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Research Article

In Vitro Antifungal, Antimicrobial Properties and Chemical Composition of *Santolina chamaecyparissus* Essential Oil in Syria

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ABSTRACT
The essential oil of the *Santolina chamaecyparissus* was isolated by herdro distillation using a modified Clevenger apparatus, and its constituents were identified and quantified by GC/MS analyses. The total composition of the essential oil was 92.09%. The main constituents of the essential oil were Artemisia ketone (15.65%). Another compounds present are as follow: Alpha-Amorphene (12.11%), Beta -Phellandrene (10.63%), Beta-Myrcene (7.42%), Nootkatone (6.97%). This study seeks to evaluate the chemical composition and its effect on the growth inhibition of microorganisms. The antibacterial was investigated in vitro against four gram-positive bacteria (*Bacillus subitus, Staphylococcus aureus, Streptococcus pyogenes, Micrococus luteus*) and six gram-negative bacteria (*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginose, Klebsellia pneumonia, Proteus vulgaris, Vibrio parahaemolyticus*), also against six fungi (*Fusarium solani, Aspergillus flavus, Penecillium variable, Mucor sp, Rhyzopus sp* and *Candida albicans*). The tested essential oil had different degrees of antibacterial and antifungal activity. The antimicrobial activities of essential oil evaluated in the present study by measuring the inhibition zones using Agar Disk Diffusion method. MIC and MBC varied from 0.4 – 1.6 mg/ml, and from 0.8 – 3.2 mg/ml, respectively, while MIC and MFC ranged from 0.4 to 3.2 and from 0.8 to 6.4 mg/ml respectively.

Keywords: *Santolina chamaecyparissus* L.; Essential oil; GC-MS; Artemisia ketone; Antimicrobial activity.

INTRODUCTION

Aromatic plants are frequently used in traditional medicine and essential oils extracted from them are widely used as antioxidants and antimicrobial agents as well as for the prevention and treatment of different human diseases[1,2]. Currently, essential oils are attracting increasing interest in the scientific community. Essential oils are complex mixtures of natural compounds comprised mostly of volatile constituents with multiple biological activities such as antimicrobial, insecticidal and antioxidant properties[3,4]. Most of the antimicrobial activity in essential oils appears to derive from oxygenated terpenoids such as alcoholic and phenolic terpenes, while other constituents are believed to contribute little to the antimicrobial effect[5,6]. Although E. oils are widely applied as natural antimicrobials, their organoleptic properties may alter the taste of food or exceed acceptable flavor thresholds[7,8]. With growing interest in the use of essential oils in both the food and pharmaceutical industries, the systematic examination of plant extracts for these properties has become increasingly important. *Santolina* species are used in folk medicine for many diseases and for ornamentation in gardens, the genus *Santolina* (*Asteraceae*/Compositae) is represented by more than 100 species widely distributed in the Mediterranean area[9]. *Santolina chamaecyparissus* L, is a hardy aromatic, dwarf fragrant, dense mound with attractive grayish-silver foliage, evergreen shrub native to the Mediterranean area growing wild and often grown in gardens for its attractive woolly silver-grey leaves born on woody stems, and for its yellow flowers[10]. It is commonly known as cotton lavender, which is a small medicinal herb, cultivated in Europe, Asia and Africa due to the antihelmintic, antispasmodic and emmenagogic properties of the infusions prepared from the leaves and flower heads. In the Mediterranean folk medicine, the flowers are used for their anaigesis, anti-inflammatory, antiseptic, antispasmodic, bactericidal, fungicidal, digestive and vulnerary properties, and is also used in phytotherapy[11]. A survey of the previous studies on the essential oils of *S. chamaecyparissus* L showed, nevertheless, that the chemical composition is highly variable, the existence of different subspecies being one of the factors responsible for these differences, along with the geographical origin of the plant material[12-14]. The present study is aimed mainly to determine the chemical composition of *S. chamaecyparissus* hydro-distilled essential oil by GC/ MS, and investigate the antimicrobial activity of the essential oil of the plant by disc diffusion and micro dilution method against some pathogen bacteria and fungi.

EXPERIMENTAL

Plant Material

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Aerial parts of *S. chamaecyparissus* were collected during the flowering stage (jun 2016), growing in the campus of Damascus University Faculty of ecological Science, Damascus (700 m above sea level, N 33°30'46", E 36°17'31"), and authenticated by the taxonomist of the Botany Department. The dirt was removed with tap water, and aerial part plant dried in the shade for about 14 days.

Essential Oil Extraction

The dried leaves (100 g) were subjected to hydro distillation using Clevenger type apparatus for 3h. The essential oil collected was dried over sodium sulphate anhydrous and a yellowish essential oil with a strong pleasant aroma was recovered with a yield of 2.1 % (v/w). The yield was calculated and recorded on the basis of dried weight material, and stored in a refrigerator at 4 °C in tightly closed amber vials, away from contamination sources and collected prior to use for analysis and various functional biological tests

Analysis of the Essential Oil (GC/MS)

Essential Oil composition was studied with gas chromatography mass spectrometry (GC/ MS). Gas chromatography analysis was carried out with an Agilent 6890 N gas chromatograph (GC) equipped with Agilent 5973 mass selective detector (MSD), Agilent Auto sampler 7683 and Agilent DB-5MS capillary column (30 m, 0.25 i.d., 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA, USA). The MS detector was operated in electron impact (EI) mode at 70 eV with interface temperature of 280ºC; the scan range was 50–550 amu. The injection port temperature was set at 250°C. GC was performed in split less mode; carrier gas was helium at a constant flow rate of 1 ml /min. The column temperature was programmed as follows: an initial temperature of 60ºC increased to 280ºC at rate of 3ºC/min. The injection volume was 1.0 µL.

Identification of Components

Relative percentage amounts were calculated from peaks total area by software apparatus. The identification of individual compounds was based on comparison of their mass spectra with those obtained from the NIST/NBS, Wiley Libraries spectra, stored in the GC-MS database. Further confirmation was done from Retention Index data generated from a series of alkane's retention indices (relatives to C8-C20 on the DB-5MS column), and published mass spectra (Adams 2007), and (V. I. Babushok, et al., 2011)[15].

Evaluation of The Antimicrobial Activity

Microbial Strains

The microorganisms used in this study were gram-positive bacteria (*Bacillus subitus, Staphylococcus aureus, Streptococcus pyogenes, Micrococus luteus*) and gram-negative bacteria (*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginose, Klebsellia pneumonia, Proteus vulgaris, Vibrio parahaemolyticus*) obtained from the Department of Medical Microbiology and Parasitology, Faculty of Medicine, Damascus university. As well as the fungi: *Fusarium solani, Aspergillus flavus, Penecillium variable, Mucor sp, Rhyzopus sp* and *Candida albicans* (yeast), obtained from the Department of Botany, faculty of science, Damascus university. The bacteria cultures were maintained on nutrient agar slant at 37±2ºC for 24 h. The organisms were then sub cultured and preserved at +4ºC in sterile bottles containing both nutrient broth and 15% sterile glycerol. While the inoculum of fungal was obtained by growing the isolates of fungal for 5 days at 28±2 °C in Sabouraud dextrose agar (SAD). To prepare the inoculum, conidia were removed from the colony surface with 3–4 ml sterile saline solution by gently scraping the surface. The stock suspension was diluted in saline solution, corresponding to 10^6 CFU/ml. The inoculants were stored at +4ºC until further use[16].

Antibacterial Screening

The disc diffusion method was employed for the determination of antibacterial activities of the essential oil in question[17]. Paper discs of 5 mm diameter were impregnated with 20 µl of the essential oil dissolved in DMSO (final concentration of 10% (v/v)) and transferred onto the Mueller-Hinton agar present in Petri dishes, which had been surface spread with 0.1 ml of bacterial suspension adjusted to 10^7 CFU/ml for *S. aureus* and 10^6 CFU/ml for the other strains[18,19]. The DMSO solvent was used as the negative control. Standard antibiotics amoxicillin (30 mg/disk), was used as positive control. After incubation at 37 ± 2°C during 24 h, the diameters of inhibition zones were measured in millimeters. Tests were carried out in triplicate.

Antifungal Screening

A suspension of the tested fungal (0.1 ml of 10^6 CFU/ml) was maintained on the Sabouraud dextrose agar plates for viability of each isolate. The antifungal activity was conducted using Agar diffusion assay as described by (Smania et al., 1995)**[20]** With some modifications**.** The molten SDA medium was poured into the sterilized petriplates and kept for solidification. After 24 h. Each fungal suspension was maintained on the medium. Then, Paper discs of 5 mm diameter prepared from Whatman Number 1 were impregnated with 20 µl of the essential oil dissolved in DMSO and were placed on the inoculated plates. The plates were incubated at 28 ± 2°C for 2-3 days, after staying at 4ºC for 2 hrs. The plates were incubated at 28 ± 2°C for 2-3 days. The results were expressed in terms of the diameter of the inhibition zone. All experiments were carried out in triplicates. Negative control was prepared using DMSO solvent. Nystatin (30 mg/mL) was used as positive controls.

Determination of the MIC, MBC and MFC

Minimum inhibitory concentration (MIC) values of essential oil were tested by two-fold serial dilution method**[21]**.The test samples of essential oil were first dissolved in 5% DMSO, and incorporated into mullerhinton broth medium in a tube to obtain a concentration of 12.8 mg/ml, and serially diluted to achieve12.8, 6.4, 3.2,1.6, 0.8, 0.4 and 0.2 mg/ml, respectively (into muller- hinton broth medium for bacteria, Sabouraud's broth medium for fungi). 10 µl of standardized suspension of each test organism (10^6 CFU/ml) was transferred to the tubes, and incubated at 37 ± 2ºC for 24 h. The MIC was determined as the lowest concentration (mg/ml) of the essential oil where no visible

growths of test organisms occur. The microorganism growth was indicated by turbidity.

The MIC end-point criterion was the lowest concentration of the essential oil at which there was no visible growth after 72 h incubation at 28 ± 2°C for fungi while the MIC of bacteria there was no visible growth after 24 h incubation at 37 ± 2ºC. To obtain the MFC (minimum fungicidal concentration), 1ml of each serial dilution was taken from each tube and spread on Sabouraud dextrose agar. Plates were incubated at 28 ± 2°C for 72 h. The MFC was defined as the lowest concentration that yielded three or fewer colonies (i.e. 99 % of the inoculum was killed)[22]. MBC (Minimum Bactericidal Concentration) is usually an extension from the MICs, where the organisms quantitatively indicate the minimum concentration when no viable organism appears in the culture[23].

Statistical Analysis

All determinations in this article were carried out in triplicates and SPSS Statistics 19.0 Software was used to evaluate one-way analysis of variance (ANOVA) at $p \leq 0.05$. Canonical Discriminate Analysis was also used to establish differences between samples, and to evaluate the importance of different variables on discrimination.

RESULTS AND DISCUSSION

Chemical Composition

The essential oil of the *S. chamaecyparissus* was extracted by the yield of 2.10 % v/w then analysis by technique of GC-MS. According to Table1, GC-MS analysis of the essential oils has led to the identification and quantification of thirty-one components, accounting for 92.09 % of the total essential oil. The most abundant of them were Artemisia ketone (15.65%). Other compounds present are as follow: Alpha-Amorphene (12.11%), Beta - Phellandrene (10.63%), Beta-Myrcene (7.42%), Nootkatone (6.97%), Alpha-Pinene (4.00%), Sabinene (3.54%), Beta-Pinene (3.42%), Germacrene B (2.55%), and Camphor (2.32%). Monoterpene Hydrocarbons (30.95%), Oxygenated Monoterpenes (24.52%), Sesquiterpene hydrocarbons (25.80%) and Oxygenated sesquiterpenes (10.82%) percent of the whole essential oil. There are many studies referring to the composition of the essential oil of the *S. chamaecyparissus* aerial parts. By surveying the data reported we found a great diversity of E. oil composition, which was effected by many factors. It can be divided the components of the oil of the various existing studies into two groups analyzed. The first group, which includes Aretmicia Kitone as a major component, such as highlighted by (Nousari et al., 2015)[24], (Demirci et al., 2000)[25], (Vernin et al., 1991)[26], (Grag et al., 2001)[27], (Pérez et al., 1992)[28] and (Yolanda et al., 2012)[29]. The second group is that possesses other key compounds such as (Samah et al., 2012)[30], (Clara et al., 2009)[31], (Ahuja et al., 2005)[32] and (Villar et al., 1986)[33]. Table 2 showed the major components. The quality and quantity of the materials forming *S. chamaecyparissus* essential oil had some differences and similarities with the cases reported in other regions. The studies of the ingredients of the essential oil of botanical populations with ecological and genetic differences can be of great importance in identifying the variety of essential oil inside the population of species.

Table 1: Percentage content of compounds in essential oil of the *S. chamaecyparissus* dried leaves

S. No.	R.I	Compositions	Area %
		Monoterpene Hydrocarbons	30.95
1.	939	Alpha-Pinene	4.00
2.	953	Camphene	0.58
3.	969	Sabinene	3.54
4.	975	Beta-Pinene	3.42
5.	991	Beta-Myrcene	7.42
6.	1031	β-Phellandrene	10.63
7.	1088	Terpinolen	1.36
		Oxygenated Monoterpenes	24.52
8.	998	Yomogi alcohol	0.83
9.	1033	Eucalyptol	0.57
10.	1054	Artemisia ketone	15.65
11.	1083	Artemisia alcohol	0.88
12.	1094	Linalool	0.43
13.	1141	Camphor	2.38
14.	1159	trans-Chrysanthemol	1.11
15.	1165	Borneol	1.27
16.	1177	Terpinen-4-ol	0.88
17.	1288	Thymol	0.52
		Sesquiterpene hydrocarbons	25.80
18.	1337	delta-Elemene	0.83
19.	1351	Alpha-Longipinene	1.00
20.	1418	Beta -Caryophyllene	0.67
21.	1454	Alpha-Caryophyllene	0.34
22.	1458	E-Beta-Farnesene	0.36
23.	1481	Germacrene D	12.11
24.	1503	Germacrene A	6.37
25.	1515	delta-Cadinene	2.55
26.	1534	Alpha-Cadinene	0.66
27.	1542	Alpha-Bisabolene, (E)-	0.91
		Oxygenated sesquiterpenes	10.82
28.	1569	Spathulenol	0.61
29.	1577	(-)-Spathulenol	2.03
30.	1729	alpha-Sinensal	1.21
31.	1811	Nootkatone	6.97
		Total	92.09

RI: retention index (Kovalts) relative to n-alkanes (C8–C20) on a non-polar DB-5

Antibacterial Activity

The in vitro antibacterial activity of essential oil of *S. chamaecyparissus* was qualitatively assessed by the presence or absence of inhibition zones. According to the results given in Table 3, essential oil showed antibacterial effect against *Bacillus subitus*, *Staphylococcus aureus*, *Micrococcus luteus* (gram positive), with their respective diameter zones of inhibition of 20, 14 and 12 mm. And showed antibacterial effect against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli* (gram negative) with their respective diameter zones of inhibition of 25.5, 19.5, and 12 mm. while no activity showed against *Streptococcus pyogens* (gram positive),

Table 2: major components of different studies.

No. re.	Country	Components
24.	Algeria	Artemisia kitone 40.33%, Z-Thyjone 9.82%, Farnesol 7.30%
25.	Turkey	Artemisia kitone 38.10%, Camphor 11.70%, β-phellandarene 9.20%
26.	France	Artemisia kitone 45.00%, Myrcene15.00%
27.	India	Artemisia kitone 32.00%, 1,8 Cineol 16.00%, Myrcene15.00%
28.	Spain	Artemisia kitone (27.80-35.00) %, T-Cidanol (23.60-4.80) %
29.	Spain	Artemisia kitone 27.19%, Dihydroaromadendrene 18.21%, β-phellandrene 7.49%
30.	Algeria	Camphor 31.10%, Cubenol 17.00%, P-Cymene 8.30%
31.	Portugal	1,8 Cineol (25.00-30.00) %, Camphor (7.00-9.00) %, Borneol (7.00-8.00) %
32.	India	Linalool 12.00%, β-ocimene 10.00%, Myrcene 10.00%
33.	Spain	Camphor 25.00%, allo-aromadendrene 19.00%

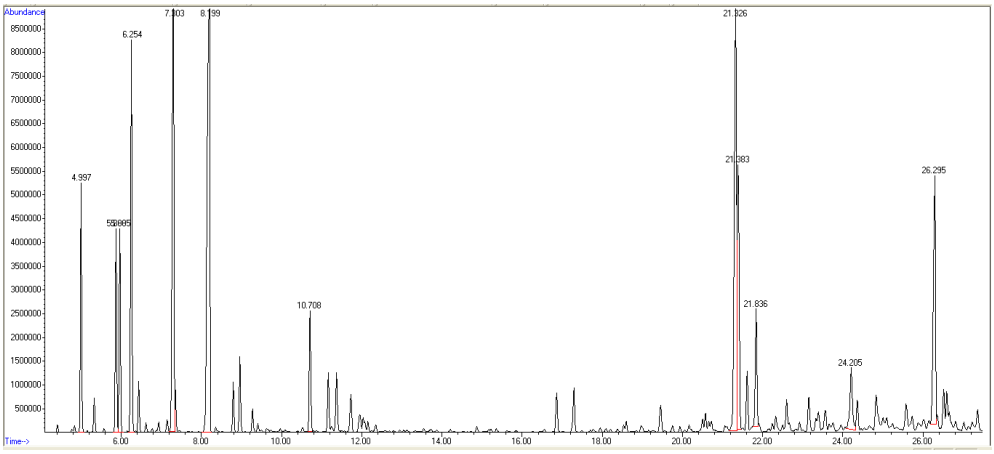

Figure 1: GC/MS Chromatogram of dried *S. chamaecyparissus* leaves essential oil

Vibrioparahaemolyticus, *Proteus vulgaris* and *Salmonella Typhimurium* (gram negative).

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the essential oil were determined, using a broth micro dilution method. The variability in the concentration of the main components present in the essential oils analyzed led us to evaluate the antimicrobial activities of the essential oil. As shown in Table 3, the MIC values for the essential oil of *S. chamaecyparissus* were found to be in the range of 0.4 – 1.6 mg/ml and the MBC values were found to be in the range of 0.8 - 3.2 mg/ml.

Antifungal Activity

The essential oil isolated from the leaves of *S. chamaecyparissus* was tested for antifungal activity against six fungal strains and their fungistatic effects were compared with the commercial antifungal Nystatine. The colony diameter (mm) of growth is inhibition shown in Table 4. The results of antifungal activity assays showed that the essential oil strongly reduced the growth of *Candida albicans* (22.3 mm) and *Fusarium solani* (20 mm), while it showed moderately activity of *Mucor sp* (12 mm) and *Rhyzopus sp* (10 mm). However, the essential oil was significantly not active against *Aspergillus flavus* and *Penicillium variabile*.

MIC values for the essential oil of *S. chamaecyparissus* were found to be in the range of 0.4 – 3.2 mg/ml. MFC varied from 0.8 mg/ml to 6.4 mg/ml, *Rhyzopus sp* (6.4 mg/l) and *Mucor sp* (3.2 mg/ml) had higher values than *Candida albicans* (0.8 mg/ml) and *Fusarium solani* (1.6 mg/ml). Inhibitory effects of the essential oil on the growth of fungal strains were lower compared to Nystatine.

A reference study shows that there is scarcity in the study of this essential oil on bacteria and fungi, it could be due to the humble bioactive impact. Most of the previous studies were about essential oil constituents. Most of the researches were similar to our study conducted by (Suresh, B. et al., 1997)[34], (Djeddi S. et al., 2012)[35], (Nouasri, A. et al., 2015)[24] and (Ruiz-Navajas Y, et al., 2012)[36].

The essential oil content of different plants varies depending on which part of the plant it is obtained from (flower, stem, leaves), the variety of the plant, its harvest season, and the method of cultivation. These differences might have been derived from local, climatic and seasonal factors. The method of extracting the plant material and its form (whether crushed or finely powdered) affected their antibacterial activity. Essential oils rich in phenolic compounds are widely reported to possess high level of antimicrobial activity, which has been confirmed and extended in the present studies. It is believed that the phenolic components of essential oils show strongest antimicrobial activity, followed by aldehyde, ketones and alcohols (Jarrar et al., 2010)[37]. In this study, the bioactivities of major component artemisia ketone 15.65% are unknown[38]. The antimicrobial activity of the essential oil of *S. chamaecyparissus* could, in part, be associated with their major monoterpens constituent Camphor and another active molecule component such as α-pinene, β-pinene, myrcene, β-phellandrene and Germacrene. α-

Table 3: Inhibition zones (mm) and Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) (mg / ml) of *S. chamaecyparissus* essential oil.

Test organisms	Essential oil	MIC	MBC	amoxicillin
gram-positive bacteria				
Bacillus subitus	20 ± 0.11	0.8	1.6	40 ± 0.11
Micrococcus luteus	12 ± 0.13	1.6	3.2	17 ± 0.21
Streptococcus pyogens	NA	NA	NA	NA
Staphylococcus aureus	14 ± 0.10	1.6	3.2	45 ± 0.13
gram-negative bacteria				
Klebsiella pneumoniae	25.5 ± 0.42	0.4	0.8	NA
Vibrio parahaemolyticus	NA	NA	NA	30 ± 0.32
Proteus vulgaris	NA	NA	NA	45 ± 0.12
Salmonella Typhimurium	NA	NA	NA	16 ± 0.31
Escherichia coli	12 ± 0.22	1.6	3.2	NA
Pseudomonas aeruginosa	19.5 ± 0.10	0.8	3.2	NA

Each result is the mean ± SD of three replicates. NA: no active

Table 4: Inhibition zone Diameter(mm) and Minimum inhibitory concentration (MIC) (mg/ml) and minimum fungicidal concentration (MFC) (mg/ml) of *S. chamaecyparissus* essential oil.

Test organisms	Essential oil	MIC	MFC	Nystatine
Fusarium solani	20 ± 0.11	0.8	1.6	25 ± 0.14
Aspergillus flavus	NA	NA	NA	28 ± 0.02
Penicillium variabile	NA	NA	NA	19 ± 0.12
Mucor sp	12 ± 0.10	1.6	3.2	25 ± 0.10
Rhyzopus sp	10 ± 0.03	3.2	6.4	30 ± 0.11
Candida albicans	22.3 ± 0.02	0.4	0.8	18 ± 0.32

Each result is the mean ± SD of three replicates. NA: no active

pinene and β-pinene have been reported to display strong antibacterial effects[39]. These components have been reported to display antimicrobial effects[40,41,42]. The essential oils containing terpenes are also reported to possess antimicrobial activity[43], which are consistent with our present study. An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death[44]. Considering the large number of different groups of chemical compounds present in essential oils, it is assumed that their antimicrobial properties are most likely not attributable to only one specific mechanism[45]. In addition, the components in lower amount may also contribute to antimicrobial activity of the essential oils, involving probably some type of synergism with other active compounds[46].

CONCLUSION

The present study is the first report in Syria which describes the chemical composition of essential oil of *S. chamaecyparissus* performed by GC-MS, and the antimicrobial activities evaluated by measuring the inhibition zones using Agar Disk Diffusion method. Although in tested sample monoterpene hydrocarbons predominate, relative to the plant chemical composition, we conclude that the essential oil is mainly represented by terpenes, alcohols, and kitones compounds and that can be used in biological assay. Generally, the essential oil of *S. chamaecyparissus* showed significant antimicrobial activity. The results of this study show a great antibacterial activity of the essential oil of *Klebsiella pneumonia*, *Bacillus subitus* and *Pseudomonas aeruginosa.* with (MIC) about 0.4 – 0.8 mg/ml, and (MBC) about 0.8 – 3.2 mg/ml. They also, show a great antifungal activity of *Candida albicans* and *Fusarium solani* with (MIC) about 0.4 – 0.8 mg/ml and MFC about 0.8 – 1.6 mg/ml. Hence, this medical aromatic plant will be a source of natural antimicrobial products. More studies are necessary to continue the characterization of different parts of essential oil of *S. chamaecyparissus.* However, further studies need to be conducted (proceeded) to obtain more information on the safety and toxicity of this essential oil.

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