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APPLICATION TRIAL OF ESSENTIAL OIL EXTRACTED FROM *CITRUS AURANTIUM* IN FOOD PRODUCTS (MINCED MEAT)

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Abstract

In recent years, humans have turned to herbal remedies to solve a variety of problems, both food-related and health-related. Synthetic food preservatives are now restricted in many developed countries because of their irreversible effects on the body. Therefore, the present study is an important contribution to the search for new beneficial preservatives from plants. The Citrus aurantium plant is widely used by the people of the Relizane region for therapeutic and culinary purposes. The extraction of essential oils from the fruit of this plant by hydro-distillation gave a yield of 0.09 ± 0.01%. The relative density at 20°C, the acid value, the ester value and the saponification value are in the order of 0.69 ±0.1, 44 ±3.0 mg KOH/g oil, 2.75 ± 0.5 mg KOH/g oil and 46.75 ± 5.1 mg KOH/g oil respectively. GC/MS analysis revealed the predominance of linalool (43 ± 1.5%) followed by limonene (19 ± 3%). In contrast, no alterations were detected in sample 3 in terms of odour, appearance and colour. A low number of total aerobic mesophilic flora (2×10^4 CFU/g) was recorded in the same sample, followed by sample 2 (4×10^4 CFU/g) and sample 1 (6×10^6 CFU/g). A total absence of Salmonella and Staphylococcus aureus in samples 3 and 2. While sample 1 appeared spoiled due to the presence of a load of 10^4 CFU/g of Salmonella. The Citrus aurantium essential oils appear to be a natural preservative that can be used and incorporated into many perishable food preparations.

Keywords: Preservatives, essential oils, Citrus aurantium. Total aerobic mesophilic flora, Salmonella, Staphylococcus aureus

1. INTRODUCTION

Worldwide, two million adults and three million children die as a result of eating contaminated food.

The contamination is usually caused by a harmful micro-organism or a chemical contaminant, resulting in food poisoning with serious consequences [1,2].

Bacteria with other microorganisms such as fungi, parasites and viruses are often the main cause of food poisoning cases; however, their eradication by chemical antibacterial substances certainly leads to the selection of resistant microbial strains [3,4].

For a long time, *Citrus aurantium L*, or bitter orange, has been used in several sectors; and especially in the food industry as a raw material for liqueurs and marmalades. Traditional Chinese medicine has recognized a wide use of its extracts to activate circulation, vital energy, appetite control, and weight loss. [5].

Essential oils are complex, natural and volatile compounds, which are often characterized by a powerful odor, produced by aromatic plants as secondary metabolites.

Steam distillation is the main method used to obtain them or by hydrodistillation, first developed by the Arabs in the Middle Ages [6].

Essential oils are natural extracts from medicinal and aromatic plants, characterized by their antimicrobial and antiseptic properties [7]. It should also be noted that many essential oils have antiviral, antioxidant, anti-toxic and anti-parasitic properties. More recently, anti-cancer properties have been noted [8].

The present research concerns the valorisation of *Citrus aurantium* from the region of Relizane, as a powerful food preservative, in the face of the problems encountered after the ingestion of significant amounts of chemical preservatives, having already marked by their irreversible effects on our body in the long term.

2. MATERIALS AND METHODS

Plant material

The material consists of the fruit of *Citrus aurantium* (C.a). This part is chosen because of its high frequency of use by the Relizanese population (0°33′21″ East, 35°44′14″ North of Algeria).

After harvesting, The samples were transported in sealed bags to the laboratory of the Department of Agricultural Sciences, Faculty of Natural and Life Sciences (University of Relizane, Algeria). The plant is then authenticated by a botanist from the same department., and by comparing its characteristics with those mentioned in the literature.

Minced meat

In order to study the antimicrobial effect of the essential oil of C.a, we chose minced meat, as it is an important source of contamination germs. The samples were purchased in a butcher's shop located near the covered market of Relizane city. The meat was then placed in sterile Whirl pak bags and transported directly to the laboratory in a cooler equipped with a cold accumulator.

Extraction of essential oils

Five grams of C.a fruit are placed in a flask. The whole is placed in a hydrodistillation set-up and boiled for about 3 hours. The vapour from the distillation is condensed in a refrigerator and then recovered. The distillate is then subjected to an extraction step (liquid/liquid) in a separating funnel, using cyclohexane (C_6H_{12}) and a release agent, sodium chloride (NaCl). The aqueous phase is removed. Then, the oils are recovered from the organic phase following the use of a rotavapor [9]. According to the AFNOR (1986) standard [10]. The essential oil yield is expressed according to formula (1).

$$Y\ \% = \frac{w1}{w0} \times 100 \qquad (1)$$

Where, Y: yield of essential oil w1: weight in grams of essential oil w0: weight in grams of plant material.

Conservation

After recovery of the essential oils, the samples are then stored away from air and light in stained and closed tubes, in order to avoid any kind of evaporation until the moment of use [9].

Physicochemical analysis of essential oils

Determination of relative density — According to the AFNOR standard,1986 [10]. A volume of 1 ml of the essential oil of C.a was weighed, and then the weight of the same volume of distilled water was weighed separately. The relative density was calibrated according to formula (2).

$$d_{20}^{20} = \mathrm{dt'} + 0{,}00068\ (\mathrm{t'} - \mathrm{t}) \qquad (2)$$

Where d_{20}^{20} is the reference density index. $\boldsymbol{dt'}$: refractive index. **t**: 20°C. **t'**: temperature at the time of measurement.

Determination of the acid value —One gram of essential oil was introduced into a flask to which 5 ml of ethanol is added. The whole is shaken vigorously and then titrated with an ethanolic solution of potassium hydroxide (KOH=0.1 mol/L). In the presence of 5 drops of phenolphthalein. The volume of KOH used for the neutralization is read directly on the burette [11].

The acid value (AV) is expressed by the formula (3):

$$AV = \frac{56.11 \times N \times V}{w} \qquad (3)$$

Where, N: Normality of potassium hydroxide, V: Volume of the ethanolic KOH solution used for the titration in ml, w: weight of essential oil.

Determination of ester value—A volume of 0.5 ml of essential oil and another 15 ml of KOH solution (0.5 mole/L) are placed in a flask connected to an ascending refrigerator. Then the whole is brought to the boil in a water bath. When the solution has become transparent due to the effect of heat, saponification is completed. After cooling, the solution is removed from the flask and placed in a beaker and titrated with HCl (0.5N) in the presence of 5 drops of phenolphthalein. In parallel, the blank is performed under the same conditions [12]. The ester index (EI) is calculated according to the relation (4):

$$EI = \frac{28.05}{w\ (V0 - V1)} - AV \qquad (4)$$

Where V0: volume in ml of HCl for the blank, V1: volume in ml of HCl for the determination, w: weight of the test sample.

Determination of saponification value —The saponification value (SV) is calculated according to the relation (5) [13].

$$SV = AV + EI \qquad (5)$$

Gas chromatography-mass spectrometry analysis (GC/MS)

A chromatograph linked to a mass spectrometer of the Shimadzu QP2010 type was used for the analysis of extracted oils. A DB-5 ms (25 m ×0.25 mm ×0.25 µm) was used as capillary column. A volume of 1 µL of the mixture of essential oil and cyclohexane was injected in splitless mode. A flow rate of 0.5 ml/min of helium as mobile phase was used during 4 min to browse the entire column. Equipment temperatures were fixed at 250°C and 280°C. In addition, the detector was programmed at 250 °C. A range of 35 to 400m/z at 0.3 s scan-1 was set to record the mass spectrum. After migration, the characterization was done by referring to a database, based on the comparison of retention time (RT) of compounds of these essential oils and other pure chemical compounds [14].

Tests in food products

The aim of this test is to analyse raw minced meat by determining the microorganisms it contains, in order to evaluate the effectiveness of the added essential oil of *C.a* as a preservative.

Sample preparation —The bulk sample is divided into 100 g parts. Two parts were mixed with an appropriate volume of EO: 5 µL and 15 µL. In contrast, a control sample was not added. The samples were placed in petri dishes and stored at room temperature in order to create the right conditions for the alteration of the minced meat.

Sensory evaluation — Particular attention was paid to colour, abnormal odour and the presence of exudate in order to monitor the spoilage of the minced meat [15].

Microbiological analysis

Total aerobic mesophilic flora (TAMF), *Staphylococcus aureus* and *Salmonella* were analyzed in the treated meat and the control.

Preparation of decimal dilutions —Ten grams of minced meat are mixed with 90 mL of buffered peptone water and the mixture is vortexed and allowed to stand for one hour at room temperature.

Decimal dilutions are prepared from the stock suspension.

A volume of 1 mL of the stock solution is placed in a tube containing 9 mL of buffered water, making the dilution to 10^{-1}.

After homogenization, a volume of 1 mL of water in the same way into another tube which in turn contains a fresh volume of 9 ml of buffered peptone water, to make the dilution $10^{-2}$and so on until the dilution $10^{-6}$is obtained [16].

Research and enumeration of TAMF—The enumeration of this microflora was carried out by the mass plating method of a 1 mL volume on PCA agar.

Two plates were plated from each dilution.

Incubation is done for 72 hours at 30°C [15]. The count is expressed as a forming unit using formula (6).

$$N=\frac{\varepsilon c}{(n1+0.1\,n2)d}\times\frac{1}{v} \qquad (6)$$

Where, ∑c: total number of colonies found; n1: number of plates chosen from the lowest dilution; n2: number of plates from the next dilution chosen; d: dilution factor; V: volume of the test sample in ml.

Testing and enumeration of Staphylococcus aureus—In petri dishes containing Chapman medium 0.1 mL of $10^{-5}$and 10^{-6} dilutions are spread on the surface by a spreader. The dishes are then inverted and incubated at 37°C for 24 hours [15].

Testing for *Salmonella*—As a pre-enrichment step, 25 g of each preparation was placed in 225 mL of buffered peptone water and incubated at 37°C for up to 20 hours. Enrichment was done by taking 1 mL of pre-enrichment broth, which was then placed in a 20 mL tube of broth (Cystine-Selenite).

Incubation was done at 37° for 24 hours.

Then a volume of 0.1 mL is spread on the surface of the Salmonella-Shigella medium [15].

Statistical study

The results are expressed as the mean ± standard deviation of three trials. Statistical analysis one- way (analysis of variance [ANOVA]) was applied to the data. A P value less than 0.05 is considered significant.

3. RESULTS AND DISCUSSIONS

Yield essential oils

Yield essential oils of C.a was found to be around 0.09 ± 0.01%. This is considered low compared to some plants already exploited industrially. According to the results quoted in the scientific literature, it is true that citrus fruits contain little essential oil. Essential oils obtained from the fruit of *Citrus reticulate*, *Citrus sinensis* and *Citrus paradisia* gave yields of 0.30 ± 0.01, 0.24 ± 0.01 and 0.20 ± 0.01 respectively [16]. The average yield of essential oils, of both *Citrus sinensis* and *Citrus aurantium* plants, calculated on the basis of dry matter found to be around 1.2% [17]. Fresh leaves essential oil (0.43%) were obtained with a higher yield than that from the skin (0.30%) and flowers (0.20%) [5]. A yield of 0.73% in essential oil from *Citrus aurantium* leaves obtained in the Chlef region [18]. The variability of essential oil yield can be related to several factors, geographical location, season, organ, species [19-20], extraction method [21]. and light as it also stimulates essential oil production [22].

Physico-chemical characteristics

The essential oils of the C.a fruit have a viscous, clear, white transparent liquid appearance. They are characterized by a refreshing odour characteristic of citrus fruits.

Physicochemical indices of essential oils of C.a

The physicochemical analyzes (relative density at 20°C, acid index, saponification index and ester index) are considered as parameters for characterizing and assessing the quality of essential oils or other fats. The results are summarized in table 1.

Table 1. Physicochemical indices of *Citrus aurantium* essential oils

Physicochemical indices	*Citrus aurantium*
Acid value (mg KOH/g oil)	44 ± 3.0
Saponification value (mg KOH/g oil)	46.75 ± 5.1
Ester index (mg de KOH/g)	2.75 ± 0.5
Density index	0.69 ± 0.1

In contrast, the relative densities at 20°C of three *Citrus* species essential oils obtained from the fresh fruit ranged from 0.815 ± 0.02 to 0.834 ± 0.02 [16]. A value of 0.863 of the relative density at 20°C was found in sour orange essential leaves [18]. The value of the relative density of

our sample is close to that found by Edogbanya et *al* (2019), who worked on the essential oil of the orange peel with D=0.67 to [23]. Relative density is a physical characteristic, widely used in the classification of essential oils; this data is still not sufficient for the identification of oils [24].

The acid value is a parameter that measures the amount of free fatty acids in the fat or oil [25], and also considered as an excellent means to determine its rancidity status [26]. Our results are strictly superior to those found by BrixiGormat et *al*, (2015), where they found a rate of 1.21 in the seeds of the same species, harvested in the Tlemcen region [27]. The acid value of the leaves of the same plant species was equal to 1.40. However, the ester index is equal to 12.25 [18].The AFNOR standard has set this index at a value less than or equal to 2 [24].

On the other hand, the saponification value was expressed as the sum of the acid number and the ester number. The amount of saponifiable units (acyl groups) per unit weight of fat gives the value of the saponification index. And consequently, a high value indicates a higher proportion of low molecular weight fatty acids. [28]. This parameter is quantified in the oil to measure the average molecular weight, it is also expressed in milligrams of potassium hydroxide per gram of oil tested (mg KOH /g of oil). Our results are low compared to those found in Egyptian *Citrus* seed oil which varied between 186.8 and 191.3 [29,30,25].

Essential oil analysis by GC/MS

The chromatographic analysis of C.a essential oils were grouped in table 2.

Table 2. Chromatographic analysis of C.a essential oils

Chemical compounds	Retention time (min)	Percentage (%)
Limonene	9.8	19 ± 3
Linalool oxide	11.8	10.7 ± 0.5
Linalol	13.2	43 ± 1.5
Terpineol alpha	18.7	11± 1
Unidentified compound	-	16.3

About the results observed the essential oils of this plant was rich in linalool with a percentage equal to 43 ± 1.5% followed by limonene (19 ± 3%). However, many compounds were not identified (16.3%). The results are very close to those observed in 2019 [9].

This can be explained by the proximity of the harvesting regions, because Mascara is located 60 km from the town of Relizane. Linalool (64.6% ± 0.04), α-terpineol (7.61% ± 0.03), (R)-limonene (6.15% ± 0.04) and linalyl acetate (5.02% ± 0.03) are characterized and found as major components in bitter orange flowers [31].

In parallel, the major components of Citrus aurantium essential oil leaves were linalyl acetate, linalool, α-Terpineol, geraniol and geranyl acetate [32].

The composition of essential oils is influenced by several conditions; climatic: temperature, light, water status and day length; cultivation: soil type, soil properties, soil fertility, sowing date, planting density, cultivation techniques and cultural practices: irrigation dose, mineral supply and fertilization. [33-36].

Biotechnological application of C.a essential oils as a preservative for beef mince.

The minced meat was subjected to sensory and microbiological analyses after being mixed with two different doses of C.a essential oils (5 µL and 15 µL). On the other hand, meat without EO is used as a negative control. The samples are coded as follows:

-Sam 1: minced meat without EO (negative control)

-Sam 2: minced meat with 5 µL of the EO added

-Sam 3: minced meat with 15 µL of EO added

Sensory analysis of minced meat —After 72 hours of incubation of the samples at room temperature, sam 3 remained intact in terms of appearance, colour and odour. However, macroscopic observation of sam 2 showed a slight degradation of colour and an unpleasant smell. Sam 1 was totally spoiled, a purplish colour, a foaming appearance while losing its consistency, and a rotten smell of the flesh (Fig 1).

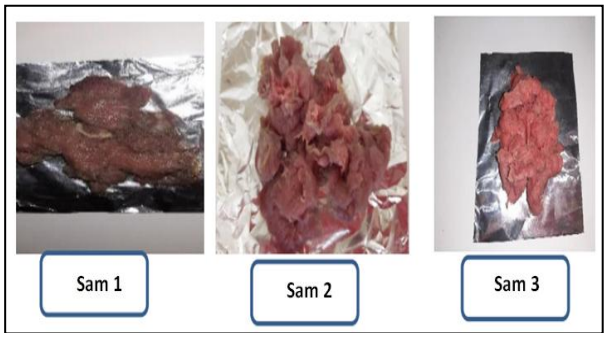

Figure 1. Macroscopic appearance of the samples

Microbiological analysis

Figure 2 showed the count of TAMF, *Salmonella* and *Staphylococcus aureus*. In view of all the results of the microbiological analysis, samples 3 and 2 are not spoiled, given the acceptable number of TAMF (2×10^4 CFU/g and 4×10^4CFU/g) respectively and the total absence of *Salmonella* and the spoiling species, *Staphylococcus aureus*, because the Algerian official journal has set threshold values for TAMF (1.5×10^6-5×10^6CFU/g), *Staphylococcus aureus* (10^3-3×10^3 CFU/g) and a total absence of any trace of *Salmonella*. Similarly, the number of 10^4 *Salmonella* per gram of sam 1, and that of the total aerobic mesophilic flora (6×10^6CFU/g) indicate that its microbiological quality and sensory quality are totally degraded. C.a essential oil fruit can be used as a powerful preservative. Indeed, the TAMF constitutes a better indicator of overall contamination and reflects the

hygienic quality of any product through the microbiological information that it provides [37]. The addition of lemon essential oil to fresh cream has a slight effect on the bacterial flora (TAMF), but significantly reduces the number of yeasts.

This essential oil could not act as a preservative in fresh cream at concentrations of 0.125%; 0.25% and 0.5%, and it could not extend the consumption date of fresh cream after 48 hours of opening the jars [38]. Limonene was described as the majority component of *Citrus aurantium* essential oil (67.04%). This constituent contributes to the antibacterial efficacy of the tested essential oil towards microbial strains [39]. Our results are in agreement with those recorded by Frassinetti et al, (2011) where gram negative bacteria are highly susceptible to essential oils extracted from bitter orange tree [40].

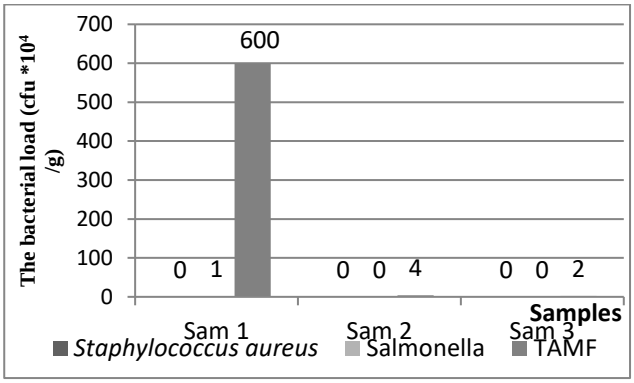

Figure 2. Enumeration of TAMF, *Salmonella* and *Staphylococcus aureus* in minced meat samples in CFU/g

4. CONCLUSIONS

The use of essential oils as antimicrobial agents has two main characteristics, on the one hand their natural origin which is a means of safety for humans and the environment and on the other hand, they do not cause any resistance to microbial germs.

The antimicrobial effect of *Citrus aurantium* essential oil can be widely used by industry as a preservative in many cosmetic, pharmaceutical and, food preparations as an alternative to chemical agents.

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