



Original Research Article

Combined effect of essential oils from Clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry), Thyme (*Thymus vulgaris* L.) and Lemon peel (*Citrus limon* (L.) Osbeck) on anti-bacterial, cytotoxic and anti-inflammatory activities

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ABSTRACT

Essential oils are natural products composed of a mixture of volatile and aromatic compounds extracted from different organs of plants that have been widely studied for their antibacterial activities against pathogens. In this study, clove, lemon peel and thyme essential oils and their mixture were assessed for their antimicrobial activities using a panel of pathogenic Gram-positive, and Gram-negative, strains. Cytotoxicity and anti-inflammatory activity were also evaluated. Lemon peel essential oil was characterized by the predominance of limonene. Eugenol was the main component in clove essential oil and thymol in thyme essential oil. Clove, lemon peel and thyme essential oils and their combination had potent antibacterial, cytotoxic and anti-inflammatory activities. This study demonstrates that the use of essential oils is an effective alternative for pathogenic bacterial control, alone or in combination with antibiotic therapy.

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1. Introduction

The use of combinations of essential oils and their isolated components are thus new approaches to increase the efficacy of EOs in food, pharmaceutical and cosmetic industries, taking advantage of their synergistic and additive effects (Bassolé and Juliani, 2012). Essential oils, also called volatile oils, have been shown to possess antibacterial, antifungal, anti-inflammatory and cytotoxic properties (Lorenzo-Leal et al., 2019). Some essential oils have been used in cancer treatment (Sylvestre et al., 2006), while others have been used in food preservation (Faid et al., 1995), aromatherapy (Buttner et al., 1996) and fragrance industries (Van de Braak and Leijten, 1999). Essential oils, or plant essences, are volatile and fragrant substances with an oily consistency typically produced by plants. They are synthesized by all plant organs, namely buds, flowers, leaves, stems, twigs, seeds, fruits,

roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008).

Thyme (*Thymus vulgaris* L.), member of the Lamiaceae family, is widely used in folk medicine for some valuable therapeutic properties, e.g., its expectorant, antitussive, antibronchitic, antispasmodic, anthelmintic, carminative and diuretic properties and antimicrobial activities (Imelouane et al., 2009). Thyme essential oil is composed of the chemical compounds α -pinene, thymol, caryophyllene (Al-Asmari et al., 2017) and carvacrol, depending on chemotype (Benzie and Strain, 1996; Mancini et al., 2015). Thyme essential oils had some anti-inflammatory and hepatoprotective properties owing to the presence of bioactive compounds, especially carvacrol and thymol (Mastelić et al., 2008). Lemon (*Citrus limon* (L.) Osbeck) is a small thorn bearing tree which belongs to the family Rutaceae. *Citrus* essential oils mainly exist in fruit peels which are usually

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discarded as waste. Thus, citrus essential oil could be manufactured at a more affordable price than plant essential oils (Tirado et al., 1995). Essential oils, also known as volatile oils, are complex mixtures of volatile constituents that are being released upon applying heat and could be considered as being used in the classical hydro-distillation, steam distillation, and those based on the application of microwave beams, e.g., microwave-assisted hydrodistillation or solvent-free microwave extraction (Mohammadhosseini et al., 2015; 2017; Wansi et al., 2018; 2019). EOs are distinguished for their medicinal properties and their fragrance since middle ages. In fact, they are used in food preservation, antimicrobial, perfumes, analgesic, sedative, anti-inflammatory, spasmolytic and medicinal uses (Bakkali et al., 2007). Limonene was the predominant compound of lemon peel essential oil (Bourgou et al., 2012).

Clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry), is a dried flower bud belonging to the Myrtaceae family considered as one of the most valuable spice. Clove essential is active against oral bacteria associated with dental caries and periodontal disease (Cai and Wu, 1996) and effective against a large number of other bacteria (Friedman et al., 2002). Previous studies have reported antifungal (Chami et al., 2005), anticarcinogenic (Zheng et al., 1992), and anti-mutagenic (Miyazawa and Hisama, 2001) and anti-inflammatory (Bachiega et al., 2012) activities which are attributed to the presence of eugenol as its major constituent (Cortés-Rojas et al., 2014).

Interestingly, phenolic monoterpenes (thymol and carvacrol) and phenylpropanoids (eugenol) in combination with other components were found to increase the bioactivities of these mixtures. Most of the studies have focused on the interaction of phenolic monoterpenes and phenylpropanoids with other groups of components, particularly with other phenols, phenylpropanoids and monoterpenes alcohols, while monoterpenes and sesquiterpenes hydrocarbons were used to a lesser extent (Bassolé and Juliani, 2012). The EOs of clove, lemon peel and thyme were well studied for their antioxidant, antibacterial and anti-inflammatory capacities owing to their richness in bioactive compounds as thymol (thyme), eugenol (clove) and 1,8-cineole (lemon peel). Research regarding the bioactivity effects of lemon peel, clove and thyme EOs in design mixture is still limited. Thus, the aim of the present study was to evaluate the antibacterial, cytotoxic and anti-inflammatory effects of clove, lemon peel and thyme essential oils and their combination.

2. Experimental

2.1. Plant material

In this investigation, three aromatic and medicinal plants were studied; clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry), thyme (*Thymus vulgaris* L.) and lemon (*Citrus limon* (L.) Osbeck). Tunisian clove was purchased from local farm market (Kef, Northwestern Tunisia) in the form of dried flower buds, and they were

stored in a dry, tightly closed bottle. 2 kg of thyme aerial parts were collected in March 2021 from Bou-kornine mountain (Northern Tunisia; Altitude 576 m; Latitude: 36°70'516" North; Longitude: 10°33'300" East). 2 kg of Lemon fruits were obtained in January 2021 from lemon tree variety 'Eureka' grown at agricultural land in Korba (Northern Tunisia; Altitude 15 m; Latitude: 36°34'42" North; Longitude: 10°51'30" East). The identification of the three species was carried out by the botanist Abderrazzak Smaoui in Biotechnologic Center of Borj-Cedria (Tunisia). Voucher specimens were deposited in the herbarium of our laboratory (*Syzygium aromaticum* Sa-LPAM-2021; *Citrus limon* CI-LPAM-2021; *Thymus vulgaris* Tv-LPAM-2021). Fresh thyme leaves and lemon peel were left to dry at ambient temperature for eight days until reaching a constant mass of the plant materials.

2.2. Essential oil extraction

2.2.1 Essential oil extraction of thyme leaf and lemon peel by Clevenger

The apparatus used for hydro distillation is of the Clevenger type Clevenger apparatus known for extracting more essential oil quantity compared to the other extraction methods. 500 g of plant material were subjected to hydrodistillation in 1500 mL of distilled water for 3 h. The condensed vapor obtained leads to the essential oil which is separated from the hydrolate (aromatic waters) by decantation after adding magnesium sulfate ($MgSO_4$) to remove traces of water. The essential oils are collected directly, using a pasteur pipette over the distillate without adding any solvent. The quantity of essential oil obtained is weighed to calculate the yield and then stored in opaque bottles at 4 °C (Zaouali et al., 2010).

2.2.2 Extraction of clove essential oil by distillation

500 g of cloves had been soaked in 4 L of distilled water in a stainless steel still, then the still was heated under pressure to bring its contents to a boil for 3 h. After condensation, the essential oil separated from the distillate by decantation after adding magnesium sulfate ($MgSO_4$) to remove traces of water. The quantity of essential oil obtained was weighed to calculate the yield and then stored in opaque bottles at 4 °C.

2.2.3. Essential oil mixture

The optimized blended essential oil made with clove, lemon peel and thyme essential oils respecting the proportions proposed by the following mixture design: 25.7% thyme essential oil and 32.3% lemon peel essential oil and 41.9% clove essential oil equivalent to 15.42 mg, 19.38 mg and 25.14 mg, respectively.

2.3. Chromatographic analysis characterization of essential oils

2.3.1. GC-FID quantification method

The analysis was carried out on Hewlett-Packard 6890 chromatograph equipped with an electronic pressure control injector, a flame ionization detector and an HP Innowax (polyethylene glycol capillary) column (30 m x 0.25 mm; 0.25 μ m). The flow of the carrier gas (N_2) was 1.6 mL/min and the split ratio was 60:1. The analysis was carried out using the following temperature program: oven temperature; isotherm at 35 °C for 10 min, from 35 to 205 °C at the rate of 2 °C/min and isotherm at 205 °C during 10 min. Injector and detector temperature were held, respectively, at 250 and 300 °C. The injection volume was 1 μ L. The quantification of each essential oil was done by the co-injection of external standard method using calibration curves generated by running GC analysis of representative compounds.

2.3.2. Identification of volatile compounds by GC/MS

The GC/MS coupling made it possible to identify volatile compounds. The principle is based on the fragmentation of compounds following their bombardment by a flow of electrons and their exposure to electric fields. The released ions will be classified according to their mass/charge ratio (m/z). The analysis is carried out by a chromatograph coupled to an Agilent mass spectrometer (5975C inert XL MSD) and electron impact ionization (70 eV). An HP-5MS capillary column (30 m x 0.25 mm, 0.25 μ m film thickness) coated with 5 % phenyl methyl silicone and 95 % dimethylpolysiloxane was used. The oven temperature was programmed at 40 °C for 1 min and then rise from 40 to 100 °C at a rate of 8 °C/min and kept constant at 100 °C for 5 min. After that, the temperature was heated to 200 °C with a rate of 10 °C/min and kept constant at 200 °C for 3 min and the final temperature was set up at 300 °C with a rate of 2 °C/min. Injector temperature was set at 250 °C. The carrier gas was helium with a flow rate of 1 mL/min; the split ratio was 100:1. Scan time and mass ranges were 1 s and 50-550 m/z, respectively. Individual peaks corresponding to the volatile components were identified by comparison of their retention indices (RI) relative to (C_8 - C_{40}) *n*-alkanes with those of literature or with those of authentic compounds available in the authors' laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley 09 NIST 2011 mass spectral library of the GC/MS data system.

2.4. Antibacterial activity by using the disk distribution method

The antimicrobial activity of the essential oils obtained was carried out by the disk diffusion method according to Yeddes et al. (2019) with a slight modification. Antimicrobial activity was tested on strains on Gram negative strains such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus aerogenes* (ATCC 13048) as well as Gram positive strains such as *Bacillus subtilis* (ATCC 6051), *Enterococcus*

faecalis (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213). The surface of the Mueller-Hinton Agar (Merck) plates was inoculated with 0.1 mL of bacterial culture suspension (10^5 CFU/mL). Then, 6 mm diameter sterile filter paper discs were placed on the surface of the plates and 10 μ L of essential oil was added to the discs. All the plates were incubated for 24 h at a temperature of 37 °C. The diameter of the inhibition zone (mm) was measured taking into account the initial diameter of the discs. The sterile disc was used as a negative control and the antibiotic streptomycin (10 μ L/disc) was used as a positive control. The tests were carried out in triplicate.

2.5. Evaluation of the anti-inflammatory activity of essential oils

2.5.1. Cellular culture

The murine macrophage cell line RAW 264.7 (American Type Culture Collection), was cultured in flasks with RPMI 1640 medium (Dominique Dutscher; w/L-Glutamine), to which was added fetal bovine serum (FBS, 10%) (Dominique Dutscher; Origin South America) and antibiotics (penicillin 100 U/mL and streptomycin 100 μ g/mL). The cells were grown at 37 °C in a humid atmosphere containing CO_2 (5%). Before each test, the RAWs (in exponential growth phase) were seeded in 24-well plates at a density of 2×10^5 cells/well. They were then incubated for 24 hours to allow them to adhere (Medini et al., 2015).

2.5.2. Evaluation of the cytotoxicity of essential oils

In order to choose a range of non-toxic concentrations, the cytotoxicity of the essential oils was evaluated using the resazurin test developed by Medini et al. (2015). In short, the RAW 264.7 macrophages previously adhered in 24-well plates were treated for 24 hours with different increasing concentrations of each essential oils. After removing the supernatant, 1 mL of a resazurin solution (2%) in PBS (Dulbecco's Phosphate Buffered Saline, Dominique Dutscher) was added to each well. After 60 min incubation, fluorescence was measured, and cell viability was calculated against a control of untreated cells according to the following equation (Eqn. 1).

$$\% \text{ of inhibition} = \frac{\text{Fluorescence (Sample)}}{\text{Fluorescence (Control)}} \times 100 \text{ (Eqn. 1)}$$

2.5.3. Evaluation of the anti-inflammatory activity by nitrite assay

Among the molecular elements involved in inflammation, nitrogen monoxide (NO) plays an important role. Our study was based on evaluating the effect of essential oils on the level of nitrite released by cells (RAW 264.7). The macrophages, adhered to the 24-well plates, were treated with four increasing concentrations of each essential oil (25, 50 and 200 μ g/mL). After 1 hour of pretreatment, the cells were stimulated with LPS (1 μ g/mL) and incubated for 24 h at 37 °C. The supernatant was



then harvested and the amount of NO produced by the cells was estimated by a colorimetric assay using Griess reagent. This test is based on a diazotization in acidic medium nitrites form a diazonium salt with sulfanilic acid which is then coupled with an amine to give an azo dye which absorbs in 540 nm. In summary, 100 μ L of the cell supernatant was incubated with 100 μ L of Griess's reagent (0.8% sulfanilamide, 0.75% N-naphthylethylene diamine in 0.5 N HCl) at room temperature for 15 minutes. The absorbance at 540 nm was measured using Varioskan Flash plate reader (Thermo Scientific) and the presence of nitrite was quantified from a standard curve of NaNO₂. The percentage inhibition is calculated relative to a control treated with LPS only without essential oil. Each test was performed three times in triplicates (Medini et al., 2015).

3. Results and Discussion

3.1. Chemical composition of lemon peel, clove and thyme essential oils

The yields of lemon peel, clove and thyme EOs were 1.30, 5.11 and 1.25% respectively, based on dry weight of plant material. GC-MS analysis of lemon peel, clove and thyme EOs and their combination are given in Table 1. Twenty-one volatile compounds were identified in lemon peel representing 99.07% of essential oil, eight compounds in clove representing 99.92% of essential oil and sixteen compounds in thyme representing 99.99% of essential oil. For the combination, twenty-six compounds were identified having 99.27% of essential oil. Lemon peel EO was characterized by the predominance of limonene (71.81%). Eugenol (87.30%) was the main component in clove essential oil and thymol (78.54%) in thyme essential oil. In fact, the combined EO was mainly rich in eugenol (32.35%), thymol (25.49%) and limonene (21.30%). There were many other reports regarding the chemical composition of lemon peel and all these researchers confirmed that limonene was the main component ranging from 29.52 to 98.40% (Ayendoun and Sossou, 1996; Mahalwal and Ali, 2003; Yoo et al., 2004; Monajemi et al., 2005; Kamal et al., 2011; Jomaa et al., 2012; Bertuzzi et al., 2013; Wu et al. 2014; Hong et al., 2017; Himed et al., 2019; Kakoos, 2019). The major component of clove essential oil is usually considered eugenol (34.10-88.58%) as reported by Prashar et al. (2006), Chaieb et al. (2007), Pinto et al. (2009), Sulistyoningrum et al. (2017). There are also numerous studies reported the essential oil composition of thyme with thymol as the main constituent ranging from 22 to 71% (Hudaib et al., 2002; Zambonelli et al., 2004; Shabnum and Wagay, 2011; Kowalczyk et al., 2020).

3.2. Antibacterial activity

The antimicrobial activity of clove, lemon peel and thyme essential oils and their combination was achieved by the disk diffusion method of Yeddes et al. (2019). Results showed that clove, lemon peel and

thyme essential oils had a significant bactericidal power against, *E. coli*, *P. aeruginosa*, *E. aerogenes*, *B. subtilis*, *E. faecalis* and *S. aureus* (Fig. 1).

For *E. coli*; *P. aeruginosa*; *E. faecalis* and *B. subtilis*, the optimized essential oil mixture (ID = 36.08 mm) exhibited considerable bactericidal activity compared to that of clove (ID = 25.58 mm), lemon peel (ID = 32.75 mm) and thyme (ID = 33.25 mm). Likewise, the essential oil mixture also exhibited considerable bactericidal activity compared to streptomycin (positive control). These results showed that the chemical composition of essential oil mixture was qualitatively and quantitatively rich in active volatile molecules owing to the potent antibacterial activity of the product obtained against certain pathogenic strains. This can be mainly due to the synergy between the active molecules such as eugenol, thymol and limonene having high levels in the EO mixture (32.35%, 25.49% and 21.30%, respectively). Indeed, according to recent work, eugenol, thymol and limonene were found to be very active molecules and endowed with a considerable antimicrobial activity (Machese et al., 2017; De Arújo et al., 2020) and their synergistic effect results in the improvement of the antibacterial effect against some strains of Gram-negative and Gram-positive bacteria. In addition, according to a recent study by Marchese et al., (2017), the interaction of eugenol and thymol has been studied by several authors against Gram-negative and Gram-positive pathogens. Indeed, with regard to Gram-negative strains, the combination of eugenol and thymol showed synergistic effects on *E. coli*, and *P. aeruginosa*, and showed an indifferent effect against *E. aerogenes* (Bassolé et al., 2010). Additionally, Pei et al., (2009) reported that the combination of eugenol and thymol had a synergistic effect against *E. coli*.

3.3. Cytotoxicity and anti-inflammatory activity

The analysis of the cytotoxicity effect of clove, lemon peel and thyme essential oils and their combination showed no toxicity for the cells with a considerable viability of 85.71%, 99.73%, 93.64% and 90.48%, respectively for a dose between 50 μ g/mL and 100 μ g/mL (Table 2). Results confirmed that these bioactive essential oils and their combination had not toxic effect and they could be used to determine their anti-inflammatory activities (Kouidhi et al., 2010; kummer et al., 2013; Rodrigues et al., 2019).

In addition, the analysis of the results of anti-inflammatory activity of the studied essential oil samples showed that the blended essential oil exhibited a considerable anti-inflammatory activity (IC₅₀ = 23.236 μ g/mL). This anti-inflammatory activity was superior to that of individual essential oils from clove (IC₅₀ = 26.696 μ g/mL), lemon peel (IC₅₀ = 65.802 μ g/mL) and thyme (IC₅₀ = 31.644 μ g/mL) (Table 2).

This significant anti-inflammatory activity of the combined essential oil could be mainly due to the synergistic effect between its main bioactive compounds, as eugenol, thymol and limonene. According to Bassolé and Juliani (2012), phenolic monoterpenes (thymol

Table 1

Chemical composition of lemon peel, clove and thyme essential oils and their combination.

Sr.Num.	Volatile Compounds*	R1a	R1b	Lemon peel EO	Clove EO	Thyme EO	Combined EO
1	Tricyclene	919	929	0.02 ± 0.01	-	-	-
2	α-Thujene	923	836	0.34 ± 0.09	-	-	0.22 ± 0.01
3	α-Pinene	934	982	1.14 ± 0.62	-	1.07 ± 0.25	0.52 ± 0.01
4	β-Pinene	937	1113	0.63 ± 0.1	-	0.16 ± 0.03	2.12 ± 0.02
5	Camphene	952	1077	0.03 ± 0.01	-	0.31 ± 0.06	0.10 ± 0.01
6	Sabinene	983	1111	5.82 ± 0.12	-	-	1.28 ± 0.02
7	β-Myrcene	991	1168	0.99 ± 0.06	-	0.58 ± 0.17	0.41 ± 0.03
8	α-Terpinene	1018	1255	1.05 ± 0.04	-	0.91 ± 0.10	0.33 ± 0.02
9	p-Cymene	1026	1277	0.23 ± 0.02	-	7.13 ± 4.03	3.59 ± 0.02
10	Limonene	1030	1031	71.81 ± 4.43	-	-	21.30 ± 1.13
11	1,8-Cineole	1033	1214	-	0.03 ± 0.01	3.50 ± 0.50	0.09 ± 0.04
12	E-β-Ocimene	1052	1022	0.5 ± 0.01	-	-	0.13 ± 0.01
13	γ-Terpinene	1059	1262	9.96 ± 0.05	-	3.44 ± 0.41	2.89 ± 0.02
14	Linalool	1098	1551	0.65 ± 0.03	-	0.39 ± 0.02	0.28 ± 0.02
15	Borneol	1165	1642	2.13±0.16	-	0.85 ± 0.17	0.61 ± 0.01
16	Terpinen-4-ol	1178	1593	-	-	0.64 ± 0.11	0.37 ± 0.01
17	α-Terpineol	1185	1711	1.22 ± 0.12	-	0.14 ± 0.03	0.42 ± 0.03
18	Camphor	1192	1498	0.58 ± 0.02	-	-	0.06 ± 0.01
19	Thymol	1266	1263	-	-	78.54 ± 4.15	25.49 ± 0.03
20	Carvacrol	1278	1283	0.02 ± 0.01	-	0.18 ± 0.02	0.14 ± 0.03
21	Bornyl acetate	1295	1601	0.01 ± 0.01	-	-	-
22	Eugenol	1330	1329	-	87.3±1.07	-	32.35 ± 1.00
23	Geranyl acetate	1383	1599	0.56 ± 0.04	-	-	0.24 ± 0.01
24	Eugenol acetate	1387	1360	-	10.4±1.02	-	5.12 ± 0.01
25	(E)-Caryophyllene	1446	1608	-	1.35±0.10	1.58 ± 0.30	0.73 ± 0.03
26	Germacrene D	1480	1685	1.35 ± 0.04	0.14±0.01	-	-
27	α-Humulene	1485	1691	-	0.19±0.02	-	0.14 ± 0.01
28	Valencene	1495	1520	0.03 ± 0.01	-	-	0.12 ± 0.01
29	Caryophyllene oxide	1578	1699	-	0.20±0.01	0.57 ± 0.09	0.22 ± 0.03
30	Chavicol	1652	1701	-	0.31±0.02	-	-
Total				99.07 ± 0.83	99.92 ± 0.78	99.99 ± 0.71	98.18 ± 2.63

*Compounds in order of elution on an HP-5 MS. aKI: Kovats index calculated on an HP-5 MS column; bKI: Kovats index calculated on HP Innwax column; EO: Essential oil.

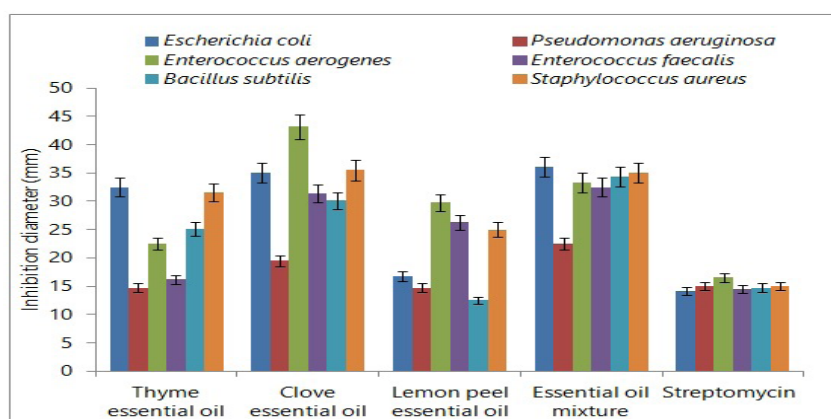


Fig. 1. Antibacterial activity of clove, thyme and lemon peel essential oil and their mixture. Values with the different letters (a-d) for each histogram color showed significant differences at $p < 0.05$.

Table 2

Cytotoxicity and anti-inflammatory activity of clove, thyme and lemon peel essential oil and their mixture.

a) Cytotoxicity evaluation					
Essential oil sample	Concentration ($\mu\text{g}/\text{mL}$)	Clove essential oil	Lemon essential oil	Thyme essential oil	Essential oil mixture
Viability (%)	3.25	93.33 \pm 0.90	-	-	103.70 \pm 1.00
	25	89.83 \pm 1.93	118.34 \pm 7.47	94.45 \pm 5.35	94.82 \pm 2.96
	50	85.71 \pm 1.96	93.07 \pm 7.54	93.64 \pm 3.22	90.48 \pm 2.07
	100	-	99.73 \pm 0.86	78.72 \pm 8.45	-
b) Anti-inflammatory activity evaluation					
IC ₅₀ ($\mu\text{g}/\text{mL}$)		26.69 \pm 0.60	65.80 \pm 0.85	31.64 \pm 0.89	23.23 \pm 0.23

and carvacrol) and phenylpropanoids (eugenol) in combination with other components were found to increase the bioactivities of these mixtures. Most of the studies have focused on the interaction of phenolic monoterpenes and phenylpropanoids with other groups of components, particularly with other phenols, phenylpropanoids and monoterpenes alcohols, while monoterpenes and sesquiterpenes hydrocarbons were used to a lesser extent. On the other hand, Lee et al. (2011) and Sumiwi et al. (2015) reported that eugenol was endowed with a considerable anti-inflammatory activity manifested by inhibition of cyclooxygenase 2 (COX-2) of the order of 58.15% (IC₅₀ = 8.85 mg/mL). Andrade and De Sousa (2013) demonstrated that thymol exhibited a considerable anti-inflammatory activity in vitro through the inhibitory power of cyclooxygenase 1 and cyclooxygenase 2 which catalyzed the synthesis of chemical mediators of inflammation. Yoon et al. (2010) and Rahman et al. (2014) found that limonene was characterized by a considerable anti-inflammatory activity through the inhibition of prostaglandin E2 production. Indeed, the inhibition of inflammation by these several bioactive compounds via the inhibition of these enzymes cyclooxygenase 1, cyclooxygenase 2 or prostaglandin E2 are responsible for the synthesis of the various chemical mediators involved in the information transmission between cells and which are at the origin of inflammatory processes.

4. Concluding remarks

The combination of clove, lemon peel and thyme essential oils enhanced the antibacterial, cytotoxic and anti-inflammatory activities. Lemon peel essential oil was characterized by the predominance of limonene. Eugenol was the main component in clove essential oil and thymol in thyme essential oil. These results represented a basis for further studies that could lead to the development of a new treatment based on the combination of these essential oils as natural bioactive products, both in food, cosmetic and therapeutical fields.

References

- Al-Asmari, A.K., Athar, M.T., Al-Faraidy, A.A., Almuhaiza, M.S., 2017. Chemical composition of essential oil of *Thymus vulgaris* collected from Saudi Arabian market. *Asian Pac. J. Trop. Biomed.* 7(2), 147-150.
- Andrade, L.N., de Sousa, D.P., 2013. A review on anti-inflammatory activity of monoterpenes. *Molecules* 1(18), 1227-1254.
- Ayedoun, A.M., Sossou, P.V., Mardarowicz, M., Leclercq, P.A., 1996. Volatile constituents of the peel and leaf oils of *Citrus limon* L. Burm. f. from Benin. *J. Essent. Oil Res.* 8(4), 441-444.
- Bachiega, T.F., de Sousa, J.P.B., Bastoss, J.K., Sforcin, J.M., 2012. Clove and eugenol in noncytotoxic concentrations exert immunomodulatory/anti-inflammatory action on cytokine production by murine macrophages. *J. Pharm. Pharmacol.* 64, 610-616.
- Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2007. Biological effects of essential oils- A review. *Food Chem. Toxicol.* 46, 446-475.
- Bassolé, I.H.N., Juliani, H.R., 2012. Essential oils in combination and their antimicrobial properties. *Molecules* 17, 3989-4006.
- Bassolé, I.H.N., Lamien-Meda, A., Bayala, B., Tirogo, S., Franz, C., Novak, J., Nebié, R.C., Dicko, M.H., 2010. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha x piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. *Molecules* 11(15), 7825-7839.
- Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* 239, 70-76.
- Bertuzzi, G., Tirillini, B., Angelini, P., Venanzoni, R., 2013. Antioxidative action of *Citrus limonum* essential oil on skin. *Eur. J. Med. Plant.* 3(1), 1-9.
- Bourgou, S., Rahali Fatma, Z., Ourghemmi, I., Saidani Tounsi, M., 2012. Changes of peel essential oil composition of four Tunisian citrus during fruit maturation. *Sci. World J.* 2012, 528593.
- Buttner, M.P., Willeke, K., Grinshpun, S.A., 1996. Sampling and analysis of airborne microorganisms. In *Manual Environment. Microbiol.* Edited by: Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stezenbach, L.D., Walter, M.V., ASM Press: Washington DC. Pp. 629-640.



- Cai, L., Wu, C.D., 1996. Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. *J. Nat. Prod.* 59, 987-990.
- Chaieb, K., Hajlaoui, H., Zmantar, T., Ben Kahla-Nakbi, A., Rouabhia, M., Mahdouani, K., Bakhrouf, A., 2007. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzygium aromaticum* L. Myrtaceae), A short review. *Phytother. Res.* 21(6), 501-506.
- Chami, F., Chami, N., Bennis, S., Bouchikhi, T., Remmal, A., 2005. Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytother. Res.* 19, 405-408.
- Cortés-Rojas, D.F., de Souza, C.R., Oliveira, W.P., 2014. Clove (*Syzygium aromaticum*), A precious spice. *Asian Pac. J. Trop. Med.* 4, 90-96.
- De Arújo, A.C.J., Freitas, P.R., Dos Santos Barbosa, C.R., Muniz, D.F., Rocha, J.E., Albuquerque da Silva, A.C., de Morais Oliveira-Tintino, C.D., Ribeiro-Filho, J., da Silva, L.E., Confortin, C., do Amaral W., Deschamps, C., Barbosa-Filho, J.M., de Lima, N.T.R., Tintino, S.R., Coutinho, H.D.M., 2020. GC-MS-FID characterization and antibacterial activity of the *Mikania cordifolia* essential oil and limonene against MDR strains. *Food Chem. Toxicol.* 136, 111023.
- Faid, M., Bakhy, K., Anshad, M., Tantaoui-Elaraki, A., 1995. Alomond paste: Physicochemical and microbial characterizations and preservation with sorbic acid and cinnamon. *J. Food Prod.* 58, 547-550.
- Himed, L., Merniz S., Monteagudo-Olivan, R., Barkat, M., Coronas, J., 2019. Antioxidant activity of the essential oil of *Citrus limon* before and after its encapsulation in amorphous SiO₂. *Sci. Afr.* 6, e00181.
- Hong, J.H., Khan, N., Jamila, N., Hong, Y.S., Nho, E.Y., Choi, J.Y., Lee, C.M., Kim, K.S., 2017. Determination of volatile flavour profiles of *Citrus* spp. fruits by SDE-GC-MS and enantiomeric composition of chiral compounds by MDGC-MS. *Phytochem. Anal.* 28, 392-403.
- Hudaib, M., Speroni, E., Di Pietra, A.M., Cavrini, V., 2002. GC/MS evaluation of thyme (*Thymus vulgaris* L.) oil composition and variations during the vegetative cycle. *J. Pharmaceut. Biomed. Anal.* 29(4), 691-700.
- Imelouane, B., Amhamdi, H., Wathelet, J.P., Ankit, M., Khedid, K., El Bachiri, A., 2009. Chemical composition and antimicrobial activity of essential oil of thyme (*Thymus vulgaris*) from Eastern Morocco. *Int. J. Agric. Biol.* 11(2), 205-208.
- Jomaa, S., Rahmo, A., Alnori, A.S., Chatty, M.E., 2012. The cytotoxic effect of essential oil of Syrian Citrus limon peel on human colorectal carcinoma cell line (Lim1863). *Middle East J. Cancer.* 3(1), 15-21.
- Kamal, G.M., Anwar, F., Hussain, A.I., Sarri, N., Ashraf, M.Y., 2011. Yield and chemical composition of *Citrus* essential oils as affected by drying pretreatment of peels. *Int. Food Res. J.* 18(4), 1275-1282.
- Kaskoos, R.A., 2019. Essential oil analysis by GC-MS and analgesic activity of *Lippia citriodora* and *Citrus limon*. *J. Essent. Oil-Bear. Plants* 22, 273-281.
- Kouidhi, B., Zmantar, T., Bakhrouf, A., 2010. Anticariogenic and cytotoxic activity of clove essential oil (*Eugenia caryophyllata*) against a large number of oral pathogens. *Ann. Microbiol.* 60(4), 599-604.
- Kowalczyk, A., Przychodna, M., Sopata, S., Bodalska, A., Fecka, I., 2020. Thymol and thyme essential oil-new insights into selected therapeutic applications. *Molecules* 25(18), 4125.
- Kummer, R., Fachini-queiroz, F.C., Estevão-Silva, C.F., Grespan, R., Leite Silva, E., Bersani-Amado, C.A., Cuman R.K.N., 2013. Evaluation of anti-inflammatory activity of *Citrus latifolia* Tanaka essential oil and limonene in experimental mouse models. *Evid. Based Complement. Alternat. Med.* 2013, 1-8.
- Lee, J.Y., Park, W., 2011. Anti-inflammatory effect of myristicin on RAW 264.7 macrophages stimulated with polyinosinic-polycytidylic acid. *Molecules* 8(16), 7132-7142.
- Lorenzo-Leal, A.C., Palou, E., López-Malo, A., Bach, H., 2019. Antimicrobial, cytotoxic, and anti-inflammatory activities of *Pimenta dioica* and *Rosmarinus officinalis* essential oils. *Biomed Res. Int.* 2019, 1639726.
- Mahalwal, V.S., Ali, M., 2003. Volatile constituents of the fruits peel of *Citrus lemon* (Linn) Burm. *F. J. Essent. Oil-Bear. Plants* 6(1), 31-35.
- Mancini, E., Senatore, F., Del Monte, D., De Martino, L., Grulova, D., Scognamiglio, M., Snoussi, M., De Feo, V., 2015. Studies on chemical composition, antimicrobial and antioxidant activities of five *Thymus vulgaris* L. essential oils. *Molecules* 20, 12016-12028.
- Marchese, A., Barbieri, R., Coppo, E., Erdogan Orhan, I., Daglia, M., Nabavi, S.F., Izadi, M., Abdollahi, M., Nabavi, S. M., Ajami, M., 2017. Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. *Crit. Rev. Microbiol.* 6(43), 668-689.
- Mastelić, J., Jerković, I., Blazević, I., Poljak-Blazi, M., Borović, S., Ivancić-Baće, I., Smrecki, V., Zarković, N., Brcić-Kostić, K., Vikić-Topić, D., Müller, N., 2008. A comparative study on the antioxidant and biological activities of carvacrol, thymol and eugenol derivatives. *J. Agric. Food Chem.* 56, 3989-3996.
- Medini, F., Bourgou, S., Lalancette, K. Snoussi, M., Mkadmini, K., Coté, I., Abdelly C., Legault, J., Ksouri, R., 2015. Phytochemical analysis, antioxidant, anti-inflammatory, and anticancer activities of the halophyte *Limonium densiflorum* extracts on human cell lines and murine macrophages. *S. Afr. J. Bot.* 99, 158-164.
- Miyazawa, M., Hisama, M., 2001. Suppression of chemical mutagen-induced SOS response by alkylphenols from clove (*Syzygium aromaticum*) in the *Salmonella typhimurium* TA1535/pSK1002 umu test. *J. Agric. Food Chem.* 49, 4019-4025.
- Mohammadhosseini, M., 2017. Essential oils extracted using microwave-assisted hydrodistillation from aerial parts of eleven *Artemisia* species: Chemical compositions and diversities in different geographical regions of Iran. *Rec. Nat. Prod.* 11(2), 114-129.
- Mohammadhosseini, M., Akbarzadeh, A., Flamini, G., 2017. Profiling of compositions of essential oils and volatiles of *Salvia limbata* using traditional and advanced techniques and evaluation for biological activities of their extracts. *Chem. Biodivers.* 14(5), e1600361.
- Mohammadhosseini, M., Mahdavi, B., Shahnama, M., 2015. Chemical composition of essential oils from aerial

- parts of *Ferula gummosa* (Apiaceae) in Jajarm Region, Iran using traditional hydrodistillation and solvent-free microwave extraction methods: A comparative approach. *J. Essent. Oil-Bear. Plants* 18(6), 1321-1328.
- Monajemi, R., Oryan, S., Haeri, R.S.A., Ghannadi, A.R., Jafarian, D.A., 2005. Cytotoxic effects of essential oils of some Iranian citrus peels. *Iranian J. Pharm. Res.* 4(3), 183-187.
- Pei, R.S., Zhou, F., Ji, B.P., Xu, J., 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Si.* 7(74), 379-383.
- Pinto, E., Vale-Silva, L., Cavaleiro, C., Salgueiro, L., 2009. Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and *dermatophyte* species. *J. Med. Microbiol.* 58, 1454-1462.
- Prashar, A., Locke, I.C., Evans, C.S., 2006. Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. *Cell Prolif.* 39, 241-248.
- Rehman, M.U., Tahir, M., Khan, A.Q., Khan, R., Hamiza, O.O., Lateef, A., Hassan, S.K., Rashid, S., Ali, N., Zeeshan, M., Sultana, S., 2014. D-Limonene suppresses doxorubicin-induced oxidative stress and inflammation via repression of COX-2, iNOS, and NFκB in kidneys of Wistar rats. *Experiment. Biol. Med.* 4(239), 465-476.
- Rodrigues, V., Cabral, C., Evora, L., Ferreira, I., Cavaleiro, C., Cruz, M.T., Salgueiro, L., 2019. Chemical composition, anti-inflammatory activity and cytotoxicity of *Thymus zygis* L. subsp. *sylvestris* (Hoffmanns. & Link) Cout. essential oil and its main compounds. *Arab. J. Chem.* 12(8), 3236-3243.
- Shabnum, S., Wagay, M.G., 2011. Essential oil composition of *Thymus vulgaris* L. and their uses. *J. Res. Develop.* 11, 83-94.
- Sulistyoningrum, A.S., Saepudin, E., Cahyana, A.H., Rahayu, D.U.C., Amelia, B., Haib, J., 2017. Chemical profiling of clove bud oil (*Syzygium aromaticum*) from Toli-Toli and Bali by GC-MS analysis. *AIP Conference Proceed.* 1862, 030089.
- Sumiwi, S., Sihombing, O.S., Subarnas, A., Abdassah, M., Levita, J., 2015. A study to predict anti-inflammatory activity of eugenol, myristicin, and limonene of *Cinnamomum sintoc*. *Int. J. Pharm. Pharmaceut. Sci.* 12(7), 51-54.
- Tirado, C.B., Stashenko, E.E., Combariza, M.Y., Martinez, J.R., 1995. Comparative study of Colombian citrus oils by high-resolution gas chromatography and gas chromatography-mass spectrometry. *J. Chromatogr. A*, 697, 501e513.
- Van de Braak, S.A.A.J., Leijten, G.C.J.J., 1999. Essential Oils and Oleoresins: A Survey in the Netherlands and Other Major Markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Rotterdam. p.116.
- Wansi, J.D., Sewald, N., Nahar, L., Martin, C., Sarker, S.D., 2018. Bioactive essential oils from the Cameroonian rain forest: A review - Part I. *Trends Phytochem. Res.* 2(4), 187-234.
- Wansi, J.D., Sewald, N., Nahar, L., Martin, C., Sarker, S.D., 2019. Bioactive essential oils from the Cameroonian rain forest: A review - Part II. *Trends Phytochem. Res.* 3(1), 3-52.
- Wu, P.S., Kuo, Y.T., Chen, S.M., Li, Y., Lou, B.S., 2014. Gas chromatography-mass spectrometry analysis of photosensitive characteristics in citrus and herb essential oils. *J. Chromatogr. Sep. Tech.* 6(1), 1-9, doi:10.4172/2157-7064.1000261.
- Yeddes, W., Nowacka, M., Rybak, K., Younes, I., Hammami, M., Saidani-Tounsi, M., Witrowa-Rajchert, D., 2019. Evaluation of the antioxidant and antimicrobial activity of rosemary essential oils as gelatin edible film component. *Food Sci. Technol. Res.* 2(25), 321-329.
- Yoo, Z.W., Kim, N.S., Lee, D.S., 2004. Comparative analyses of the flavors from Hallabong (*Citrus sphaerocarpa*) with lemon, orange and grapefruit by SPTe and HS-SPME combined with GC-MS. *Bull. Korean Chem. Soc.* 25(2), 271-279.
- Yoon, W.J., Lee, N.H., Hyun, C.G., 2010. Limonene suppresses lipopolysaccharide-induced production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines in RAW 264.7 macrophages. *J. Oleo Sci.* 8(59), 415-421.
- Zambonelli, A., D'Aulerio, A.Z., Severi, A., Benvenuti, S., Maggi, L. Bianchi, A., 2004. Chemical composition and fungicidal activity of commercial essential oils of *Thymus vulgaris* L. *J. Essent. Oil Res.* 16, 69-74.
- Zaouali, Y., Bouzaine, T., Boussaid, M., 2010. Essential oils composition in two *Rosmarinus officinalis* L. varieties and incidence for antimicrobial and antioxidant activities. *Food Chem. Toxicol.* 11(48), 3144-3152.
- Zheng, G.Q., Kenney, P.M., Lam, L.K., 1992. Sesquiterpenes from clove (*Eugenia caryophyllata*) as potential anticarcinogenic agents. *J. Nat. Prod.* 55, 999-1003.