extract. While *Pseudomonas* sp., *E. coli*, and *Bacillus subtilis* were all inhibited by an acetonic extract of *T. ammi*.

Shahidi (2004) reported the antibacterial activity of methanolic extract of *T. ammi* seeds against *P.aeruginosa*, *Bacillus pumilus*, *Staphylococcus aureus*, *S. epidermidis*, *E. coli*, *Klebsiella pneumoniae*, and *Bordetella bronchiseptica*. Furthermore, the acetonic extract displayed antibacterial

efficacy against *Enterococcus faecalis, E. coli, K. pneumoniae, P. aeruginosa, Salmonella typhi, S. typhimurium, Shigella flexneri*, and *S. aureus*.

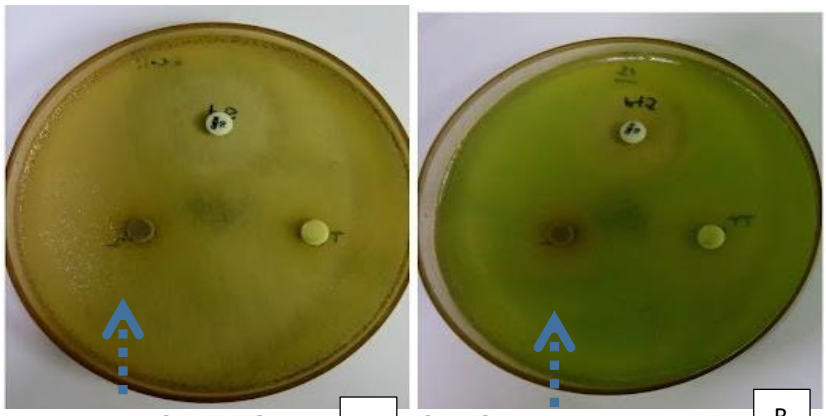


Figure 1: Antibacterial activi Ethanolic extract againts Gran positive and Gram negative bacteria

Table 3: Antimicrobial activity of extracts

Sample	*P. aeruginosa* ATCC27853	*S. aureus* ATCC25923
Ajwain Seed Extract	No zone	10 mm
Gentamicin control	27 mm	34 mm

d) GCMS Analysis of *T. ammi* Ethanolic Extract

The Ethanolic extract of *T. ammi* was sent for GCMS analysis. The large range of compounds in the extract was determined by the peak and retention times. The high number of peaks showed the complexity of compounds in the plant extract. Some compounds were identified in high concentration in the extract at specific retention time.

GCMS analysis was performed on the Ajwain seed extract as the sample was in larger quantity. The GCMS report on the X axis showed the Retention Time taken for each of the analytes to pass through the column and reach the mass spectrometer detector. Amongst the important analytes Terpinene showed retention time at 15.8 mins with 0.02% area. Thymol showed retention time at 19.71 mins with 43.833% area with highest number of counts detected by the mass spectrometer, Hexadecanoic acid showed retention time at 23.23 mins with 42.864 % area. Literature search showed that Thymol has known antibacterial, antifungal and antioxidant and free radical scavenging properties. Similarly Hexadecanoic acid and Terpinene have anti inflammatory properties. The higher Intensity counts on the Y axis demonstrated by Thymol and Hexadecanoic acid could also be due to higher affinity to the detector. However relevant standards with known concentrations were run to ensure accurate counts. The detailed results are shown below.

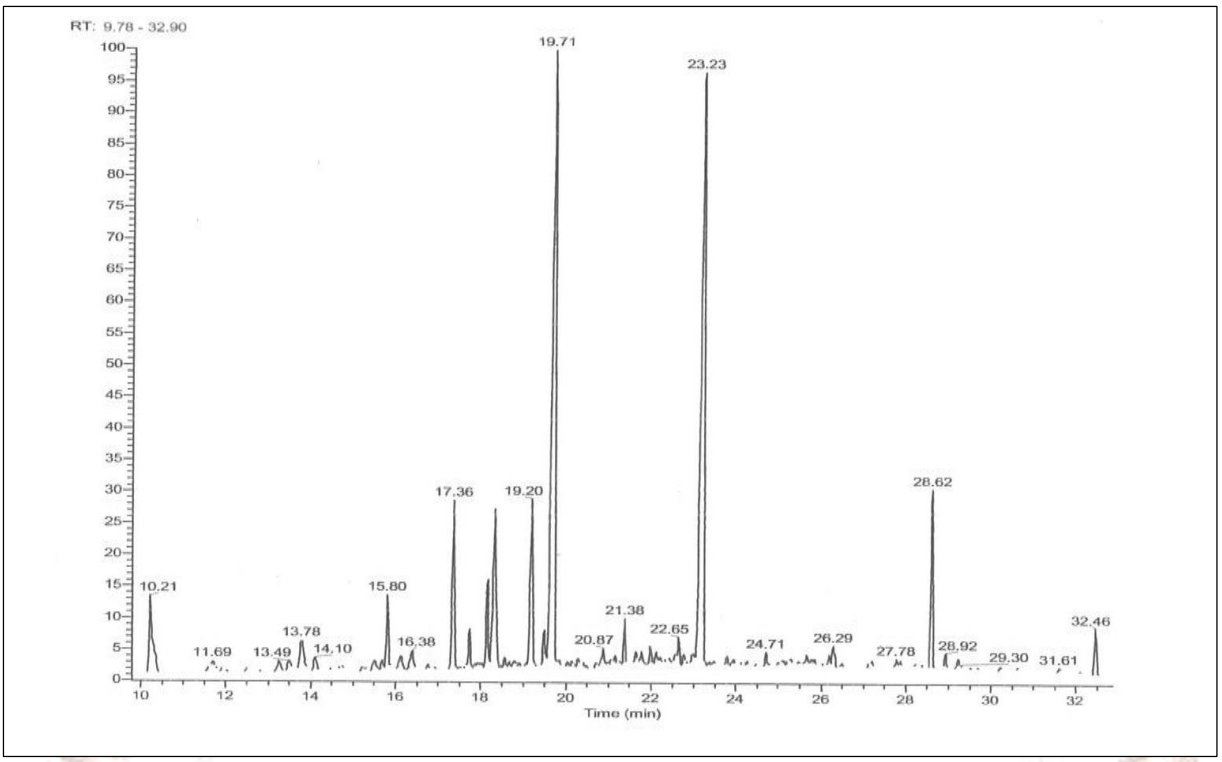


Figure 2: GC-MS Spectra of ethanolic extract of sample

The prominent compounds eluted were Thymol and Hexadecanoic acid. Chemically thymol is 2-isopropyl-5-methylphenol. Similarly in hexadecanoic acid the total number of carbon atoms to the number of carbon-carbon double-bonds) is 16:0. It is a saturated fatty acid.

From GC-MS analysis, the extract of *T. ammi* seeds contains biological activities compounds such as a-cymene act as antioxidant, anti-inflammatory, antitumor, ç-terpinene has antitrypanosomal activity, n-hexadecanoic acid (palmitic acid) is reported to be an antioxidant, nematicide, and a pesticide , 3,5-dimethyl anisole act as antimicrobial, anti-inflammatory, and antioxidantcis-13-octadecenoic acid act as anti-inflammatory, antiandrogenic, anticancer, preservative, and hypocholesterolemic.

Some researchers have evaluated the antimicrobial activity of ajwain oil. Thymol and carvacrol were found to be more effective in killing bacteria. The antibacterial properties of natural products, such as essential oils and their components, are widely explored by both industrial and academic fields. The antibacterial activity of the EOs is dependent on the composition and concentration, type, and dose of the target microorganism. The high antibacterial potential of cumin essential oil compared to Ferula essential oil has already been identified due to the high ratio of phenolic monoterpene compounds to other monoterpenes.

Table 4: List of identified compounds of methanolic extracts of Ajwain Seed.

S. No	Identified Compounds	Biological Role/ Pharmacological Activity
1	Cymene	antioxidant, anti-inflammatory, antitumor
2	Pinene	anti inflammatory
3	Terpinene	anti inflammatory, antitrypanosomal activity
4	Phenol,2,4 bis(1,1-dimethylethyl)	antioxidant, anticancer, antifungal
5	Ethyl thio-penta-1,5,dien-3-ol	not reported
6	3,5-dimethylanisole	antimicrobial, anti-inflammatory, and antioxidant
7	Isoretinine a	acne
8	Thymol	antioxidant, free radical scavenging, anti inflammatory, analgesic, antispasmodic, antibacterial, anti tumor
9	Hexadecanoic acid	anti inflammatory, antioxidant, nematicide, and a pesticide
10	8,11-octadecadienoic acid, methyl ester	antiinflammatory, antiandrogenic, anticancer, preservative, and hypocholesterolemic
11	2-cyclohexyl-2,5-cyclohexadiene-oxime	antioxidant, antibacterial
12	Isothiocyanic acid, methyl ester	antibacterial

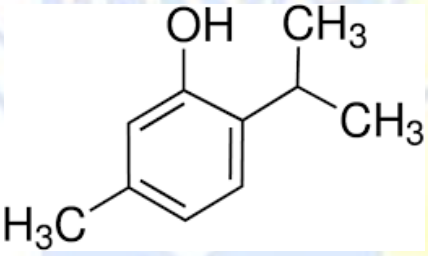


Figure 3 : Chemical structure of thymol

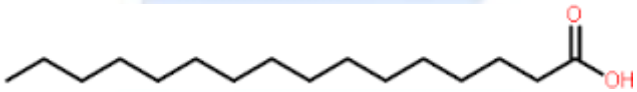


Figure 4 : Chemical Structure of hexadecanoic acid

IV. Conclusion:

T. ammi is well known medicnal plant used in our day to day life. The ethanolic extract shows antioxidant and antimicrobial property which makes it more prominent drug. The chemical analysis suggests that many molecule are present in the extract which are already explored with their drug potential.

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