Phytochemical study and evaluation of the biological activities of essential oils from aromatic plants (*Citrus Limon, Salvia officinalis and Cymbopogon citratus*): formulation of food detergent-disinfectant

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Abstract— The complex chemical composition of essential oils (EO) attributes very interesting and highly appreciated biological activities. Three evaluated EOs of aromatic plants (*citrus limon, Salvia officinalis* and *Cymbopogon citratus*) by Two methods (hydro and steam) distillation with yields between 0.20 and 0.84%. Majority composition of mono terpenes, hydrocarbons and terpene alcohols was detected. Antibacterial and antifungal activities was detected on the strains studied with a concentration of 0.5% of the OE of limon and Silvia and 0.1% for citratus. By the DPPH method, the antioxidant activity of the EOs of Silvia and citratus showed a significant antioxidant effect and good insecticide effect. The mean idea is the formulation of food detergent-disinfectant (FDD) by mixture design methodology (DOE) using Minitab 17. Five products are effective and meet the requirements of current quality standards for FDD with proved effectiveness by surface test

Index Terms— essential oils, *Citrus limon, Salvia officinalis, Cymbopogon citratus*, food disinfectant detergent, DOE.

1 INTRODUCTION

THE discovery and adoption of chemicals for hygiene has led to a wide variety of cleaning and disinfection products. The long-term use of these products presents a danger for humans and the environment because they are not biodegradable. In addition, the rules of organic production, including the non-use of chemicals, as well as the obligation of cleaning and disinfection in accordance with the principles and standards of the organic world. This has led us to look for specific alternatives, in particular those that preserve a healthy, biodegradable and non-toxic environment.

Nowadays, essential oils are of interest to more and more demand by chemists and biologists. Essential oils, with their broad spectrum of action for a large number of bacterial, fungal and insect species, are a very promising alternative, without being a source of danger for human health or pollution for the environment [1]. In addition, it is considered as the best source of fragrances and aromas [2]. It is in this perspective and in order to find a solution to this invasion of chemicals and to meet the requirements of rigorous principles and standards of the organic world, we are interested in the study of essential oils from three organic plants (*Citrus limon, Salvia officinalis and Cymbopogon citratus*).

Our research is based on three objectives:

- Know the photochemical properties and the biological activities of essential oils (EO) extracted from the plants examined,
- Apply the results obtained by integrating the EO into the formulation of an ecological Food Detergent-Disinfectant (FDE) using a mixture formulation supported by a well-studied DOE experiment plan, provided by the Minitab 17 software.
- Finally, study the physicochemical and microbiological stability of the product and apply it to cleaning as well as the study of the evaluation of its effectiveness.

2. MATERIALS AND METHODS

2.1. The studied species

We worked on three different plants (fig. 1).

Many researchers proved they contain valuable compounds such as essential oil.

Citrus limon zest; Leaves and flowers of *Salvia Officinalis;* Aerial part of *Cymbopogon citratus*.

Fig. 1: The three studied species (A, B, C) used in the extraction of essential oils.

2.2. Essential oil extraction

For the extraction of EO, two methods are applied in this work: A Hydrodistillation was established using a Clevenger type device. Formation of water vapor, by an assembly that helps us recover the plant's EO from plant material by passing a stream of water vapor through the plant sample. The distillate separates by unequal density into two immiscible phases. No organic solvent is used during this protocol. The essential oil is collected in opaque bottles, which is stored in the refrigerator at 4°C and well protected from light.

2.3. Yield estimation and physicochemical characterization

The yield is defined as the ratio between the mass of essential oil obtained and the mass of plant material used.

The physicochemical characterization (odor, color and appearance) is carried out using a recognition of specialist in sensory analyzes, of the refractive index obtained with a refractometer and of the acid index through an experimental measurement in triplicate.

2.4. GC chromatographic analysis

A perfect knowledge of the chemical composition of essential oils (EO) is necessary to highlight a possible local specificity for its characterization and to assess their quality. In order to identify the main constituents of *Citrus limon, Salvia officinalis* and *Cymbopogon citratus* EO, we used the gas chromatographic (GC) method. GC is an analytical method applied to compounds that can be vaporized by heating without decomposition [3]. It is the separation technique most used in the field of characterization of essential oils (EO), because it allows the individualization of the constituents of a sample of the order of the milligram or the microgram.

2.5. Biological activities of essential oils

Antibacterial activity and antifungal activity

Due to their antibacterial action developed over several years, the use of EO is a serious and promising substitute for the treatment of infectious diseases. In this context, we are therefore interested in the study of the antimicrobial activity of the three essential oils on certain pathogenic germs.

The choice of bacteria is focused on four strains common in human pathology. These species are often responsible for food toxi-infections or food poisoning (FTI), thus constituting a major public health problem due to their natural resistance to various antibacterial agents. Table 1 shows the Bacterial Strains Tested as following.

TABLE 1
THE BACTERIAL STRAINS TESTED ARE AS FOLLOWING

Bacteria	Group
Escherichia coli ATCC 25922	Gram negative
Salmonella typhi ATCC 14028	Gram negative
*Bacillus cereus*ATCC11778	Gram positive
Staphylococcus aureusATCC 25923	Gram positive

The fungicidal power has been examined for *Saccharomyces cereviseae*, commonly known as "baker's yeast". It is an agent of degradation of food and raw materials (2nd cause of the losses of the food products of vegetable origin) [4].

The methods used to study the interaction between essential oils and microbial species are based on the diffusion of these oils in culture media to inhibit the growth of a pathogenic microorganism. Indeed, in 100 ml flasks of the medium, increasing concentrations of about 0.1, 0.5 and 1% of the EOs are added (each oil apart).

Insecticide Activity

Essential oils currently represent an alternative solution for the protection of stored products. In this context, we tested our three essential oils on the insect *Sitophilus zeamais* shown in figure 2. It is one of the most destructive pests of cereals and other stored products processed and unprocessed in sub-Saharan Africa. *S. zeamais* causes qualitative and quantitative damage to stored products, with a weight loss of between 20 and 90% for untreated stored maize [5].

Fig. 2*: Sitophilus zeamais*

There are various types of tests for the insecticidal activity of EO. In this present work, we performed three tests

Repellent activity

The insect repellency effect of the EO was evaluated using the preferential filter paper method described by Jilani et al [6]. The method consists in cutting the filter paper into circles of 9 cm in diameter which will be divided into two parts:
- Part 1 impregnated with a 200 µl solution of acetone.
- Part 2 impregnated with a 200 µl solution of acetone mixed with the dose of the tested EOs: 0.0314 µl/cm² and 0.125 µl/cm².

The two parts are attached by sticky tape in Petri dishes and then the insects are placed in the middle of the boxes. Each test dose is repeated 5 times. After treatment at 15, 30, and 60 minutes, the measure is read by counting the number of *Sitophilus zeamais* present in each half-circle. The repulsion percentage is calculated using the following formula in Eq 1.

$$\boldsymbol{PR = (Nc - Nt)/(Nc + Nt)} \qquad (1)$$

PR: Repulsion percentage, Nc : Number of insects present on the part treated with acetone only, Nt: Number of insects present on the part treated with EO.

The average repulsion percentage is calculated and assigned to one of the different repulsive classes according to the classification of Mc Donald et al [7].

Fumigant Activity

Fumigation with essential oils is studied with a wide range of crop pests. The results show that the toxic effects depend on the insect species, the plant and the time of exposure to the essential oil [8]. The fumigant activity of the extracted essential oils is tested on adults of *S. zeamais*. The experiment consists of placing 10 insects of the species in spittoons of 40 ml of volume. HE is deposited with a micropipette on Wattman paper 3 cm in diameter, hung on the lid and tightly closed. The concentrations are 0, 25, 50, 100 and 200 µl/l of air at the rate of 5 repetitions for each one. Insect mortality is determined after 24 hours. The assessment of the effectiveness of EOs on the insects used is calculated using Abbott's formula [9] in Eq 2.

$$\boldsymbol{Mc = (Mo - Mt) / (100 - Mt)} \qquad (2)$$

Mc: corrected mortality rate, Mo: mortality rate in treated boxes, Mt: Mortality rates observed among controls

Antioxidant Activity

Several methods have been put in place to evaluate the effectiveness of the antioxidant to scavenge free radicals (DPPH). As a result, the demonstration of the antioxidant power of the extracted essential oils was carried out by a chemical test which corresponds to the measurement of the reducing power by the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) [10]. The percentages of inhibition of free radicals $DPPH^+$ was calculated according to the formula in Eq 3.

$$\boldsymbol{\%I = (A0 - Ai) / A0} \qquad (3)$$

%I percentage of inhibition, A_0: Absorbance of the control, A_i: Absorbance of the sample.

2.6. Treatment of Corn Seeds with Sage EO

In order to ensure the effectiveness of the EO in the protection of corn seeds, we carried out a seed treatment test as shown by the following protocol:

A sample of 15 g of corn is taken and 0.1 g of Kaolin is added to it to cover the corn seeds and facilitate the diffusion of the EO. Four doses are used in this EO sage test: 25, 50, 100 and 200µl / l of air. Respecting the requested formulation and the mixture of ingredients with an addition of insects 20 insects per spittoon then storage for a week. Under the same conditions, we put untreated corn seeds and seeds mixed with Kaolin to serve as controls.

In this research, we used the mixture design in the experimental design (Minitab 17) to optimize the formulation of the EO concentrations of the products tested (Mixture design Plan). After each experience, the Bactericidal potency of the different germs on the products tested and their pH Measurement were measured. The relationships between the different combinations and products were analyzed through the Minitab 17.0 to select the optimal EO concentrations combination of the products tested.

2.7. Statistical design

The acquired data was processed to calculate statistical values such as the mean and standard deviation (SD) using Microsoft Excel 2010 (Microsoft 365). For data analysis, Minitab 17 software was used. The assumptions of normality and constant variance were checked and confirmed. Mixture design analysis and analysis of variance (ANOVA) were used to determine the regression coefficients, the statistical significance of the model terms, and to fit the mathematical models of the experimental data that aimed to optimize the overall region for all response variables. A second order polynomial model was used to predict the response variables as shown in Eq 4.

$$\boldsymbol{Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3} \qquad (4)$$

Y is the predicted dependent variable; β_0 is a constant regression coefficient, β_1, β_2, and β_3 are the regression coefficients for the linear effect terms; and $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ and β_{123} are the regression coefficients of two and three-factor interactions effect terms, respectively. X_1(Lemon EO), X_2(Sage EO) and X_3 (Lemongrass EO) are the factors (independent variables) (Table 2). The adequacy of the model was predicted through the regression analysis (R^2) and the ANOVA analysis. The relationship between the independent variables and the response variables (Bactericidal, Fungicidal Power of Essential Oils and pH) was demonstrated by the Mixture design graph. Multiple graphical and numerical optimizations of the experimental data were done to identify the optimum Mixture conditions to achieve the desired optimum of Bactericidal, Fungicidal Power of Essential Oils and a neutral pH. Verification of predicted response conditions that would give the desired response was determined based on the superimposed contour graph of different responses.

2.8. Formulation of an Ecological food Detergent-Disinfectant (FDD)

Design of the experiment

In this study, Lemon EO, Sage EO and Lemongrass EO were used as mixture starters, ranging from 0 to 100%, as shown in Table 2. The Mixture design method in the experimental design, provided by the software Minitab (Ver. 17.0, U.S. Federal Government Commonwealth of Pennsylvania, USA), was used to optimize the formulation of the above the EO concentrations of the products tested (Mixture design Plan). Generally, the mixture design is used to study the relationships between the proportion of different variables and responses. A Simplex Lattice Design is developed and the P-value is the probability that the magnitude of a contrast coefficient is due to random process variability.

The Formulation of our Ecological food Detergent-Disinfectant (FDD) is Based on Essential Oils and Monitoring of its Stability. Due to the various physicochemical, antioxidant, antibacterial, antifungal and insecticidal properties, it is interesting to benefit from these multiple advantages. In this context, we propose to

formulate an ecological food detergent-disinfectant (FDD) for cleaning food industry equipment such as cutting boards, worktops, utensils devices…

Ingredients

Sodium bicarbonate is classified as a biological ingredient by the European Union and it is not classified as dangerous according to the European directive 67/548 / EEC and CLP1272 / 2008 [11]. It is a product with multiple benefits.

Lemon vinegar discovered for thousands of years and used in household products for its degreasing, disinfecting, descaling and anti-limescale properties. It is also a deodorant.

Lemon hydrosol: It pleasantly perfumes cosmetic preparations by bringing a very fruity touch. As a result, we believe that it benefits from its properties by incorporating it into our manufacturing formula.

Essential Oils

According to our analysis, we decided to vary the concentrations within a range of 0.1 to 1% for each of the essential oils to be tested and we measure the bactericidal, fungicidal, antioxidant and insecticidal power for each experiment (Table 2).

TABLE 2
THE EO CONCENTRATIONS OF THE PRODUCTS TESTED (MIXTURE DESIGN PLAN)

Run	Lemon EO (X_1)	Sage EO (X2)	Lemongrass EO (X3)
P_1	0.8	0.1	0.1
P_2	0.1	0.8	0.1
P_3	0.1	0.1	0.8
P_4	0.45	0.45	0.1
P_5	0.45	0.1	0.45
P_6	0.1	0.45	0.45
P_7	0.33	0.33	0.33
P_8	0.58	0.21	0.21
P_9	0.21	0.58	0.21
P_{10}	0.21	0.21	0.58

To optimize our formulation, we have chosen the concept of a mixing plan using the Minitab 17 Software, which allows the implementation of the matrix of experiments. This software is widely used in the modeling and optimization of industrial processes [12]. We chose "The Simplex Lattice Design" shown in figure 3 for the construction of the mixing plan, ensuring a perfect distribution of the uniformity of the ten mixtures in the experimental field by following the different powers sought for this ecological disinfectant.

Final Product

pH is an important criterion for characterizing a detergent. The pH of our products is measured using a pH meter.

The evaluation of the bactericidal and fungicidal activity of our products is carried out according to the dilution-neutralization method according to the Tunisian standard NT 49.02 1988. The method consists in putting in contact for a defined time (5 min) a bacterial suspension at temperature given (20°C or 32°C) with different concentrations of the

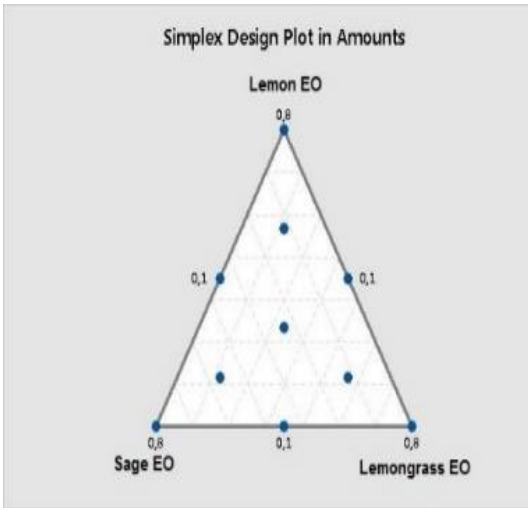

Fig. 3: Simplex Lattice Design

product. At the end of this contact time, the antimicrobial properties of the disinfectant are neutralized with a neutralizer. The number of surviving bacteria in the neutralized mixture is then compared to the enumeration of the bacteria initially present. A series of controls was carried out under the same conditions but including an equal volume of sterile distilled water instead of the product.

Study of Product Stability

A poorly formulated detergent does not remain stable over time. We, therefore, conducted an accelerated stability study for 20 days, checking pH, odor and color as well as microbiological parameters at different temperatures according to time.

2.9. Product Application

The cleaning and disinfection steps are according to the type of detergent [13] in 6 points in the case where the detergent and the disinfectant are each separately, or in 4 points in the case where the detergent and the disinfectant constitute a mixed product: In our case, these steps are performed as following:

- Preparation: Dispose of waste,
- Cleaning and Disinfection: Clean with water, then spray the FDD (shake well before spraying), leave a contact time (10 minutes), exert a mechanical action with a cloth.
- Rinsing: Rinse with water.
- Drying: Using disposable paper

We applied this plan for the cleaning and disinfection of the laboratory washbasin, a trolley and the steam distillation installation in CTAB at Chott Mariem (Tunisia).

2.10. Evaluation of Cleaning and Disinfection Effectiveness: Surface Test

The control of cleaning and disinfection in the IAA, especially the evaluation of the effectiveness of control of the hygiene of the surface, must be first visual, accompanied by

a microbiological test in the second place. The surface test (surface sampling) is applied to see the bacteria and mold existing industrial equipment.

3. Results and Discussion

3.1. Essential Oil Yield

Fig. 4 shows that the EO hydrodistillation yield of Citrus limon bark is 0.84 ± 0.14%. This result is comparable to other studies [11,12] which found variable proportions of approximately 0.3 to 0.6% and 0.7% respectively. In addition, Guimarães et al. [14] showed that the yield of the EO of the bark of *C.limon* silt is 0.18 ± 0.004%. On the other hand, some researchers [15] gave the yield is 2%. In addition, for Sage, our results are superior to those determined in other research. Souguir et al. [16] whose yield percentages vary between 0.23 and 0.28%. For Lemongrass, our results are lower than those mentioned in other research whose percentages vary between 0.6 to 0.65% [17]. We Note that the distillation yield is variable, in the same plant, depending on the season according to other research [18]. Steam training involves recovering the EO contained in plant excretory cells by means of water vapor.

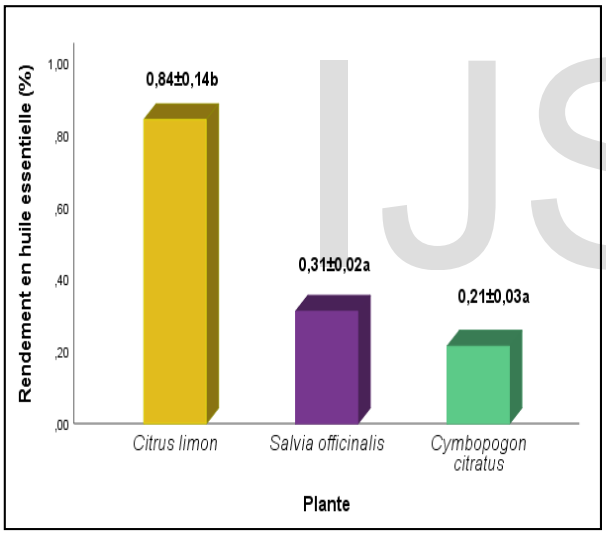

Fig. 4: EO Yield Obtained for Ripe Lemon, Sage and Lemongrass by Hydrodistillation (n = 3)

The results shown in Figure 5 show that the EO yield of ripe lemon, sage and lemongrass are 0.47, 0.29 and 0.20% respectively

The Physicochemical Characteristics of Essential Oils and Organoleptic characterization by carrying out the sensory analysis of the essences gives the following results in Table 3.

The table 4 recapitulates for the three essential oils of the various plants by giving the majority components as well as their percentages while recalling their corresponding properties.

By comparing our results with those cited in the literature, we note variability in the percentages of the main constituents. According to the study of Djenane [12] for example, the chemical composition of Lemon EO identified by GC-MS analysis shows that the major components are the same as us (Limonene, β-Pinene, and γ-Terpinene) with different percentages (51.40%, 17.04% and 13.46%) respectively. For sage, our results are in agreement with other research [19] whose main compounds are α-thuyone (26.49% - 10.58%), 1.8 –cineole (31.89% - 8.58%), camphor (40.14% - 2.10%).

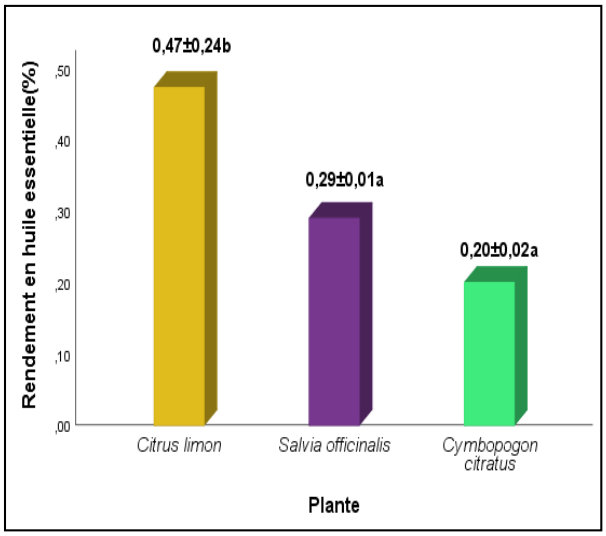

Fig. 5: EO Yield for Ripe Lemon, Sage and Lemongrass Obtained by Steaming (n = 3)

TABLE 3
PHYSICOCHEMICAL CHARACTERISTICS OF ESSENTIAL OILS AND ORGANOLEPTIC CHARACTERIZATION

	Odor	Color	Appearance	Refractive index at 20°C	Acid index
Lemon	Feature, fresh	Light yellow to green	liquid	1.470	0.88
Sage	Spicy	Pale yellow	liquid	1.462	0.46
Citronella	Lemon	pale yellow	limpid	1.479	4.34

On the other hand, for Lemongrass, our results differ from other research [20] that notes that the main chemical constituents are (Geraniol (27.13%), Citronellol (12.67%), and Citronellal (32.77 %). While we noted that Geraniol does not constitute a major constituent (represents 3.13%) and that α-terpineol is among the major constituents (29.28%) against (0.54%) by that research [20]. On the other hand, for the other two constituents (Citronellal and Citronellol) our results show that they are higher by 41.32% and 15.52% respectively.

This may be due to the influence of several factors (climatic conditions, extraction method) and the chromatographic method (CPG / GC-MS).

TABLE 4
MAJOR CHEMICAL COMPONENTS OF ESSENTIAL OILS OF C. LIMON, S. OFFICINALIS, AND C. CITRATUS OBTAINED BY GC CHROMATOGRAPHY

EO	Majority components	%	Proprieties
Lemon	Limonene	67.67	Used in cleaning products for its refreshing smell and its dissolving effect. Limonene can be used as a metal degreaser, wetting agent and cleaning agent, as well as an aroma in soaps, and household cleaning products. No toxic effect, neither hepatic nor on development, reproduction, nor mutagenic nor carcinogenic, on the contrary, several studies on animals report that limonene can inhibit the formation of tumors [21].
	γ-Terpinene	9.88	Antioxidant properties [22], anti-inflammatory and antibacterial activities [23].
	β-Pinene	6.78	Known for its antiseptic, antibacterial, antifungal and antiviral properties [24].
Sage	α-Thuyone	25.93	Effective insecticide that can eliminate odors [25].
	1,8-cineole	20.61	A monoterpene of refreshing and spicy smell. It is also indispensable in products. It has anti-fungal, antioxidant, anti-infectious, bactericidal and antiviral properties [26].
	Camphor	11.97	One of the most powerful natural antivirals known. They are powerful anti-infective molecules with a broad spectrum of action against bacteria, viruses and fungi. Camphoris also an insecticide [27].
Lemongrass	*Citronellal*	41.32	It has insect repellent properties [28]. Research shows that citronellal has strong antifungal [29].
	α-*terpineol*	29.28	An alcohol that gives off a pleasant scent. It is also a powerful anti-infectious antioxidant, antiviral, bactericidal, fungicidal, virucidal and insect repellent [26].
	Citronellol	15.52	An organic compound that gives off a pleasant lemony scent. Used in cosmetics as a perfume or masking agent, that is to say, to mask the odors of other components of the product. It is a very powerful repellent against insects and is, therefore, present in most insecticides. It also has fungicidal, virucidal and insect repellent properties [26].

TABLE 5
BACTERICIDAL AND FUNGICIDAL POWER OF ESSENTIAL OILS

		Control	**Lemon EO**			**Sage EO**			**Cetronella EO**		
Strains	**Concentration (%)**	0	0.1	0.5	1	0.1	0.5	1	0.1	0.5	1
1	*Escherichia coli*	0.5 10^3	>10^3	0	0	>10^3	2	18	0	0	0
2	*Salmonella typhi*	1.5 10^3	>10^3	0	0	>10^3	140	26	15	0	0
3	*Bacillus cereus*	0.5 10^3	>10^3	0	0	>10^3	0	0	0	0	0
4	*Staphylococcus aureus*	1.5$10^3$	>10^3	0	0	>10^3	0	0	0	0	0
5	*Saccharomyces cereviseae*	1.5 10^3	>10^3	0	0	>10^3	0	0	0	0	0

To say that an EO has a bactericidal and /or fungicidal power, the number of colonies must be : (Px ≤ N '/ 10] for each see).
N ': corresponds to the number of colonies in the control.

3.2. Biological Activities

According to Table 5 we note that the tested bacteria were sensitive to the three Eos studied. In fact, the *C. Limon*, *S. Officinalis* and *C. Citratus* species exerted an important antimicrobial effect against the strains studied. The EO of *C. Citratus*, reveals an interesting antibacterial activity against the 4 strains at a low concentration of 0.1%. For *Salmonella typhi*, this bacterium showed some resistance to the tested EO. In addition, the Hes of *C. limon* and *S. officinalis* are clearly effective against the 4 strains from a concentration of 0.5%. According to the results of the antifungal activity, it is clear that *Saccharomyces cereviseae* is sensitive to the three EOs studied. In fact, a concentration of 0.5% HE of *C. limon* or *S. officinalis* resulted in an absence of growth of the yeast. In addition, a concentration of 0.1% HE of *C. Citratus* caused the absence of growth of the yeast.

Regarding the antioxidant activity shown in table 6 and considering the results of the determination of the IC_{50} values, we deduce that the EO of sage has the highest antioxidant capacity compared to the other species studied. This could be related to its higher content of free radical scavengers acting as antioxidants, including 1.8 cineole. Thus, the OE of *C. Citratus* occupies the second position by a good antioxidant power that causes a 91.88% inhibition of free radicals for a concentration of 100 µl/ml. As for the lemon EO, it is also a powerful antioxidant because of its composition rich in monoterpenes.

The capital letters indicate the difference between the strands tested for the same concentration; Lowercase letters indicate the difference between concentrations for the same sample; Averages with the same letters are not significant and are different at $p < 0.05$

We can say that our essences are endowed with a significant antioxidant power, in particular the EO of Sage that is the most effective antioxidant with an IC_{50} of 18.24 µl/ml. This value is close to that of ascorbic acid. In addition, the EO of Lemongrass also has an antioxidant power with an IC_{50} of 23.65µl/ml, these two essences (Sage and Lemongrass) show an interesting antioxidant effect (92.76 and 91.88% for 100 µl/ml with a non-significant difference) close to that obtained by ascorbic acid (99.53% with deference significant).

On the other hand, the EO of Lemon tree brings back the stable free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) to yellow-colored diphenylpicrylhydrazine with an IC_{50} of 41.72 µl/ml showing an antioxidant activity lower than that of ascorbic acid and EO tested.

Our results are largely in agreement with those found in the literature. For Sage, in some researches [30] on EO and extracts of *S. Officinalis* recorded a strong trapping activity of the DPPH radical, and explained them by the high content of 1.8 cineole, which is a major component of this oil.

Other researchers [31] have shown that *C. Citratus* EO has important antioxidant properties. This activity, which is attributed to EO extracted from *C. limon*, appears to be related

TABLE 6
EVALUATION OF THE ANTIOXIDANT ACTIVITY OF *C. LIMON*, *S. OFFICINALIS* AND *C. CITRATUS* (N = 3)

Sample	Concentration (µl/ml)	Inhibition (%)	IC_{50} (µl/ml)
Ascorbic acid	25	96,24±0,21Da	16,19±0,09
	50	97,07±0,17Db	
	75	98,43±0,13Dc	
	100	99,53±0,08Cd	
Citrus limon	25	41,13±3,98Aa	41,72±0,04
	50	59,24±0,19Ab	
	75	66,85±0,28Ac	
	100	70,12±0,76Ac	
Salvia officinalis	25	82±1,03Ca	18,24±0,08
	50	85,4±0,66Cb	
	75	90,75±0,19Cc	
	100	92,76±0,28Bd	
Cymbopogon citratus	25	64,83±0,29Ba	23,65±0,58
	50	74,64±0,29Bb	
	75	89,99±0,38Bc	
	100	91,88±0,38Bd	

to its composition. Wei and Shibamoto [32] noticed an important antioxidant activity of essential oils rich in monoterpenes (limonene α- and β-pinene).

To characterize the three Eos of *C. limon*, *S. officinalis*, and *C. Citratus* against *S. zeamais*, the determination of the mean percent repellency and the repellant class are necessary.

The results obtained were illustrated in the following Table 7.

According to the Table 7, we can conclude that both oils (sage and lemon) contain active biological sources with high repulsive potential; it will be possible to use them for the pest control of stored products. On the other hand, for the EO of *C. Citratus*, it was necessary to apply a concentration of 0.125 µl/cm² to a moderately repellent effect. Therefore, we can say that we have more resistance to *C. Citratus*. In the majority of cases, insects have moved to untreated control. In addition, when the dose is increased, the insects move towards control. These studies also show that the repellent effect is closely related to the structure and dose of the test molecule.

TABLE 7
AVERAGE REPULSION PERCENTAGES AND REPULSIVE CLASSES (N = 5)

EO	Dose (µl/cm²)	Time (min)	Repulsiveness (%)	Average (%)	Repulsive class	Effect
Lemon	0.0314	15	64[a]	60.02±25.47	IV	repulsive
		30	32.78[a]			
		60	83.27[a]			
	0.125	15	83.69[a]	83.98±0.90	V	Very repulsive
		30	85[a]			
		60	83.27[a]			
Sage	0.0314	15	66.16[a]	78.72±12.63	IV	Repulsive
		30	96[a]			
		60	74[a]			
	0,125	15	86[a]	83.08±12.41	V	Very repulsive
		30	93.77[a]			
		60	69.47[a]			
Lemongrass	0.0314	15	8[a]	36.33±20.53	II	Slightly repulsive
		30	56[a]			
		60	45[a]			
	0.125	15	44[a]	55.06±16.40	III	Moderately repellent
		30	49.20[a]			
		60	72[a]			

Averages with the same letters are not significant and are different at p <0.05.

On the other hand, time exposure has no effect since there is no significant difference between the repulsive averages of our essences at 15 minutes, 30 minutes and/or 1 hour.
The results of the insecticidal activity of the fumigant of our EO on adults of *S. zeamais* are reported in the Table 8.

TABLE 8
PERCENT MORTALITY OF SITOPHILUS ZEAMAIS AFTER EXPOSURE TO DIFFERENT EO (N = 5)

EO	Dose (µl/l of air)	Mortality after 24h (%)
Lemon	0	0[a]
	25	0[a]
	50	0[a]
	100	0[a]
	200	2±4.47[a]
Sage	0	0[a]
	25	16±11.40ab
	50	30±21.21b
	100	60±15.81c
	200	84±19.49[d]
Lemongrass	0	0[a]
	25	6±8.94ab
	50	8±4.47ab
	100	18±14.90b[c]
	200	26±20.73c

Averages with the same letters are not significant and are different at p <0.05

The examination of Table 8 shows that sage essential oil is more effective against *S. zeamais* adults after a 24-hour exposure. This efficiency varies according to the concentrations. Lemongrass EO ranks second when compared to the insecticidal efficacy of sage and lemon.
In fact, only 16% of insects were killed by a dose of 25 µl/l of air, whereas a dose of 200 µl/l of air causes 84% mortality. The EO of Lemongrass occupies the second position if we compare its insecticidal efficacy with that of Sage and Lemon. In addition, according to Table 8, the EO of Lemongrass kills 26% of insects against 84% by the oil of Sage and only 2% by the EO of Lemon for a dose of 200 µl/l of air. Likewise, the fumigant effect of the essence of Citronella varies according to the concentrations.
In addition, the EO of Lemon does not show efficacy except for a low percentage of mortality for a dose of 200 µl/l of air, perhaps this percentage can be increased if the dose is increased. This variation in toxicity is related to the difference in the chemical composition of essential oils according to other authors [33].
The evaluation of the insecticidal activity by fumigation of our essences is determined by the LD_{50} for our insect. After probit analysis, the results were illustrated in Table 9. The analysis of this Table 9 proves that the essential oil of *S. officinalis* has an LD_{50} of the order of 102.75 µl/l of air and an LD_{90} of the order of 200 µl/l. For *C. Citratus* and *C. limon*, lethal doses cannot be calculated because a 50% mortality rate was not achieved for both oils, confirming the high toxicity of *S. officinalis* oil in *S. zeamais* adults.

TABLE 9
DETERMINATION OF LD_{50} LETHAL DOSES AND LD_{90} FOR DIFFEREN INSECTS TREATED FOR 24 HOURS

EO	LD_{50}	LD_{90}
Lemon	-	-
Sage	102.75 µl /l of air (82.075-129.2)	200 µl/ l of air (165.075-263.575)
Lemongrass	-	-

3.3. Treatment of Corn with Sage EO

According to the previous results, the EO of Sage has a significant insecticidal activity; therefore, this oil is chosen to carry out the treatment of corn kernels. We note that *S. zeamais* attacked the corn kernels in the case of the controls (controls and Kaolin controls) as well as in the spittoons treated with EO as shown in the following Figure 6.

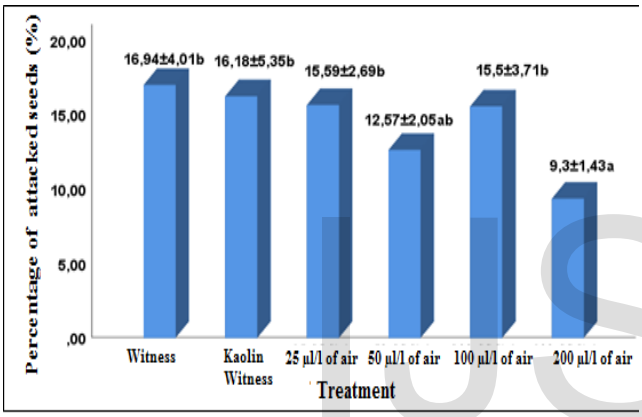

Fig. 6: Treatment of Corn Kernels with Sage HE (n = 5)

In figure 6, we note that Data analysis reveals that Sage EO has a protective effect for corn kernels. Indeed, we notice that the highest percentage of the grains attacked by *S. zeamais* is that of the control followed by kaolin control, starting from a concentration of 25 µl/1 of air; the percentage of the attacked grains begins to decrease. We can conclude that Sage EO can protect stored corn kernels against *S. zeamais* attack. Treatment with essential oil of sage resulted in a decrease in the percentage of attack. The results obtained are presented in the following figure 7.

Fig. 7: Maize grains attacked by *S. zeamais*

3.4. Final Product

The bactericidal Potency of the products tested and the measurement of the pH of the products tested for the ten experiments presented in Table 2 was carried out for the various strains studied as answers or experimental measurement necessary for the statistical calculations and the determination of the various mathematical models with their corresponding regression coefficients. These results are summarized in Table 10 witch shows that our products have a neutral pH since, according to disinfection standards, products with a pH between 6 and 8 take the neutral criterion [34]. This shows that our products can be considered as multi-use products according to the Brussels Association [35]. In addition, our results ensure that there is no risk of metal corrosion when used to clean the machines. The results of the bactericidal activity of our products are reported in Table 10.

Of the 10 products tested, only five products have bactericidal and fungicidal activity. Indeed, in comparison with the control, with the neutralizer and by the application of the formula of computation, one can conclude that in order for a product to have a bactericidal and fungicidal power, it is necessary that the number of colonies is: ($Px \leq N'/10$) for each seed. We notice that Gram (+) bacteria are more sensitive than Gram (-) bacteria, because the number of colonies of these bacteria is always lower than that of other bacteria. The high resistance of Gram (-) bacteria is partly related to the complexity of the cell wall of these microorganisms which contains a double membrane, unlike the simple membrane structure of Gram (+) bacteria [31]. A mathematical model giving the responses studied as a function of the contributions of each of the components used as well as the coefficients obtained by interaction of components 2 to 2. Examination of this Table 10 shows that the variation of the components has an effect on the parameters studied. This effect can be positive such as the case of (sage *lemongrass) for example or else negative such as the case of (lemon * sage * lemongrass). The regression coefficients will allow us to determine the regression equations for each parameter as Eqs. (5) to (10): Lemon: (c), Sage: (s) and Citronella (cit)

pH:

$Y=7.662\ (c) + 7.668\ (s) + 7.202\ (cit) - 0.93\ (c)(s) - 0.85\ (c)(cit) + 0.43\ (s)(cit) - 0.19\ (c)(s)(cit)$; $R^2=87.63\%$ (5)

Escherichia coli:

$Y=1014\ (c) + 409\ (s) - 671\ (cit) + 4653\ (c)(s) + 609\ (c)(cit) + 8887\ (s)(cit) - 46455\ (c)(s)(cit)$; $R^2=93.69\%$. (6)

Salmonella typhi:

$Y=999\ (c) + 389\ (s) - 741\ (cit) + 4845\ (c)(s) + 819\ (c)(cit) + 9188\ (s)(cit) - 48271\ (c)(s)(cit)$; $R^2= 93.79\%$ (7)

Bacillus cereus:

$Y = 1007\ (c) + 418\ (s) - 591\ (cit) + 4229\ (c)(s) + 526\ (c)(cit) + 8659\ (s)(cit) - 43328\ (c)(s)(cit)$; $R^2= 91.04\%$. (8)

Staphylococcus aureus:

$Y=1030\ (c) + 416\ (s) - 597\ (cit) + 4579\ (c)(s) + 536\ (c)(cit) + 8596\ (s)(cit) - 45310\ (c)(s)(cit)$; $R^2= 94.74\%$. (9)

Saccharomyces cereviseae:

$Y=986\ (c) + 356\ (s) - 755\ (cit) + 5227\ (c)(s) + 786\ (c)(cit) + 9691\ (s)(cit) - 52324\ (c)(s)(cit)$; $R^2= 92.70\%$.(10)

TABLE 10
BACTERICIDAL POTENCY OF THE PRODUCTS TESTED AND PH MEASUREMENT OF THE PRODUCTS TESTED

Adapted strain	*E. coli*	*Salmonella typhi*	*Bacillus cereus*	*S. aureus*	*Saccharomyces cereviseae*	pH
N	2.510³	3.4 10 ³	2.6 10 ³	2.9 10 ³	3.2 10 ³	*
N'	2.4 10 ³	3.0 10 ³	2.2 10 ³	2.6 10 ³	3.0 10 ³	*
P_1	>10³	>10 ³	>10 ³	>10 ³	>10 ³	7.5
P_2	>10³	>10 ³	>10 ³	>10 ³	>10 ³	7.58
P_3	40	11	53	79	0	7.23
P_4	>10 ³	>10 ³	>10 ³	>10 ³	>10 ³	7.44
P_5	64	54	82	80	0	7.26
P_6	>10³	>10 ³	>10 ³	>10 ³	>10 ³	7.45
P_7	96	71	98	88	0	7.39
P_8	116	104	120	200	0	7.28
P_9	>10 ³	>10 ³	>10 ³	>10 ³	>10 ³	7.42
P_{10}	79	49	312	124	10	7.35

N: Number of germs in the test bacterial suspension, $P_1$to P_{10}: Product 1 to product 10;
N ': Number of germs in the test bacterial suspension with Neutralisant

3.5. ANOVA and validation of the models

The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using ANOVA, which is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for testing hypotheses on the parameters of the model. The statistical significance of models equations was evaluated by the F test and the model F values implied that the models were significant. There was only a 0.01% chance that the models'F values could occur due to noise. The P values were also very low (P < 0.001), indicating the significance of models. Statistical analyzes were performed using multiple regressions and ANOVA with multiple regressions from Minitab 17.0 software to fit the first order equation.

The results of the empiric model of Bactericidal Potency of the products tested and pH measurement of the products tested in the form of ANOVA are calculated respectively. The fitting of the models was checked by the coefficient of regression R2 which were equal to 0.876 for pH and from 0.910 to 0.947 for Bactericidal Potency indicating that the variability in the response could be explained by models.

3.6. Results Discussed by the Mixing Plan

Contour graphs

According to Henry's curves of residual values, the points go with the line, which explains an experimental procedure managed by the same phenomenon with a strong correlation and a significant linearity giving a significant and adequate model. A graph figure 8 displays a 2-dimensional view, which allows the establishment of the response values and the desired operating conditions. Let us take the example of contour graph for pH shown in figure 8.

With the example when the pH = 7.23 which corresponds to a combination of: 0.23% Lemon, 0.11% Sage and 0.65% Lemongrass, the highest values are on the left (dark blue color), can reach 7.6 (> 7.55). The intermediate values are presented by a degradation of the two colors (green and blue). Finally, whatever the concentrations of our product, we are always in the desirable conditions for the pH.

According to the results obtained during the evaluation of the bactericidal and fungicidal activity of the products tested by the dilution-neutralization method, we concluded that a product is known to be effective and has a bactericidal power against *E. coli* when the number of colonies that appear is less than 240 CFU shown on figure 9.

In conclusion, our optimal zone is the one with a number (< zero) and between (0-200) CFU. Take the example when the growth of *E. coli* is approximately zero CFU, which corresponds to a combination as following: 0.36 % of Lemon, 0.28 % of Sage and 0.35 % of Lemongrass

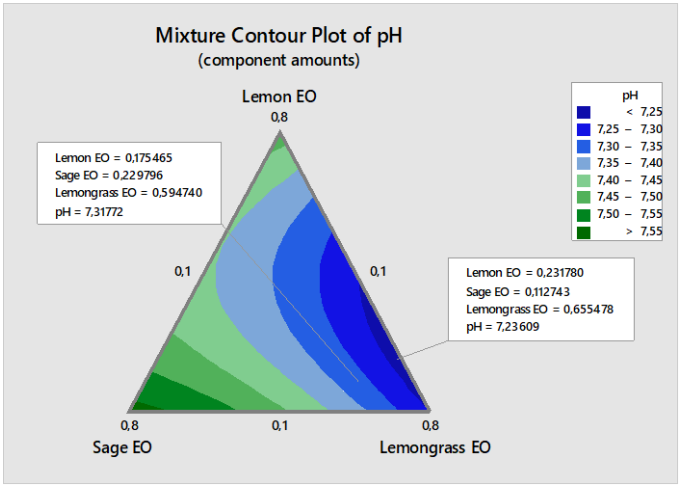

Fig. 8: Contour Graph for pH

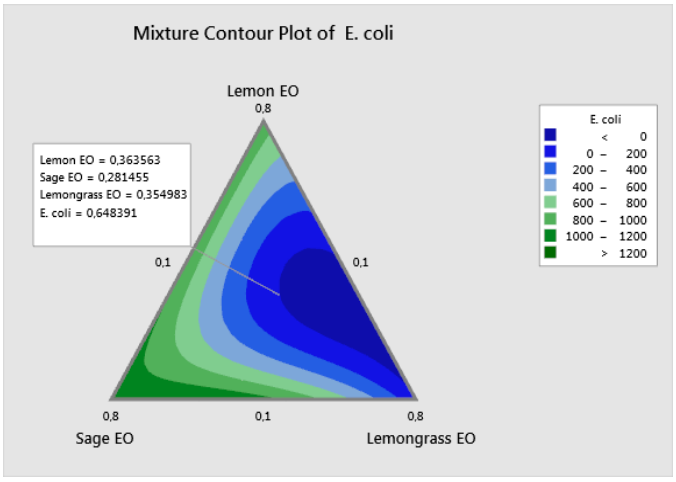

Fig. 9: Contour Graph for *E. coli*

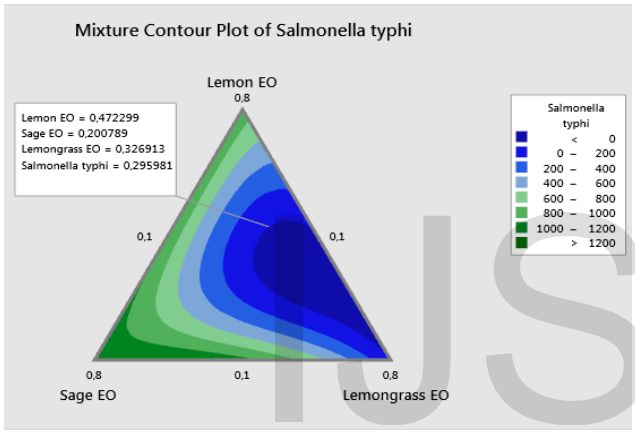

Fig. 10: Contour Graph for *Salmonella typhi*

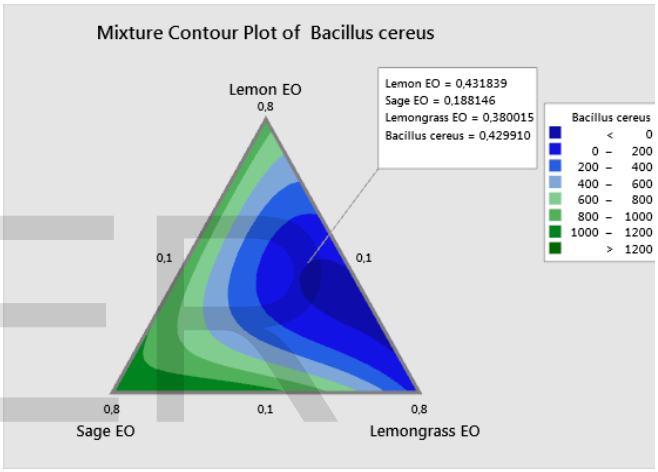

Fig. 11 : Contour Graph for *Bacillus cereus*

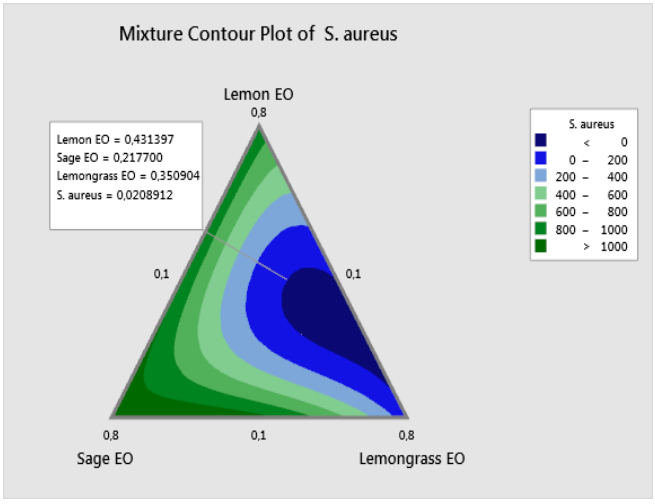

Fig. 12 : Contour Graph for *Staphylococcus aureus*

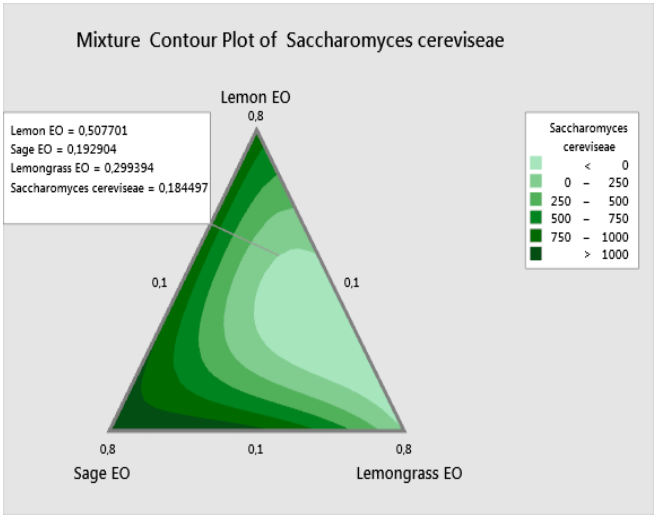

Fig. 13 : Contour Graph for *Saccharomyces cereviseae*

For *Salmonella typhi*, the number of colonies must be less than 300 CFU, so the optimal areas are those with blue and dark blue color shown in figure 10. This is the case when the growth of *Salmonella typhi* is approximately zero CFU, which corresponds to a combination as following: 0.47% of Lemon, 0.21% of Sage and 0.32% of Citronella.

For *Bacillus cereus*, the number of colonies must be less than 220 CFU, so the optimal areas are those with a blue and dark blue color shown in figure 11. This is the case when the growth is of the order of zero CFU, which corresponds to a combination as following: 0.43% Lemon, 0.18% Sage, and 0.38% Lemongrass

For *S. aureus*, the number of colonies must be less than 260 CFU, so the optimal areas are blue and dark blue shown in figure 12. This is the case when the growth is of the order of zero CFU which corresponds to a combination as following: 0.39% of Lemon, 0.15% of Sage and 0.46% of Lemongrass.

For *Saccharomyces cereviseae* According to the results obtained during the evaluation of the bactericidal and fungicidal activity of the products tested, a product is said to be fungicidal on *Saccharomyces cereviseae* when the number of colonies, which appear, is less than 300 CFU. By conclusion, the optimal areas are those with green and light green color. Take the example when the growth of *Saccharomyces cereviseae* is approximately zero CFU (Fig. 13) which corresponds to a combination as following 0.51% of Lemon, 0.19% of Sage and 0.30% of Citronella.

Superimposed contour graph

According to Tunisian standard NT 49.02 1988, to say that a product has bactericidal power, the number of colonies must be ($Px \leq N\ '/\ 10$) for each germ. That is, under the following conditions at the same time: pH between 6 and 8, *E. coli* <240 CFU, *Salmonella typhi* <300 CFU, *Bacillus cereus* <220, *S. aureus* <260 CFU and *Saccharomyces cerevisiae* <300 CFU

Drawing the graph of superimposed contours allows us to determine the optimum zone, which meets all optimums answers (Y) and the desired conditions, giving the figure 14, and shows the white color area of which presents the optimum of the parameters studied (the five germs and the pH). This interesting result gives the manager more control over the process with simplicity and helps in decision-making and decision support.

According to the figure 14, the optimal formulation will be as following:

For pH =7.29 ; *E. coli* = 115 CFU ; *Salmonella typhi* = 92 CFU ; *Bacillus cereus* = 184 CFU ; *S. aureus* = 144 CFU ; *Saccharomyces cerevisiae* = 51 CFU. The optimum Corresponds to the formulation of 0.2% of Lemon, 0.2% of Sage, 0.6% of Lemongrass.

Analysis of the results optimization curve

According to the figure 15 opting for an average desirability of 0.783 to reach an optimum of pH (Y=7) and a target value (Y= 1) for the five *E. coli*, *Salmonella typhi*; *Bacillus cereus*; *S. aureus* and *Saccharomyces cerevisiae* which corresponds to 0.1% Lemon, 0.1% Sage and 0.8% Lemongrass show as following in figure 15.

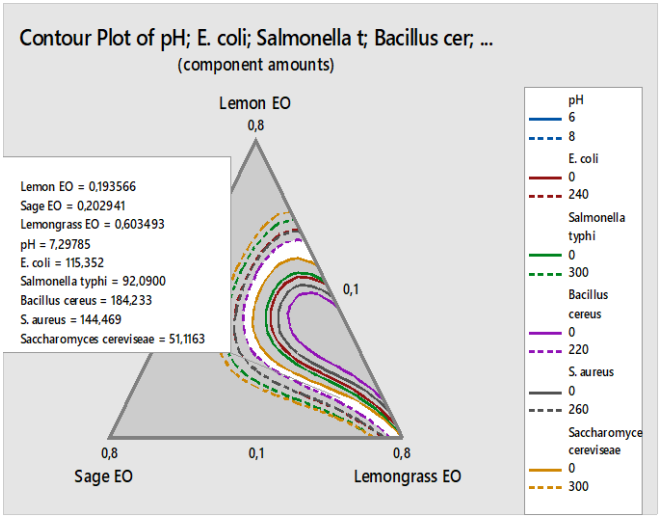

Fig. 14: Superimposed Contour Graph: (pH, *E. coli*, *Salmonella Typhi*, *Bacillus cereus*, *Staphylococcus aureus and Saccharomyces cereviseae*)

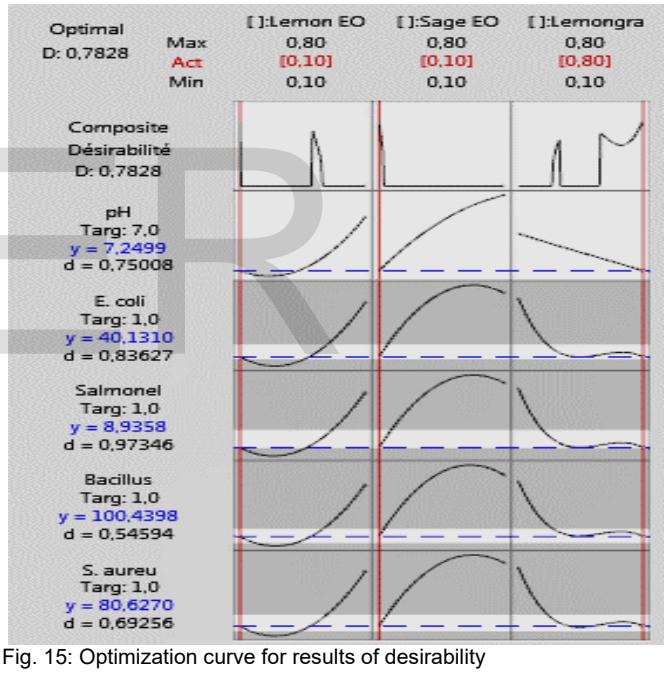

Fig. 15: Optimization curve for results of desirability

3.7. The Stability of the Product

All results of the physical and microbiological analyzes obtained during the study we conducted on the stability of our FDD are presented in this section. Three physical parameters are evaluated during the 20 days. The results obtained are presented in the following two Tables 11 and 12:

The Examination of theses tables shows that temperature can influence our FDD. On the one hand, there is a slight change in color for the vials placed at 37 °C and 44.5 °C; this indicates that the increase in temperature may affect the color of the product. On the other hand, the smell remains strong and characteristic of Citronella for bottles placed at room temperature, or placed at 4°C. On the other hand, the smell becomes less intense for those exposed to the sun, placed at 37 °C and 44.5 °C.

Our results in Table 12 shows that pH varies with incubation temperature over time. This slight variation in pH values could be due to the decomposition of citric acid present in lemon vinegar. Moreover, this variation did not affect the quality of our FDD since the various values obtained are in agreement with the standards (between 6 and 8).

Concerning the microbiological stability, after 72 hours of incubation on LPT media at 30 °C and GC at 25 °C. for 5 days, a total absence of the total seeds was detected for all the products except for the product number 3 which shows a development of aerobic germs (N = 50); this could be due to lack of hygiene at the laboratory during handling. A total absence of yeasts and molds for all products was recorded.

Following the application of the product, we come to notice a perfect clean accompanied by a significant brilliance. Moreover, the assurance of the effectiveness of our plan of cleaning and disinfection; our FDD is confirmed by the results obtained following the application of the surface test. Indeed, we notice a total absence of colonies on the agar plates.

3.8. Estimated Cost

By referring to the market price of essential oils and according to our optimized recipe using our extracts of essential oils, the price of a 500 ml bottle costs about $ 2.41 (or 6.685 Tunisian Dinar) in addition to the packaging costing $ 0.37 (or 1.020 Tunisian dinar). The total cost will be $ 2.78 (or 7,705 Tunisian dinar).

TABLE 11
EVALUATION OF THE COLOR AND ODOR STABILITY OF THE TESTED PRODUCTS

Parameter	Control (The day of preparation)	For 20 days				
		Ambient Temperature Flasks	Bottles exposed to the sun	Bottles placed at 37 °C	Bottles placed at 4 °C	Bottles placed at 44.5 °C
Color	Dark yellow	no change of the initial color	no change of the initial color	a slight change in color (yellow to brown)	no change of the initial color	a slight change in color (yellow to brown)
Odor	High characteristic odor of lemongrass	High characteristic odor of lemongrass (no change of smell)	from the 17th day the smell becomes less intense	from the 12th day the smell becomes less intense	High characteristic odor of lemongrass (no change of smell)	from the 7th day the smell becomes less intense

TABLE 12
PH MEASUREMENT OF PRODUCTS TESTED FOR STABILITY STUDY

Product	Sample number	pH_0	pH_{20}
Bottles at room temperature	1	7.39	7.40
	2	7.38	7.38
	3	7.39	7.4
Bottles exposed to the sun	4	7.41	7.47
	5	7.37	7.42
	6	7.37	7.40
Bottles placed at 37 °C	7	7.37	7.48
	8	7.34	7.45
	9	7.32	7.41
Bottles placed at 4 °C	10	7.45	7.4
	11	7.41	7.32
	12	7.41	7.30
Bottles placed at 44.5 °C	13	7.38	7.50
	14	7.34	7.42
	15	7.32	7.46

4. Conclusion

The EO of ripe lemon, sage and lemongrass were obtained by two distillation methods: hydrodistillation, the yields of which vary between 0.21% and 0%. , 84% respectively and steam stripping which gives 0.27, 0.29 and 0.20% respectively. The characterization of the collected species gave satisfactory results, in accordance with pharmacopoeia standards and in agreement with previous work. Gas chromatography (GC) gives monoterpenes, hydrocarbons and terpene alcohols as major components proving antibacterial, antifungal, antioxidant and insecticidal efficacy.

An antibacterial effect against the four strains studied (Escherichia coli, *Salmonella typhi*, *Bacillus cereus* and *Staphylococcus aureus*) is demonstrated. The EO concentration (0.5%) of *C. limon* EO and *S. officinalis* resulted in a total absence of growth of the four above-mentioned strains. EO of *C. Citratus* showed high bactericidal activity from 0.1% up to 1%, Gram (-) bacteria being more resistant than Gram (+) bacteria.

For the antifungal activity of our EOs on the yeast *Saccharomyces cereviseae* shows their fungicidal power. Indeed, the EO of *C. Citratus* caused a total absence of yeast growth from a concentration of 0.1% while the other two EOs have a fungicidal power from a concentration of 0.5. %.

The antioxidant activity of the EOs of sage and lemongrass is greater than that of lemon, and this by comparison with that obtained by ascorbic acid with percentages of inhibition of the order of 92.76 and 91.88% for 100 µl/ml for Sage and Lemongrass respectively.

Insecticidal activity showed that C. limon and *S. officinalis* have a strong repellency than *C. Citratus*. For the activity of the fumigant, the EO of sage is the most effective against *S. zeamais*, we applied it for a treatment of corn seeds which confirmed the toxicity of this oil for the insect by showing a low insect attack on seeds.

Our EOs can be considered as promising agents for the innovation of new methods and means of environmental protection and the assurance of human security. Hence the idea of manufacturing an Ecological Food Detergent-Disinfectant named "Safe-Natural Cleaner" intended for cleaning and disinfection of food industry instruments, mainly "BIO" certified industries. This innovative, value-added product is designed for fight against the invasion of chemicals that have adverse effects on people and the environment.

Minitab 17 made it possible to minimize the number of experiments for an optimization of the experimental time and the possibility of optimizing the recipe with more control of the process, simplicity and decision support. Storage at room temperature away from the sun and / or at 4°C is the best storage condition for our product for good storage stability. The surface test by our FDD for cleaning and disinfection of some instruments has proven its effectiveness.

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