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Study of the antibacterial and antioxidant properties of the essential oils of four citrus varieties, *Citrus sinensis*, *Citrus reticulata*, *Citrus limon* and *Citrus maxima* (Rutaceae) consumed in Gabon

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Abstract

Our study focused on the study of the antibacterial and antioxidant properties of citrus essential oils (EO) of *Citrus sinensis*, *Citrus reticulata*, *Citrus limon* and *Citrus maxima*. The extraction of essential oils from the four citrus species was carried out using the hydrodistillation method using an extraction device adapted to the Laboratory. The antioxidant activity of different citrus fruits has been evaluated by neutralizing the DPPH radical. The following yields of EO were obtained: 0.74% and 0.80%, respectively for the EO of the sweet orange from Morocco and the wild orange from Cameroon; 0.2%, for EO of Mandarin; 0.18% and 0.13% respectively for the EO of *lemon* and Grapefruit. Microscopic observations revealed the presence in fermented milk, apart from lactic acid bacteria of the presence of Micrococcus *spp* Gram + bacteria from the family of Micrococcaceae been used for antibacterial testing. The results showed that HE has antibacterial activity with regard to the diameters of the halos. It appears that all citrus fruits have different activities. The EO of *C. lemon* has the best antioxidant activity followed by that of the Wild Orange with a diameter of the inhibition zone of 13 ± 1.41 and 9.5 ± 1.41mm respectively.

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Introduction

Medicinal plants are nowadays an important source of new bioactive molecules with therapeutic potential (Kosalec, S. *et al.*, 2005). One of the major originalities of medicinal plants lies in their capacity to produce very diverse natural substances. In fact, alongside the classic or ubiquitous primary metabolites, i.e. those found in most cellular reactions (carbohydrates, proteins, lipids, nucleic acids), they frequently accumulate so-called secondary metabolites, the physiological function is not always obvious but represents an important source of bioactive molecules usable by Man in fields as different as pharmacology or the food industry (Boukri N., 2014). These metabolites, produced in very small quantities, are accumulated in different organs and sometimes in specialized cells of the plant (Boudjouref M., 2011). Essential oils represent a very interesting group of secondary metabolites.

These are complex mixtures made up of several tens or even more than a hundred compounds, mainly terpenes (compounds whose basic unit is monoterpene made up of 10 carbon atoms and formed from two isoprene units) and aromatic compounds (Haib El., 2011; Seddik, M. 2011), with antimicrobial and antioxidant properties of which they represent a very good source of food preservative, and many other important biological properties (Dung, N.T. *et al.*, 2008). The objective of this work is to evaluate the antibacterial and antioxidant properties of the essential oils of citrus fruits consumed locally, *Citrus sinensis*, *Citrus reticulata*, *Citrus limon* and *Citrus maxima*.

Material and methods

Context of the Study and Plant Material

Context of the study

Our study took place in Libreville in Gabon, in the province of the Estuary between July 2018 and January 2019 period in line with the abundance of fruit on the market stalls of Libreville.

Sampling

Our samples were purchased in the urban food markets of Mont-Bouët, Petit-Paris and Venez-voir located in the 3rd Arrondissements of the Gabonese capital. The fruit sampling was brought to the Multidisciplinary Sciences Laboratory (LAPLUS) of the École Normale Supérieure.

Plant material

The plant material chosen in this study is represented by the peel of the fruits of four citrus fruits of the Rutaceae family, including orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), lemon (*Citrus limon*) and grapefruit (*Citrus maxima*). All the fruits after their purchases were sent then washed to the Multidisciplinary Laboratory of Sciences before extracting the different essential oils.

Citrus essential oil extraction

Device used for the extraction of essential oils

The essential oils were extracted by hydrodistillation using a suitable Clevenger device for 4 hours. Freshly harvested citrus epicarps are directly immersed in a pot containing distilled water.

The water is brought to a boil, the vapors are condensed by passing through a refrigerant and are recovered in a separating funnel or two phases are observed.

The lower phase (hydrosol) is collected in a beaker by decantation; the upper phase (organic phase) is the essential oil. To remove traces of water, we used magnesium sulfate as a moisturizer. At the end of each experiment, the essential oil recovered is stored in the refrigerator and protected from light in a tightly closed bottle. The extraction yield is calculated by the following method (Afnor, 2007): Yield (%) = (Mass of essential oil obtained/mass of citrus epicarp used) x100.

Antibacterial activity

The antibacterial activity was carried out by the method on discs; the culture is seeded on the surface of a Mueller-Hinton agar, which is a basic medium for the cultivation of non-demanding bacteria. Discs, made from Whatman filter paper previously sterilized in an autoclave of 6 mm in diameter soaked in

essential oils, are placed on the surface of the agars or culture medium.

The bacterial strains used

The analysis of fresh and turned yogurt allowed us to identify three bacterial strains isolated from milk, notably lactic bacteria (lactobacillus and streptococcus) and micrococcus spp. The presence of micrococcus spp in our milk is not a source of danger. In fact, the Micrococci are mainly found on the skin of mammals and in this case we could have contamination during the preparation of our yogurt or even during the manipulations in the laboratory. They are also found on meat, dairy products, soil and water. Generally non-pathogenic, Micrococci can behave as opportunistic pathogens in immuno-compromised people. The bacterial strains used in the evaluation of the antibacterial activity of citrus essential oils as part of our study were isolated in the Microbiology Laboratory of the Institute for Research in Tropical E*cology (IRET), from Canaan milk*. After isolation and purification, these bacterial strains were stored at 4 ° C in a refrigerator. These strains are classified as non-pathogenic being commensals.

Culture media

To obtain young colonies of bacteria in order to isolate them and to highlight the antibacterial and antioxidant activities of essential oils, several media were used: the Tryptone Casein Soybean Medium; EMB medium (methylene blue eosin agar or Levine medium); the Muller-Hinton Medium.

Isolation media

To isolate the bacteria present in Canaan's milk, we used two culture media: the Eosine Blue medium of Methylene, originally recommended by Levine, is used to isolate and identify Escherichia coli and Enterobacter, as well as intestinal bacteria with Gram negative in pharmaceuticals, dairy and other food products. It is also used for water control as an isolation and identification medium after culture in a liquid medium; Tryptone Soybean Casein medium, which is specially designed to highlight hemolytic reactions and to promote the growth of particularly demanding aerobic and anaerobic germs. It is also used to practice the CAMP test or for the confirmation of Legionella in waters.

Seeding

The inoculation of the culture media on the surface was carried out using a loop, for this approximately 0.5 µL of the suspension to be inoculated in the Petri dishes containing the agars previously prepared under the strict aseptic conditions, that is to say sheltered from all forms of contamination.

Isolation of bacterial strains

The four quadrant method (also called the streak or zigzag method) is used for most samples because it will allow colonies isolated from different germs to be obtained. It provides a semi-quantitative estimate of the quantity of germs contained in the various samples. The quadrants are made by zigzagging the quarters of the agar with a variable amount of sample.

Multiplication of isolated strains

The selected strains were placed in micro-tubes with a capacity of 1.5µL of water previously sterilized in an autoclave and then left in the high security at room temperature. The study of the morphology of the bacteria consists in a macroscopic description of the colonies obtained during the culture of these microorganisms and a preparation in the fresh state makes it possible to examine the mobility of the bacteria and to detect their form, the associations and the mobility of bacteria. Standard catalase, ONPG and oxidase tests were used.

Biochemical characterization test of isolated bacterial strains

The biochemical characterization test of the bacterial strains extracted from Canaan's milk was done thanks to the API Staph gallery. API Staph is a standardized system for the identification of the genera Staphylococcus, Micrococcus and Kocuria including miniaturized biochemical tests and a database. The list of bacteria that can be identified with this system is given in an identification table at the end of the leaflet.

Study of the antibacterial power of citrus essential oils

The bacterial growth inhibition test is a laboratory technique designed to test the sensitivity of the strain

of the bacteria that has been extracted from Canaan's milk to the metabolites produced by them.

Sensitivity of bacteria in solid medium

The sensitivity of bacteria was studied using the standard disc or antibiogram method. Standard diffusion methods or antibiograms are the most used by diagnostic laboratories. Discs of Whatman paper impregnated with essential oils to be tested are placed on the surface of an agar medium previously sown with a pure culture.

Evaluation of bacterial activity

To assess the antibacterial activity of essential oils, we used the disc diffusion method. Although it is recognized as reliable and reproducible, it is mainly used in the preliminary stage to more in-depth studies, because it allows access to essentially qualitative results.

The technique used is a modification of the method of (Amanda J. *et al.,* 2002). It consists in using paper discs impregnated with the different substances to be tested. The discs are placed on the surface of an agar uniformly seeded with a suspension of the bacteria to be studied. Each essential oil diffuses from the disc within the agar and determines an inhibition zone there according to its concentration.

The bacteria grow on the entire surface of the agar except where they meet a sufficient concentration of antibiotic to inhibit their growth. There is thus observed around the discs a circular zone free from colonies, called inhibition zone.

The larger the diameter of this area, the more sensitive the strain to the essential oil. The smaller it is, the more resistant the bacteria. For the technical performance of the work, sterile 6 mm diameter filter paper discs were placed on the surface of each box. After incubation, the inhibition diameter was measured in millimeters with the disc included.

Antioxidant activity evaluation

The methods used to assess the antioxidant activity of essential oils are relatively few and generally involve the coloring or discoloration of a specific reagent in the presence of an antioxidant agent. For our study, we opted for the method which uses DPPH• (2,2-diphenyl-1-picryl-hydrazyl) as a stable radical of purple color absorbed at 517nm.

This measurement was carried out according to the method described by Brand-Williams and colleagues (Brand-Williams W. *et al.,* 1995). The reduction of an alcoholic solution of the stable radical species DPPH• in the presence of a hydrogen-donating antioxidant (HA) results in the formation of a non-radical form $DPPH_2$. This reduction is followed by the measurement of the decrease in its absorbance at 517nm in the presence of an antioxidant.

Statistical analyzes

After collection, the data was first entered into Excel 2016 software and then analyzed using Statview 05 software for comparison of means.

Results

Extraction of essential oils

Organoleptic characteristics of essential oils

The organoleptic parameters of the essential oils obtained by the hydrodistillation of the five citrus species selected for our study are summarized in Table 1.

Table 1. Organoleptic and quantitative characteristics of the essential oils of five citrus fruits.

Essential Oil origine	Color	Odor	Aspect	Fresh Weight (g)	EO (g)
Orange from morocco *(Citrus sinensis)*	Clair yelow	high		2121.79	15.62
Wild orange *(Citrus sinensis)*	Transparency	high		1360.81	10.89
Mandarine *(Citrus reticulata)*	Transparency	high	Limpid liquide	1880.64	3.75
Citron *(Citrus limon)*	Transparency	high		2106.11	3.74
Pamplemousse *(Citrus maxima)*	Yelow	Douce		2315.07	2.90

EO: essential oil

Yield of essential oil extractions

The yields of essential oils of the different citrus fruits are grouped in Fig. 1

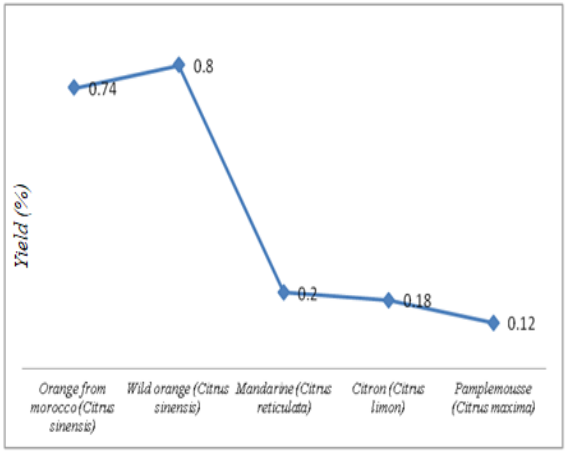

Fig. 1. Citrus essential oil production yields.

Antibacterial activity

Macroscopic observations

Under conditions favorable to the growth of lactic acid bacteria after an incubation of 24 h at 37° C in an oxygen-depleted atmosphere, the results obtained from the culture on an enriched agar result in the appearance of small colonies.

The colonies have a white coloring. Among these colonies, macroscopic observation of the culture medium in which we seeded the bacteria derived from normal fermented milk revealed the presence of a few colonies of yellowish coloration.

Microscopic observations

Gram stain of bacteria isolated from normal milk

Gram staining, carried out from the appeared colonies, shows the presence of Gram negative bacteria. They are in the form of a straight bacillus, curved, comma, with regular contours.

Microscopic observations, after gram staining, allowed us to observe in normal fermented milk (that is to say that has not yet passed the expiration date) the two lactic acid bacteria: *Streptococcus thermophilus* (in the form shell, spherical, Gram + bacteria) and *Lactobacillus bulgaricus* (Fig. 2).

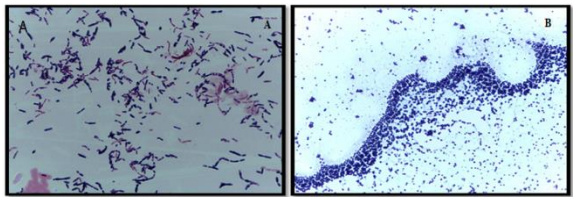

Fig. 2. Observation of bacteria from normal (A) and contaminated (B) milk with MO, objective 100.

Catalase, ONPG and Oxydase test

The catalase tests revealed that our bacterial strain is sensitive to the catalase test (Catalase +). The ONPG tests revealed that there was no ONP (yellow colored product) in the medium, for this purpose our tested bacterial strain did not hydrolyze the ONPG. Oxydase tests have revealed that our tested bacterial strain is negative for the catalase (Oxydase -) test.

Biochemical characterization test for bacterial strains

The result of the biochemical characterization tests of our bacterial strain allowed us to affirm that the bacteria studied do not participate in the fermentative process of milk (degradation of glucose into lactic acid). Indeed, all the verification tests were negative.

Evaluation of antibacterial activity

The in vitro evaluation of the antibacterial activity of the essential oils of the citrus fruits tested is carried out using the disc diffusion method. The measurement in millimeters, of the diameter of the zones of inhibition of the cultures, makes it possible to evaluate the effectiveness or not of the essential oil on the germs tested. In general, the essential oils tested exerted an antibacterial activity. Through our results, crude orange essential oils from Morocco and lemon have shown a greater inhibitory effect against the strains tested. The aromatograms method is the technique chosen to assess the antibacterial activity of lemon essential oil. To this end, a scale for measuring antimicrobial activity was used by Ponce (Ponce A. G. *et al.*, 2003) (Fig. 3).

Antioxidant activity

The antioxidant activity of citrus essential oil was evaluated using the DPPH radical scavenging method by comparing with that of ascorbic acid.

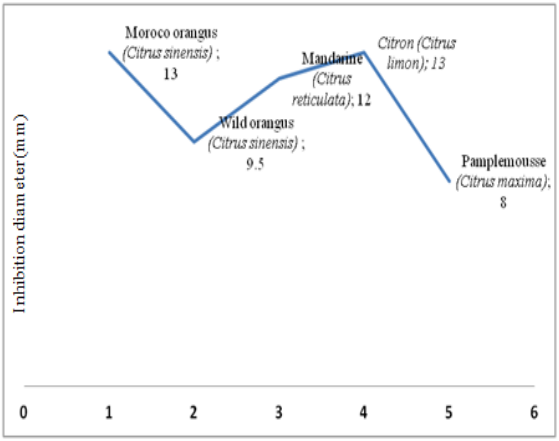

Fig. 3. Diameters of the inhibition zones (mm) of citrus essential oils.

Sensitive (+) : diameter between 8 and 14 mm ; Very sensitive (++) : diameter between 14 and 19 mm

The results obtained show that essential oils have a capacity to trap the DPPH° radical, as we also note that the percentage of inhibition of the radical is proportional to the concentration of the different essential oils used. The neutralization of the DPPH radical by extracts from different parts of citrus fruits has been attributed, by several authors, to the presence of phenolic compounds which easily give up protons to reduce it (Burt S. 2004). The antioxidant activity of lemon essential oil was assessed using the method of DPPH radical trapping by comparing with that of ascorbic acid. In general, the antioxidant activity of lemon essential oil has been found to be very high at high concentrations. It is observed that the essential oil of lemon exhibits a higher activity than that of ascorbic acid.

The results show that the DPPH radical has an intense purple coloration which disappears on contact with a proton-donating substance followed by the appearance of the yellow coloration. This change in color highlights the antioxidant power of our oils by their ability to trap the free radical. These results also show a proportional increase in the inhibition percentages of the free DPPH radical as a function of the different concentrations of the total oil; which made it possible to obtain logarithmic curves. This result indicates that our essential oils have strong antioxidant activities (Table 2).

Table 2. antioxidant tests.

HE	IC_{50}	Regression Equation	R^2	IAA
Ascorbic acid	6.78	y = 7.2626x + 0.7862	0.989	0.738
Citrus sinensis	1.44	y = 34.551x + 0.3943	0.530	3.483
Citrus limon	0.74	y = 68.201x – 0.5838	0.850	6.741

IAA: Anti-oxidant Activity Index; IC_{50}: Concentration inhibiting 50% of DPPH.

Discussion

The device for extracting essential oils by hydrodistillation set up within the Laboratory of Life and Earth Sciences (LaSciViT) made it possible to extract essential oils from our different citrus varieties found in the markets of the capital Libreville (Gabon). The extraction yields vary considerably depending on the different samples and the citrus species. Regarding the extraction yield of the essential oil of Mandarin (*Citrus reticulata*), the results show a yield of about 0.2%. These values obtained are very low compared to the few rare works carried out on the peel of Mandarin fruits which were able to reach a yield four times higher than ours, that is to say approximately 0.88% (Bouguerra A. *et al.,* 2014).

Regarding the yield of essential oil from the peel (skin) of lemon (*Citrus limon*), the results show the same deficit about 0.4%, that is to say 0.62% or even 0.7% found by a study (Yamina B. M. *et al.,* 2018; Djamel D. , 2015) compared to ours which is 0.18%. By comparison with the results obtained from genetically modified products as shown by certain studies (Soumaya B. *et al.,* 2012). Ahmed S *et al.*, 2006) which obtained respective yields of 1.30% and 1.12% much higher from natural products. The results on the extraction of grapefruit essential oils give a yield of 0.13%. These results are not far from those of Hellal which are 0.18% (Hellal Z., 2011).

Only the yield of grapefruit essential oil remains comparable to that found by other authors. This oil would have many other properties that allow it to be more stable. Extractions carried out on the bark

(epicarp) of wild and sweet oranges gave yields of 0.8% and 0.74% respectively. These results seem to indicate that wild oranges are very rich in essential oils and like grapefruit essential oil; the space-time conditions do not influence the yield of the essential oil of wild orange. Citrus extraction rates do not match the results from the literature. These differences can be explained by various factors such as their products are grown in approved research centers where these fruits are freshly harvested while ours are bought in the local market. The time between the harvest period and that of treatment is very short. This helps maintain biological properties, in this case essential oils. Furthermore, by comparison, the time separating the harvest period from that of the use for the extraction of these oils is very long. Besides the physiological differences between these cultures, there are several parameters, the types of distillation apparatus used, as well as the quantities that have been distilled. The authors (Zrira and Bejilali, 1991, 1992) agree with these results. According to them, the yield, physical characteristics and chemical composition of essential oils can be also influenced by various factors such as species, harvest period, age of the plant; the humidity of the plant material, the part subject to distillation and finally the essential oil extraction technique.

The results of antibacterial tests revealed, by measuring the diameters of the zones of inhibition, that the essential oils of citrus fruits have antibacterial activity. The tests were carried out by the method of diffusion on paper soaked in essential oil and the inhibition diameters formed around this paper 24 hours after incubation at 37°C. These tests were carried out on bacteria extracted from fermented milk (normal milk and contaminated milk). Microscopic observations revealed the presence of Lactobacillus bulgaricus and *Streptococcus thermophilus* but also of Micrococcus spp. However, the presence of *Micrococcus spp* could indicate contamination of the milk. Micrococci are mainly found on the skin of mammals and in this case we could have contamination during the preparation of our yogurt or even during laboratory manipulations. They are also found on meat, dairy products, soil and water. However, Micrococci can behave as opportunistic pathogens in immuno compromised people.

However, our results could admit certain limits with regard to the identification of bacteria by biochemical characterization tests, because the API system Staph, used in our work, is intended for the identification of the species mentioned in the database and alone. It cannot be used to identify other microorganisms or to exclude their presence. Only pure cultures containing a single type of microorganism should be used. To this is added the validation tests. The evaluation of the antioxidant activity of citrus essential oils leads to the determination of the antioxidant activity indices (IAA) through the determination of the concentration which inhibits 50% of our DPPH (IC_{50}). The discoloration of the ethanolic solution of DPPH highlights the anti-free radical and antioxidant power of these oils by their capacity to trap the free radical. The lowest index of antioxidant activity for ascorbic acid is obtained (IAA = 0.74). This result shows that ascorbic acid has a moderate antioxidant activity. The essential oil of *Citrus sinensis* with an IAA index = 3.48 has a high antioxidant activity, on the other hand, that of *Citrus limon* (IAA = 6.74) has a very high antioxidant activity. Indeed, the antioxidant activity is considered low if IAA <0.5, moderate if 0.5 <IAA <1, high if 1 <IAA <2 and very high if IAA> 2 (Hellal Z., 2011). These results also show a proportional increase in the inhibition percentages of the free DPPH radical as a function of the different concentrations of the total oil allowing the logarithmic curves to be obtained. This result indicates that our essential oils have strong antioxidant activities.

Conclusions

This work relates to the evaluation of the antibacterial and antioxidant activities of citrus essential oils consumed locally for the conservation of Canaan milk. The sampling of citrus fruits in the markets of the Libreville capital allowed the testing of four citrus species on bacteria isolated from fermented milk.

The extraction of essential oils by hydrodistillation was carried out using a Clevenger type device using a suitable device. The yield values of the essential oils of the citrus peels were 0.13%, 0.18%, 0.20%, 0.74% and 0.80% (i.e. the yields of the essential oils of *Citrus maxima*, *Citrus limon*, *Citrus reticulata* respectively and *Citrus sinensis*).

The in vitro determination of the antibacterial power of essential oils was carried out by the method of diffusion of essential oils on Whatman paper. The five essential oils tested alone showed antibacterial activity by evaluating the activity.

The antioxidant activity of citrus essential oils was evaluated and compared using the following methods: the DPPH method. It has been noted that essential oils have shown antioxidant activity.

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