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# Exploring the Essential Oil of *Illicium verum* from Palestine: An Investigation into Composition, Antimicrobial Properties, and Anticancer Potential

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## ABSTRACT

One of the most important sources of pharmaceuticals is medicinal plants. The evergreen star anise*, Illicium verum*, is an important herb that grows throughout the southwestern region of Asia. *I. verum* oil has long been used to treat otalgia and rheumatism, as an antimicrobial for coughs, toothaches, and sinusitis, and as a preservative for food. *I. verum* EO is also used to cure diarrhea, flatulence, and spasms. The objectives of the current study are to determine the chemical constituents of *I. verum* essential oil (EO) and to evaluate its antibacterial and anticancer effects *in vitro*. The EO was extracted using a hydrodistillation process combined with a cleavenger device. GC-MS analyses of EO revealed the presence of 31 phytochemical compounds in *I. verum* EO, of which E-Anethole 88.38% was the major component, followed by Shisofuran 2.88% and Limonene 2.01%. The remaining minor ingredients represent around 7.7%. Antimicrobial activity against a series of microbial strains was determined using the microdilution technique. The findings revealed that *I. verum* EO inhibited the growth of all bacteria tested to varied degrees. The *I. verum* EO had the highest antimicrobial activity against *Candida albicans,* with a minimum inhibitory concentration (MIC) of 6.25 mg/ml. Moreover, the *I. verum* EO had MIC of 100 mg/ml against Methicillin-Resistant *Staphylococcus aureus, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Escherichia coli*. Furthermore, the MIC against *Proteus vulgaris* was 50 mg/ml. The anti-cancer effects of EO were examined using the CellTilter 96®Aqueous One Solution Cell Proliferation (MTS) Assay and measuring the inhibition index on LX-2, 3T3, Hela, and MCF-7 cells. The cytotoxicity results showed the best $IC_{50}$ was obtained against LX-2 cells (57.265 ± 2.51 µg/ml), followed by HeLa cells (90.499 ±1.87 µg/ml), 3T3 cells (118.819 ± 3.014 µg/ml), and MCF-7 cells in that order. The findings of this study suggest that *I. verum* EO is a rich source of pharmacologically active ingredients that can be confirmed and examined therapeutically for their therapeutic potential and the development and design of new natural medicinal formulations.

**Keywords:** *Illicium verum*, essential oil, GC-MS, antimicrobial, anticancer.

## INTRODUCTION

Ancient people used plant secondary metabolites for various purposes, including disease treatment, food preservation and flavoring, and insect eradication. The World Health Organization estimates that about 80% of people worldwide use plant extracts for medicinal purposes (1). Synthetic drugs frequently have a variety of side effects that can seriously harm human health, even though synthetic chemicals have largely replaced plant compounds due to their quicker results. Efforts are being undertaken to substitute synthetic medications with secondary metabolites found in plants to improve human health and prevent various ailments (1, 2). Furthermore, the demand for natural bioactive compounds has increased due to growing awareness of the potential harm caused by synthetic additives (3). The majority of antitumor drugs currently being used are derived from natural sources, which makes the search for new anticancer medications

imperative, given the prevalence of cancer worldwide (4, 5). EOs produced from plants have been used in traditional medicine for the treatment and prevention of numerous ailments, cosmetics, cuisines, and food supplements, as well as dentistry practice and hygiene items. EO produced from plants have been used in traditional medicine for the treatment and prevention of numerous ailments, cosmetics, cuisines, and food supplements, as well as dentistry practice and hygiene items. *I. verum* EO has long been used to treat otalgia and rheumatism, as an antimicrobial for coughs, toothaches, and sinusitis, and as a food preservative. Star anise oil is used to cure diarrhea, flatulence, spasmodic symptoms, and ease colic (6).

*I. verum*, Star anise, a medium-sized evergreen plant from the Magnoliopsida family, has been utilized as a traditional herbal medicine due to its vigorous antibacterial, antioxidant, and insecticidal properties(7, 8), analgesic, and sedative activities (9). Aromatic plants like anise seeds and star anise have a long history of use in folk and conventional medicine and the pharmaceutical industry(10). The fruits of *I. verum* are commonly used as a spice in the food industry, and in traditional Eastern Asian medicine, they were used to treat stomach pain and sepsis (11, 12). The main chemical components found in *I. verum* include phenylpropanoids, flavonoids, neolignans, monoterpenoids, and sesquiterpenoids. The phenylpropanoid substance *trans*-anethole is the primary component of the EO extracted from *I. verum* fruit (9, 13). The main component of *I. verum* EO is *trans*-anethole (12, 14). Previous studies suggested that it has trans-anethole's insecticidal, antimicrobial, and antioxidant properties (15-18). It is extensively used in food, perfume, and pharmaceutical industries due to its sweet flavor and aromatic scent (13). It is important to note that the chemical constituents of *I. verum* EO from Palestine, as well as its biological and pharmacological properties, have not been documented in the Middle East. The objectives of the current study are to determine the chemical constituents of *I. verum* EO and to evaluate its antimicrobial, and anticancer effects *in vitro*.

## MATERIALS AND METHODS

### *Chemical reagents*

All the experiments in the current study were carried out utilizing commercially available chemicals and reagents unless otherwise stated.

### *Equipment*

Gas Chromatograph (Clarus 500-Perkin Elmer, Singapore), Mass Spectrometer (Clarus 560D-Perkin Elmer, Singapore), microscope (MRC-IX-73-inverted, China), incubator (Nüve, 06-3376, Turkey), vortex (Heidolph company, 090626691, Germany), autoclave (MRC, A13182, USA), grinder (Molineux I, China), and a microplate reader (Unilab, 6000, USA) were utilized in the current investigation.

### *Herbal material*

*I. verum* fruits were brought from the Bedia, Nablus/Palestine herbal store in June 2022. The taxonomical characterization of the plant was established in the Pharmacy Department at An-Najah National University and deposited under the voucher specimen number (Pharm-PCT-2791). Later on, *I. verum* fruits were cleaned, rinsed at least three times with distilled water and then dried in the shade at 25±2 °C and 55 ± 5 RH of humidity for three weeks, which were kept in special papery bags for further experimental work (19).

### Extraction of *I. verum* EO

The EO was extracted utilizing the hydrodistillation procedure pronounced by Jaradat et al. (20). Briefly, 0.1 kg of the dried stars was suspended with 1 L of distilled water, and the EO was extracted using a Clevenger device operating at atmospheric pressure for 180 min at 100 °C with a hydrodistillation rate of 0.54 ml/min. The obtained EO was chemically dried using calcium carbonate and stored at 2 °C in the refrigerator until further use, and the obtained yield was 1.01%.

### GC-MS characterization *I. verum* EO

The separation was achieved by Perkin Elmer Elite-5-MS fused-silica capillary column (30 m x 0.25 mm, film thickness 0.25

µm), while Helium was used as a carrier gas at a standard flow rate of 1.1 mL/min. The temperature of the injector was adjusted at 250 oC with an initial temperature of 50 °C, initial hold of 5 min, and ramp 4.0 °C/min to 280 °C. The total running time was 62.50 min, and the solvent delay was from 0 to 4.0 min. MS scan time was from 4 to 62.5 min, covering a mass range of 50.00 to 300.00 m/z. The chemical ingredients of the EO were characterized by comparing their mass spectra with the reference spectra in the MS Data Centre of the National Institute of Standards and Technology and by matching their Kovats and retention indices with values reported in the literature (20-21). In addition, the EOs Kovats and retention indices with values were compared with 20% of HPLC grade reference essential oils that were purchased from Sigma-Aldrich (Germany) (21, 22).

**Antimicrobial activities of *I. verum* EO**

The fungal and bacterial isolates used in this study were from ATCC (American Type Culture Collection) and from selected MRSA species (Methicillin-Resistant Staphylococcus aureus), that were obtained from the Palestinian area at clinical settings and exhibited multi-antibiotic resistance. The screened microorganisms included three Gram-positive bacteria (Methicillin-resistant Staphylococcus aureus (MRSA), *Enterococcus faecium* (ATCC 700221) and *Staphylococcus aureus* (ATCC 25923)), three Gram-negative bacteria (*Shigella sonnie* (ATCC 25931), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) and two fungal strains (*Epidermophyton floccosum* (ATCC 10231) and *Candida albicans* (ATCC 90028)).

The microdilution assay was utilized to assess the antimicrobial activity of a *I. verum* EO against bacterial and yeast strains. A total of 8.8 g of Mueller-Hinton broth were dissolved in 400 mL of distilled water under heat and boiled for 1 min. The obtained solution was autoclaved and kept at 4 °C until use. 100 µL from the broth was placed in each well of the microdilution tray. Then, 100 µL of the EO was added to the first well and the mixture was shacked. After that, serial dilutions up to well #11 were performed. Well #12 contained no EO and was considered a positive control for microbial growth. A fresh bacterial colony was picked from an overnight agar culture and was prepared to match the turbidity of the 0.5 McFarland standards to provide a bacterial suspension of $1.5\times10^8$ CFU (colony forming unit)/mL. The suspension was diluted with broth by a ratio of 1:3 to a final concentration of $5\times10^7$ CFU. Then, 1 µL of the bacterial suspension was added to each well except well #11, which was a negative control for microbial growth. Finally, the plate was incubated at 35 °C for 18 h. The microdilution method for the yeast *C. albicans* was performed as described above, except that after matching the yeast suspension with the McFarland standard, it was diluted with NaCl by a ratio of 1:50, followed by 1:20, and 100 µL was placed in the wells. The plate was incubated for 48 h instead of 18 h.

To investigate anti-*Epidermatophyton floccosum* mold activity of the *I. verum* EO, an agar dilution method was performed. Sabouraud dextrose agar (SDA) was prepared, of which 1 mL was placed in each tube and kept in a 40 °C water bath. 1 mL of the EO was mixed with 1 mL of SDA in the first tube, and serial dilutions were performed in six tubes, except tube #6, which did not contain any plant material and was considered a positive control for microbial growth.

After the SDA was solidified, the spores of the mold culture were dissolved using distilled water containing 0.05% Tween 80 and scratched from the plate for comparison with McFarland turbidity. From that suspension, 20 µL was pipetted into the six tubes, except tube #5, which was considered a negative control. The tubes were incubated at 25 °C for 14 days. The MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism (21, 23).

**Cell culture and cytotoxicity assay**

HeLa, 3T3, LX-2, and MCF-7 cervical adenocarcinoma cells were grown in RPMI-1640 media with 10% fetal bovine serum, 1% Penicillin/Streptomycin antibiotics, and 1% l-glutamine. Seeding a density of $2.6 \times 10^4$ cells per well, the cells were kept in a humidified atmosphere at 37°C with 5% $CO_2$. The Cell-Tilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay was used to assess

cell viability after 48 h of exposure to various concentrations of EO for 24 h, as per the manufacturer's instructions (Promega Corporation, Madison, WI). At the end of the treatment, 20 µL of MTS solution was added to each well for every 100 µL of media, and the plates were incubated at 37 °C for 2 h. The spectrophotometer reading for absorbance was taken at 490 nm (24).

**Statistical examination**

The cytotoxicity data was analytically averaged from triplicate tests. Results were presented as means ± SD. GraphPad Prism, version 6.01, was used for the statistical analysis.

**RESULTS**

***Phytochemical composition of I. verum EO***

We decided to investigate the chemical constituents and potential medicinal properties of *I. verum* EO based on our expertise in isolation techniques, identifying natural compounds from plants, and evaluating their biological activities. Our goal was to look for potential drug leads from natural sources. To accomplish this, we used a microwave-ultrasonic apparatus for isolation and GC-MS to identify and estimate the phytochemical composition. The obtained yield of the EO was 1.72±0.97%. GC-MS analyses indicated the presence of 32 phytochemical compounds in anise star EO, of which E-Anethole 88.38% was the major component, followed by Shisofuran 2.88% and Limonene 2.01% as revealed in percentage of the area under the curve Table 1 and Figure S1.

**Table** (**1**)**:** *I. verum* EO phytochemical composition.

| No. | EO components | R.T | (R.I) | Area | %of area |
|---|---|---|---|---|---|
| 1 | α-Pinene | 9.776 | 937 | 1410148 | 0.13 |
| 2 | β-Pinene | 11.74 | 974 | 467677 | 0.04 |
| 3 | Myrcene | 12.35 | 987 | 407776 | 0.04 |
| 4 | Pseudolimonene | 12.93 | 1000 | 329130 | 0.03 |
| 5 | Isosylvestrene | 13.152 | 1005 | 1377479 | 0.13 |
| 6 | α-terpinene | 13.51 | 1014 | 214526 | 0.02 |
| 7 | p-Cymene | 13.84 | 1022 | 2379945 | 0.22 |
| 8 | Limonene | 14.05 | 1027 | 21617976 | 2.01 |
| 9 | 1,8-Cineole | 14.18 | 1030 | 2504547 | 0.23 |
| 10 | *Trans*-ocimene | 14.83 | 1045 | 90633 | 0.01 |
| 11 | γ-Terpinene | 15.3 | 1056 | 436775 | 0.04 |
| 12 | Linalool oxide B | 15.87 | 1069 | 158956 | 0.01 |
| 13 | Terpinolene | 16.43 | 1083 | 1037471 | 0.10 |
| 14 | Meta-Cymenene | 16.53 | 1085 | 512813 | 0.05 |
| 15 | p-Cymenene | 16.62 | 1087 | 588023 | 0.05 |
| 16 | Linalool | 16.78 | 1091 | 891268 | 0.08 |
| 17 | Linalool | 17.05 | 1097 | 8388065 | 0.78 |
| 18 | Terpinen-4-ol | 20.21 | 1178 | 2266188 | 0.21 |
| 19 | Estragole | 20.78 | 1193 | 14932049 | 1.39 |
| 20 | Shisofuran | 20.87 | 1195 | 31020480 | 2.88 |
| 21 | 4-methoxybenzaldehyde | 21.71 | 1218 | 2782972 | 0.26 |
| 22 | Cis-p-anethole | 22.87 | 1250 | 3088590 | 0.29 |
| 23 | 4-methoxybenzaldehyde | 22.96 | 1253 | 15351606 | 1.42 |
| 24 | E-Anethole | 24.24 | 1288 | 952234624 | 88.38 |
| 25 | Methyl o-anisate | 26.04 | 1342 | 313840 | 0.03 |
| 26 | α-Ylangene | 27.01 | 13.70 | 162694 | 0.02 |
| 27 | Anisyl methyl ketone | 27.13 | 1374 | 234974 | 0.02 |
| 28 | α-Cedrene | 28.33 | 1411 | 293097 | 0.03 |
| 29 | β-caryophyllene | 28.56 | 1418 | 491453 | 0.05 |
| 30 | Propiophenone | 29.45 | 1447 | 321692 | 0.03 |
| 31 | E-Methyl isoeugenol | 33.46 | 1573 | 216292 | 0.02 |
| **Total** | | | | | **99%** |

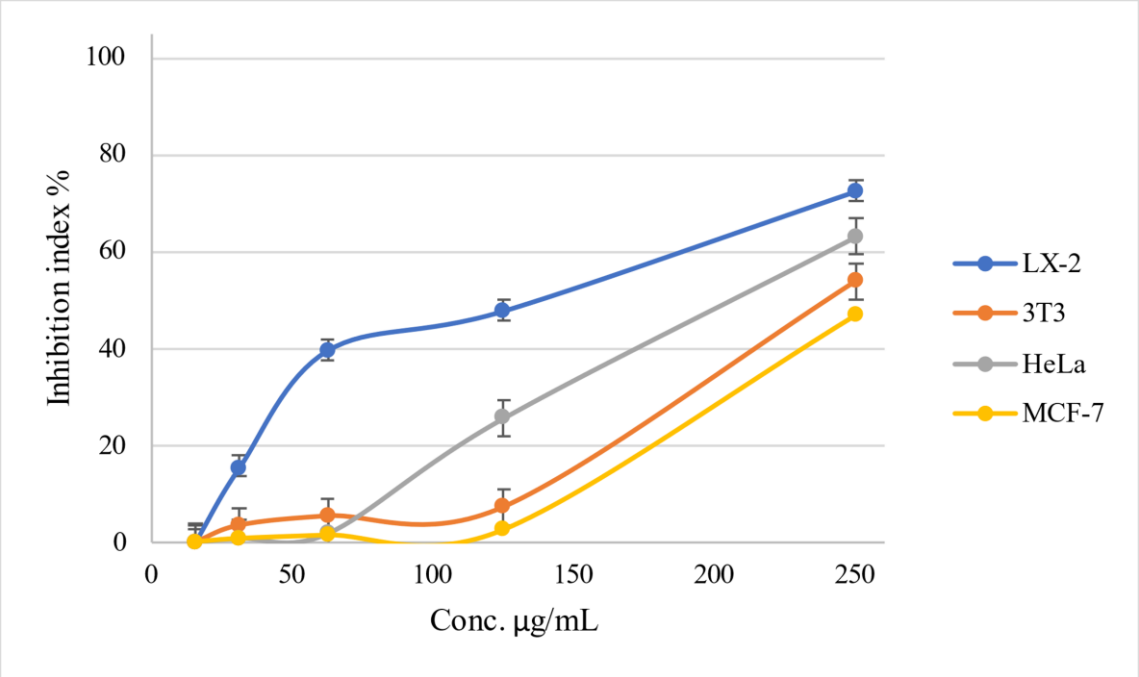
### Antimicrobial activity

The microdilution assay was used to determine the antimicrobial potential of *I. verum* EO against Methicillin-Resistant *Staphylococcus aureus*, *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, and *Candida albicans*. The EO inhibited the growth of all of the studied bacteria to different degrees. The highest antimicrobial activity was recorded against *Candida albicans* with MIC of 6.25 mg/ml, then *Proteus vulgaris* with a MIC of 50 mg/ml, while the EO has a MIC of 100 mg/ml against MRSA, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*,and *E. coli*.

**Table (2):** Antimicrobial MIC values of Anise star EO.

| Microorganisms | | MIC (mg/ml) |
|---|---|---|
| Methicillin-resistant *Staphylococcus aureus* (MRSA) | | 100 |
| *Staphylococcus aureus* | | 100 |
| *Klebsiella pneumoniae* | | 100 |
| *Escherichia coli* | | 100 |
| *Proteus vulgaris* | | 50 |
| *Pseudomonas aeruginosa* | | 100 |
| *Candida albicans* | | 6.25 |

### Cytotoxic effects




| | LX-2 | 3T3 | HeLa | MCF-7 |
|---|---|---|---|---|
| IC50 | 57.265 ±2.51 µg/ml | 118.819 ±3.014 µg/ml | 90.499 ±1.87 µg/ml | 131.171 ±2.47 µg/ml |

**Figure (1):** The inhibition index of *I. verum* EO on LX-2, 3T3, Hela, and MCF-7 cells, as well as the EO $IC_{50}$ on various cell types. The best $IC_{50}$ was obtained against LX-2 cells (57.265 ± 2.51 g/ml), followed by Hela cells (90.499 ±1.87 µg/ml), 3T3 cells (118.819 ± 3.014 µg/ml) , and MCF-7 cells in that order.

## DISCUSSION

Plant secondary metabolite research is a global strategy for the discovery of new therapeutic agents because plants contain a diverse range of biologically active compounds. Because of their healing properties, medicinal plants are widely used in traditional medicine. It is critical to link scientific studies with clinical observations and traditional knowledge in order to create an inventory of their biological activities. This study aimed to evaluate the biological activity

of *I. verum* EO. The chemical constituents and antimicrobial and anticancer properties have not yet been reported of local *I. verum* EO. The purpose of the study was to examine the chemical constituents and possible medicinal properties of *I. verum* EO. Anethole 88.38% was the major ingredient, followed by Shisofuran 2.88% and Limonene 2.01%. The remaining minor ingredients represented 7.7%. The predominant component of the EO extracted from the fruit of *I. verum* is the phenylpropanoid *trans*-anethole. Various investigations indicate that the average amount of *trans*-anethole in EO of *I. verum* is between 72 and 92 percent (9, 13, 24). The experiments confirmed that the amount of *trans*-anethole in the EO is dependent on the extraction technique used. Wang *et al*. (25) evaluated the impact of steam distillation (SD), solvent extraction (SE), and supercritical fluid extraction (SFE) on volatile content (SFE). The content of *trans*-anethole was as follows: 70.61% (SE), 77.31% (SFE) and 74.96% (SD).

The antimicrobial activity of *I. verum* EO revealed that *I. verum* EO inhibits the growth of all bacteria tested to varying degrees, with the highest antimicrobial activity against *C. albicans;* the MIC was 6.25 mg/ml. The EO had a MIC of 100 mg/ml against MRSA, *S. aureus, K. pneumoniae, P. aeruginosa,* and *E. coli*, while the tested EO had a MIC of 50 mg/ml against *P. vulgaris*. Previous work showed that the antimicrobial activity of *I. verum* EO against two gram-positive bacteria, *S. aureus* (MRSA) and *S. pyogenes*, as well as three gram-negative bacteria, *E. coli* and *P. aeruginosa* had an MIC from1-1,6 mg/mL (26). The antimicrobial activity of *I. verum* EO may be attributed to *Trans*-anethole, Kwiatkowski *et al*, found that *trans*-anethole at a concentration of 4% showed an antimicrobial activity against *S. aureus.* In addition, the study showed that *trans*-anethole enhanced the effectiveness of mupirocin when used in combination *trans*-anethole (27). The mechanism involved in *trans*-anethole action come from the work of Hançer *et al,* trans-anethole display inhibitory activity against quorum sensing as was observed by a blue ring around *Escherichia coli* QSIS1. Furthermore, *trans*-anethole used at a concentration of 6 mM decreased the expression of *P. aeruginosa* elastase gene (*lasB)* by about 57% and *P. aeruginosa* PAO1 virulence factor production (28). The anti-cancer effect *I. verum* EO can be attributed to *Trans*-anethole which has anti-proliferative and pro-apoptotic effects on MCF-7 and MDA-MB-231 cell line (29). There is a lack in literature on the effect of *I. verum* EO or its major constituents *trans*-anethole on other cell line tested in the current work. The effect of *trans*-anethole on cell cycle involve the p53 and mitochondrial intrinsic pathway**,** whereas *trans*-Anethole induced cell cycle arrest at the G0/G1 phase with the generation of reactive oxygen species and reduction in mitochondrial membrane potential (30).

## CONCLUSION

This study has provided initial data supporting the importance of *I. verum* EO from Palestine in traditional medicine. The extracted EO exhibited anticancer and antimicrobial properties, highlighting the EO's main composition, with *trans*-anethole as the main constituent. This study draws attention to *I. verum* EO as a potent source of bioactive molecules. Future research on *I. verum* EO should focus on identifying the molecular targets and signaling pathways involved, emphasizing *in vivo* studies.

**Ethics approval and consent to participate:** Not applicable

**Consent for publication:** The authors give the Publisher the Author's permission to publish the work.

**Availability of data and materials:** The corresponding author's data supporting this study's findings are available upon reasonable request.

**Author's contribution:** Malik Alqub: conceptualization, writing-original draft, formal analysis, investigation, methodology, supervision, validation, visualization, and writing review & editing. Mohammed Hawash, Nawaf Al-Maharik, Mustafa Ghanim: formal analysis, validation, visualization, and writing review & editing. Osayd Masri, Masa Arafat, Aida Jamous: methodology, investigation ,formal analysis and writing.

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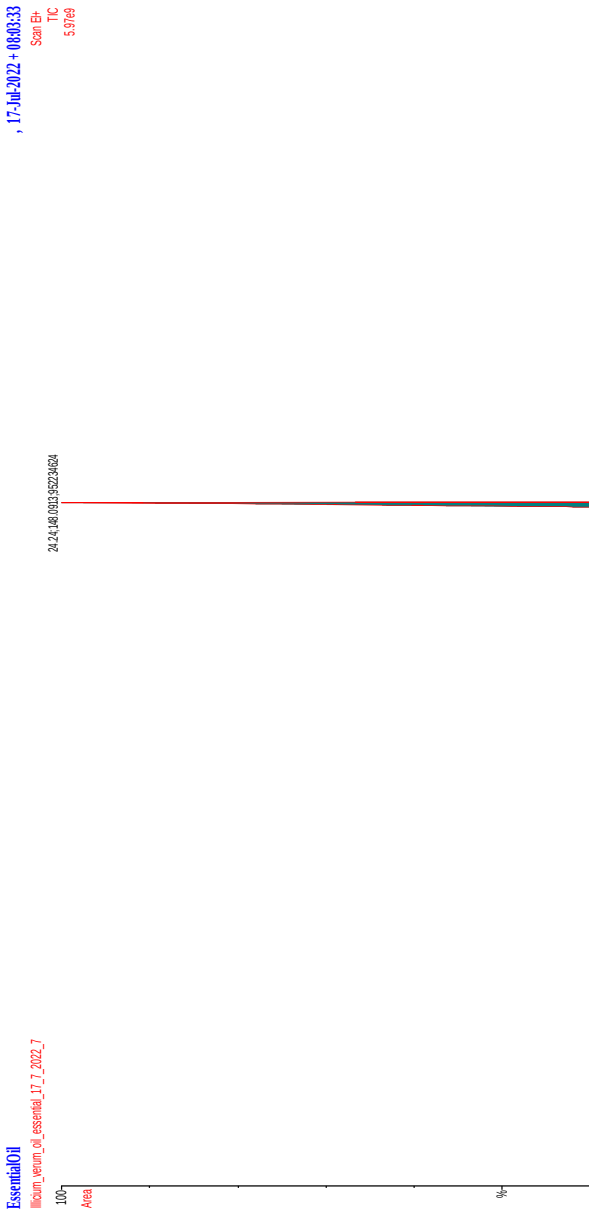
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