Determination of chemical composition and evaluation of Antioxidant, and Antimicrobial activities of Clove Oil obtained from Syzygium Aromaticum Moroccan species

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Article History:
Received on: 05 Apr 2020
Revised on: 01 May 2020
Accepted on: 13 May 2020

Keywords:
Antifungal activity, Antioxidant activity, essential oil, phenolic compounds, Syzygium aromaticum

ABSTRACT
The composition, antimicrobial and antioxidante activities of essential oil obtained from Syzygium aromaticum (Moroccan species), were studied. The extraction was carried out by hydro-distillation and analyzed by gas chromatography-mass spectrometry (GC-MS). The antioxidant effect was evaluated using two in vitro antioxidant assay using DPPH and FRAP methods, 2,2-Diphenyil picrylhydrazyl free radical (DPPH) and Total reducing ability determination by Fe3+–Fe2+ transformation Method (FRAP). And the antimicrobial effects of essential oils were evaluated by the disk diffusion method. The determination of composition showed that Twenty-four compounds were identified in the extracts of clove oil by gas chromatography/mass spectrometry. The constituents major of clove buds was Eugenol with percentage (63, 68 %). The result shown that clove oil had an important effect antioxidant in two in vitro assays including reducing power FRAP and DPPH radical scavenging. The result has been compared to that of some natural antioxidant. Based on the measurement of the diameter of inhibition, a moderate to high antimicrobial activity, according to oils was revealed against all yeast strains and moderate against bacteria strains. The Eo has showed a considerable antioxidant effect and antimicrobial activity against bacterial and fungus strains, deserving further investigation for clinical application in the treatment of fungal infections.

INTRODUCTION
The incidence of fungal infections and nosocomial infections has increased dramatically in recent years especially in patients with impaired immunity at the same time, the majority of conventional antifungals have a lot of problems in terms of toxicity, drug interactions, lack of fungicidal efficacy, cost and the emergences of resistant strains. Therefore, an intention to natural remedies with plants with therapeutic virtues which drives an intense search for new drugs more effective and less toxic (Kaufman et al, 2006).

Clove Oil have been a longe history of employment as natural microbial agents in many pharma-
Clove oil of *S. aromaticum* has been described in many references as having useful Antiseptic, Analgesic and Anesthetic effects and is widely employed in dentistry. Clove EO were employed for inhibitory activity against various spoilage microorganisms of intermediate moisture foods (*Matan et al.*, 2006). In 2007, it has been known and proved as antifungal effects against dermatophytes (*Park et al.*, 2007). Clove oil is known for its use to protect against the oxidative degradation of food by free radicals (*Gulcin*, 2004). There are two classes of antioxidants, synthetic and natural. synthetic antioxidants such as butylated hydroxyanisols (BHA) and butylated hydroxytoluens (BHT) have been widely used in the food industry, although their use has begun to be questioned because of their toxicities (*Scalbert et al.*, 2005). Thus, the development and use of more effective natural and safer antioxidants obtained from botanical sources, particularly medicinal plants, is desirable (*Scalbert et al.*, 2005).

This work is performed in the objective to investigate the antimicrobial and antioxidant effects of clove EO (*Syzygium aromaticum*).

**MATERIALS AND METHODS**

**Collection of Plant**

*S. aromaticum* was collected from the region of Rabat and sale, Morocco, dried and kept at room temperature.

**Extraction and identification of Essential oil**

Clevenger apparatus have been used to extract clove oil by hydro distillation for 3 H, according to the method recommended in the *pharmacopoeia* (*Européenne*, 1996). Yields were 15%, they were determined on the basis of the dry weight of the plant. GC-MS used to identify the composition.

**Chemicals**

The antioxidant effect of *S. aromaticum* oil was assessed using two reagents: free radical-scavenging activity with DPPH and FRAP, and BHA, BHT and Ascorbic acid were used as reference antioxidant.

**Antimicrobial activities**

**Fungal and Bacterial strain**

The antibacterial activity was evaluated against: *Pseudomonas aeruginosa* (Gram negative bacteria); *Staphylococcus epidermedis Meti-R* and two *Staphylococcus Meti-S* (Gram-positive bacteria).

**Antifungal activity was evaluated using fungal organisms**

clinical strains of *Candida albicans* and *non-albicans*, recurrent clinical straine of oral, cutaneous candidiasis and others. The identification of fungal isolates was made by microbiology methods after stored in Sabouraud dextrose.

Antibacterial positive control performed using 10 and 15 μg gentamicin discs.

Control antifungals were dissolved in 10% dimethylsulfoxide (DMSO) to study their antifungal activity on isolated yeast strains. Fluconazole 150 mg capsules and Terbinafine 250 mg tablet were added after grinding to 2 ml of 10% DMSO.

**Dispersion technique on agar medium**

Antimicrobial power of the various extracts on the growth of yeasts was evaluated by the diffusion method on agar medium. In this method, a petri dish containing 15 ml of Sabouraud chloramphenicol agar for antifungal specie and Mueller Hinton for bacterial specie, the medium are inoculated by flooding with a suspension containing colonies taken from a fungal culture and diluted in physiological saline, then a sterile disc of Whatman paper (6 mm) soaked with an amount of pure essential oil (5, 10, 15 μl) is deposited in the center of the agar. This method was made according to the method of (*Hazzit et al.*, 2009; *Européenne*, 1996).

**Antioxidant activity**

**Measurement of antioxidant activity**

**DPPH * assay**

To determine the anti-oxidative power of extracts using DPPH, the extract are allowed to react (DPPH●) in a methanol solution. In its radical form, DPPH● absorbs at 517 nm, and upon reduction by an antioxidant, the absorption disappears.

The method described by Popovici in 2009 with few changement. 50 μl of each different concentration (15 to 300 μg/ml) has been added to 1.95 ml of DPPH (1.97 mg/ml) solution in methanol. Test mixture were vortexed after incubated at room temperature in the darks for 30 min; thereafter, the absorbance values were measured at 517 nm against a blank in a Rayleigh UV 1800 spectrophotometer.

The inhibition of DPPH radical (I%) was calculated.
d using the following formula,

\[ I\% = (1 - \frac{A_e}{A_o}) \times 100 \]

A0: is the absorbance without sample and Ae: is absorbance with the sample

**FRAP assay**

This method employed to determine the reducing capacity of clove oil. It is based on a redox reaction in which an easily reduced oxidant (Fe3+) is used in stoichiometric excess and antioxidants act as reductants (Benzie and Strain, 1996). Some technic involves the capacity of clove oil to reduce the ferri cyanide complex to the ferrous ferrous form (Oyaizu, 1986). The ferric ion reduction antioxidant (FRAP) assay (Fe3 +) was used to determine the reducing power of clove oil. For the execution of this method, we prepare concentrations of clove oil between (150–15 μg/mL) in 1 mL of distilled waters were mixed with Sodium Phosphate Buffer (2.5 mL, 0.2 M, pH 6.6) and [[K3Fe(CN)6] potassium ferricyanide] (2.5 mL, 1%). The sample was incubated at 50°C for 20 min. Aliquots (2.5 mL) of trichloro-acetic acid (10%) were added to the mixture. Then, 2.5 mL of this solutions was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixtures indicates an increase of reduction capability.

**Statistical analysis**

The result collected was examined using Origin Pro (version 9.0) and recorded as Mean ± Standard deviation and. The analysis was performed using ANOVA procedures. \( P < 0.05 \) was considered significant.

**RESULTS AND DISCUSSION**

**Chemical composition of essential oils**

The result of identification showed that 24 components representing 100% of the volatile oil with high amounts of: eugenol (63.68 %) Table 1.

**Antioxydant activity**

**DPPH assay**

The results obtained were detailed below.

The result is mean of three parallel measurements.

In this technic, when an antioxidant is added to the radicals there is a degree of decolorisation owing to the presence of antioxidants which reverses the formation of the radicale DPPH. The EO was able to reduce the stable radical to the yellow colored diphenyl- picrylhydrazine based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction (Oyaizu, 1986).

The change of color from purple to yellow, indicate that the absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

The result shows a significant decrease \( (p < 0.05) \) in the concentration of DPPH radical due to the scavenging ability of clove oil and standards. AA and BHA were employed as positive control for radical scavengers. The scavenging effect of clove oil and standards on the DPPH radical decreased in the order of clove oil > BHA >AA, which were 77,16, 68,03 and 60,41%, with the concentration off 250 μg/mL, respectively (Table 2).

The effect of increasing of free radical scavenging activity of clove oil is proportional with concentration increasing \( (r^2: 0.9982) \). EC-50 values for clove oil was found as 25 μg/mL. Lower EC-50 indicates a higher DPPH free radical scavenging activity. (24).

**FRAP assay**

The Yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples.

The ability of electrons donations reflecting reducing effect, of bioactive compounds was associated with antioxidant activity (Siddhuraju, 2002; Arabshahi-Delouee and Urooj, 2007). Using the potassium ferricyanide, this assay show that EO had effective reducing effect compared to the standards (BHT, Ascorbic acid). The estimation of the reductive ability of clove oil, the Fe3+-Fe2+ transformation was investigated in the presence of clove oil using the method of (Oyaizu, 1986). Using different concentrations (25- 300 μg/mL), clove oil shows powerful reducing ability \( (r^2: 0.975) \) and these differences were statistically significant \( (p < 0.05) \). The reducing power of clove oil, Ascorbic acid, BHT, Ascorbic acid increased steadily with increasing concentration of samples. Reducing power of clove oil and standard compounds exhibited the following order: Clove oils > BHT > Ascorbic acid. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

**Antimicrobial activities**

The result of in vitro antimicrobial activity of S. aro-
Table 1: Qualitative and quantitative composition of EO of *S. aromaticum* (%).

| S/NO. | Name of constituent | Area % |
|-------|---------------------|--------|
| 1     | Eugenol             | 63.68  |
| 2     | Phenol-2-methoxy-4-a(2-propenyl)- acetates | 13.28  |
| 3     | Phenol-2-methoxy-3-(2-propenyl)- | 10.11  |
| 4     | Caryophyllenes.     | 5.97   |
| 5     | 1,4,7, -Cyclododecatriene, 1,5,9,9-tetramethyl-, Z, Z, Z- (Z)-1-Methyle-4-(6-methylhept 5-en-2-ylidene) ccyclohex-1-ene | 2.29   |
| 6     | Phenol–2-methoxy-3-(2-propenyl)- | 0.71   |
| 7     | Caryophyllene oxide | 0.54   |
| 8     | Naphthalène, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-méthylene-1-(1-methyl-ethyl)-, (1. alpha.,4a. beta.,8 a. alpha.)- | 0.28   |
| 9     | 10,10-Dimethyle-2,6-dimethylenebicyclo [7.2.0] undecan-5. beta. -ol | 0.12   |
| 10    | (1R,3E,7E,11R)-11,5,5,8-Tetramethyl -12-oxabicyclo [9.1.0] dodeca-3,7-dienes- | 0.10   |
| 11-24 | Others              | 0.68   |

Table 2: DPPH radical-scavenging of EO from *S. aromaticum*.

| Sample       | Concentration µg/ML | Scavenging effects on DPPH (%) | DPPH IC 50 (µg/ML) | IC 50 |
|--------------|---------------------|-------------------------------|--------------------|-------|
| S. aromaticum| 15                  | 42.28                         | 25                 |
|              | 25                  | 49.81                         |                    |       |
|              | 50                  | 54.29                         |                    |       |
|              | 100                 | 58.90                         |                    |       |
|              | 250                 | 77.16                         |                    |       |
|              | 530                 | 83.42                         |                    |       |
| AA           | 15                  | 23.65                         | 180                |
|              | 25                  | 28.53                         |                    |       |
|              | 50                  | 34.65                         |                    |       |
|              | 100                 | 60.41                         |                    |       |
|              | 250                 | 68.94                         |                    |       |
|              | 300                 |                                |                    |       |
| BHA          | 15                  | 23.74                         | 170                |
|              | 25                  | 26.94                         |                    |       |
|              | 50                  | 27.94                         |                    |       |
|              | 100                 | 32.96                         |                    |       |
|              | 250                 | 68.03                         |                    |       |
|              | 300                 | 70.77                         |                    |       |

*essential oils shown in below (Tables 3 and 4).

The values shown in the table are the average of three measurements.

According to our result, the highest effect of activity was observed against different *Candida* species, with the strongest inhibition zones being 40 to 45 on *C. tropicalis* species and *C. Parapsilosis* for 10 µl of essential oil (Table 3). By comparing mean growth inhibition diameters of *Candida albicans*, *Candida tropicalis* and *Candida parapsilosis* strains with Fluconazole and Terbinafine strains, the result is significant (P < 0.05).

Analyzing the mean inhibition diameters for *Candida glabrata* strains, the difference is significant statistically with terbinafine (P <0.05) and non-significant for a single *candida glabrata* strain with fluconazole (p = 1). Our results are comparable with those reported by the team of (de Oliveira et al., 2009) which demonstrated that Eugenol, which is the main compound of essential EO clove is considered a very good growth inhibitory agent. Species...
Table 3: Antifungal activity of S. aromaticum EO express by diameter of inhibition.

| Yeast            | Oils 5 µl/disc | Oils 10 µl/disc | Oils 15 µl/disc | Fluconazole 10 µl/disc | Terbinafine 10 µl/disc |
|------------------|----------------|----------------|----------------|------------------------|------------------------|
| C. Albicans      | 22 ± 2         | 24.3 ± 1.1     | 26.6 ± 0.5     | 19 ± 1                 | 30 ± 1                 |
| C. Albicans      | 21.6 ± 2       | 24.6 ± 0.5     | 40 ± 1         | 26 ± 1                 | 21 ± 1                 |
| C. Albicans      | 27.3 ± 2.5     | 26 ± 0.5       | 39 ± 1         | 29 ± 1                 | 22 ± 1,7               |
| C. Albicans      | 28.3 ± 2.8     | 31.3 ± 1.5     | 40 ± 1         | 19 ± 2                 | 19.3 ± 0.5             |
| C. Albicans      | 25.3 ± 1.5     | 27.3 ± 0.5     | 29.3 ± 1.1     | 23.3 ± 0.5             | 20 ± 1,7               |
| C. Albicans      | 24.4 ± 2.0     | 27.3 ± 2       | 39.3 ± 1.1     | 16 ± 1                 | 20 ± 1                 |
| C. Tropicalis    | 21.3 ± 3       | 20 ± 1,1       | 23 ± 0.5       | 17.3 ± 0.5             | 27 ± 0.5               |
| C. Tropicalis    | 22 ± 2.6       | 27 ± 1         | 29 ± 1.7       | 20 ± 1                 | 24.6 ± 0.5             |
| C. Tropicalis    | 32 ± 6.8       | 43 ± 1.7       | 44 ± 1         | 30 ± 2                 | 33.7 ± 1.5             |
| C. Tropicalis    | 27.3 ± 2.5     | 29 ± 1         | 35 ± 1.5       | 26.3 ± 1.5             | 30 ± 1                 |
| C. Tropicalis    | 25 ± 1.5       | 26 ± 0.5       | 29 ± 1.5       | 23.3 ± 1               | 28 ± 1                 |
| C. Tropicalis    | 22 ± 1         | 26 ± 0.5       | 29 ± 1.5       | 27 ± 1,7               | 30 ± 1                 |
| C. Glabrata      | 27 ± 2.5       | 22 ± 1         | 29.7 ± 0.5     | 22.6 ± 1.5             | 29 ± 1                 |
| C. Glabrata      | 22 ± 3         | 25 ± 1         | 30 ± 1         | 22.6 ± 0.5             | 28 ± 1,1               |
| C. Glabrata      | 24 ± 1         | 27 ± 1         | 27 ± 1.5       | 30 ± 1                 | 28 ± 1                 |
| C. Parapsilosis  | 26 ± 1,7       | 27 ± 1         | 31 ± 1.5       | 28.6 ± 0.5             | 30 ± 0.5               |
| C. Parapsilosis  | 39 ± 1.15      | 39 ± 1.7       | 44 ± 1.5       | 30 ± 1                 | 35 ± 0.5               |

The values shown in the table are the average of three measurements. Mean ±RSD

Table 4: Antibacterial activity of S. aromaticum essential oils express by diameter of inhibition.

| Bacteria                     | Oils 5 µl/disc | Oils 10 µl/disc | Oils 15 µl/disc | Gentamicin 10 µg/disc | Gentamicin 15 µg/disc |
|------------------------------|----------------|----------------|----------------|-----------------------|----------------------|
| Staphylococcus Meti-S1       | 0              | 11 ± 0,5       | 13 ± 1         | 15 ± 1                | 25 ± 0,5             |
| Staphylococcus Meti-S2       | 11 ± 1         | 15 ± 0,5       | 16 ± 0,5       | 26 ± 0,5              | 28 ± 1               |
| Staphylococcus epidermidis   | 16 ± 0,5       | 25 ± 0,5       | 25 ± 0,5       | 30 ± 0,5              | 31 ± 1               |
| Multi-resistant Pseudomonas aeruginosa | 0             | 10 ± 0,5       | 12 ± 3         | 20 ± 0,5              |

The values shown in the table are the average of three measurements. Mean ±RSD

of the genus *candida sp* which also pose resistance problems to reference antifungals (Ahmad *et al.,* 2005; de Oliveira *et al.,* 2009).

A mild to moderate antimicrobial activity observed with a maximum inhibition diameter of 20 observed on *Staphylococcus epidermidis Meti R* at 10 µl of EO, the slight effect was observed on *Pseudomonas aeruginosa* (Table 4). By comparing the average growth inhibition diameters of *Staphylococcus Meti-S1*, *Staphylococcus Meti-S 2* and *Pseudomas aeruginosa* strains with that of gentamicin, the difference is statistically significant. For *Staphylococcus epidermidis Multi-resistant* the differences are not significant (p> 0.05).

In another study who mentioned that the EO of *Syzygium aromaticum* exhibited antibacterial activity against a large number of *methicillin-resistant S. epidermidis* and *S. aureus*, (Palombo and Semple, 2002) this result are in agreement our results. The oil also appears to be effective against both Gram-positive and Gram-negative microorganisms (Zaika, 2007; Smith-Palmer *et al.,* 1998).

In previous studies the antibacterial effect of EO of *S. aromaticum* has been demonstrated against pathogenies bacteria, such as *Camylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus*. (Burt and Reinders, 2003; Feres *et al.,* 2005).
According to previous study, the activities proven are related to the importance of the phenolic hydroxyl groups (Aligiannis et al., 2001; Nostro et al., 2004).

CONCLUSIONS

Clove oil proved to be an effective antioxidant by comparing the results obtained with that of the reference antioxidant. This EO oil may be employed to reduce or prevent the oxidation of lipids in foods products and in particular in pharmaceuticals.

The inhibitory power of fungal species growth and bacterial resistant species has shown us that EOs of Syzygium aromaticum may be an effective alternative to conventional antifungals.

The findings of result valorize Moroccan S. aromaticum as an endemic medicinal plant which can be employed a source of biological active compounds.

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