



Original Research Article

Assessment of the antimicrobial activity of essential oils from some Beninese medicinal plants :Influence of different tweens

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Medicinal plants have an important place in the therapy mechanism of Benin's population. According to the World Health Organization, 75% of African populations use plants for treatment. Many researchers used to work with tweens. Thus, this work aimed to study the influence of the use of different types of tween in the evaluation of the antimicrobial activity of four essential oils. It helped also to determine the minimum inhibitory and bactericidal concentrations of those oils including their chemical compositions. Essential oils from some plants were obtained. The sensitivity of germs namely *Micrococcus luteus* ; *Staphylococcus aureus* ATCC 29213 ; *Proteus mirabilis* ATCC 24974 ; *Pseudomonas aeruginosa* ATCC 27853 ; *Candida albicans* IP 4872 to the various essential oils by microdilution technique was tested. Two chromatographic analyzes were performed for each oil on a gas chromatograph electronically controlled pressure. Results and Implications: Tween 60 has the best features, tweens 20 and 40 at 5% do not allow having a good dispersion of the essential oils in liquid media and a good spread in agar media. Furthermore, tween 80 seems to have a synergistic action with the essential oils although it shows good results. The chemical analysis showed several major compounds: myrcene (11.48%), neral (33.53%), geranial (43.10%), estragole (97.10%), limonene (61.40%), 1,8-cineole (61.60%). The minimum inhibitory concentrations (MIC) of the oils range between 0.078 and 10 mg / ml and the Minimum Bactericidal Concentrations (CMB) range from 0.078 to 10 ml /ml.

Key words: Essential oil, Tween, medicinal plants, antimicrobial activity, mouthwash.

INTRODUCTION

Medicinal plants have an important place in the therapeutic resources of the humanity (WHO, 2002). This therapeutic

use of the power of plants to treat human diseases is very old and evolved with the history of humanity (Bourkhiss et

al., 2007). Nowadays, traditional medicine plays an important role despite the progress of modern medicine. The herbal medicines, essential health care throughout the world since the early days of mankind, are still widely used and have considerable importance in international trade. In addition, since 1977, the World Health Organization launched an active program for the promotion and development of traditional medicine, based solely on the use of medicinal plants (Gomez, 2003). Therefore, it uses a multitude of plants with different properties. Many of them have undergone extensive scientific studies and their therapeutic properties have been proven (Baudoux, 2002). Thus, the useful properties of *Citrus aurantifolia* Christm. Swing (Rutaceae), *Clausena anisata* Willd. (Rutaceae); *Cymbopogon citratus* DC. Stapf (Poaceae); *Eucalyptus camaldulensis* Dehn. (Myrtaceae) in traditional medicine have been demonstrated by Baudoux (2002). Others, unfortunately still remain poorly known or unknown. The declaration of Abuja (Nigeria) in April 2001 made of the research in the field of traditional medicine a priority for Africa. So many medicinal plants research work were carried out. About 75% of the African population use herbs for treatment (Pousset, 2004). In Benin, more than 80% of the population resort to traditional medicine for their health care needs (Sekoussounon, 2012). These plants are used in the form of decoction, pickled, brewed but can also be used in the form of essential oils (Quenum et al., 2003). These essential oils are easy to use and are mostly effective (Quenum et al., 2003). Today, for economic reasons or because of scarcity of natural sources, industry synthesizes a certain number of essential oils. It is still quite understood that many substances are extracted using techniques such as steam distillation and solvent extraction (Hmamouchi, 1992).

Several methods were used to test their antimicrobial properties in Benin, but unfortunately no research has been conducted on the effectiveness or the influence of surfactants used in the detection of antimicrobial activity of the extracts. This work aimed to study the influence of the surfactants in the evaluation of antimicrobial properties of medicinal plants in order to develop an effective method of detection of the antimicrobial activity of extracts from medicinal plants of Benin.

MATERIALS AND METHODS

Materials

Collection of plants

The leaves of the plants have been harvested in Porto Novo, Djèrègbe and Adjarra. These sites are located in the department of Ouémé.

Plants

Citrus aurantifolia Christm. Swing (Rutaceae), *Clausena*

anisata Willd. (Rutaceae); *Cymbopogon citratus* DC. Stapf (Poaceae); *Eucalyptus camaldulensis* Dehn. (Myrtaceae) were used.

Microorganisms

ATCC (American Type Culture Collection) and IP (Institut Pasteur de Strasbourg) reference strains namely: *Micrococcus luteus* ATCC 10240; *Staphylococcus aureus* ATCC 29213; *Proteus mirabilis* ATCC 24974; *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* IP4872 were used to antimicrobial and antibacterial studies.

The strains obtained from the Laboratory of Molecular Typing and Microbiology, Faculty of Science and Technology, University of Abomey-Calavi and the National Quality Control Laboratory of the Ministry of Health of Benin. They were stored at +4 ° C.

Methods

This work was a cross-sectional study of experimental kind which has been carried out over a period of 6 months (November 2014-April 2015). During that period, we got in the harvest of plants, extraction of essential oils, microbiological tests and the determination of their chemical composition.

Extraction of essential oils

The oils extraction was performed by steam distillation in a Clevenger type apparatus (Clevenger, 1928) as described by Verma et al. (2010). The essential oils recovered are weighed. Thus the oil yield of each extraction was calculated (Oussalah et al., 2007).

Chromatographic analysis of essential oils

Two chromatographic analyzes were performed for each oil on chromatograph in gaseous phase electronically controlled by the pressure kind HEWLETT PACKARD (HP5870 series) brand DELSI IGC 121C equipped with a capillary column CP WAX 52 CB (25 mm in length and 0,3 mm inside diameter) equipped with a flame ionization detector (FID) set at 250 ° C and supplied with a mixture of gas H₂/air. It is provided with a split-split less injector set at 240 ° C. The injection mode is split (leakage ratio 1/60 = 1 ml / min flow rate: 30 ml / min at a pressure of 1 bar). The carrier gas used was nitrogen with a flow rate of 1 ml / min.

Determination of Minimum Inhibitory Concentration by microdilution

Preparation of emulsion from essential oil

3920 µl of Mueller Hinton broth or 1000 µl Sabouraud were placed in the tube. 80 µl of the essential oil containing Tweens (20, 40, 60, 80) at 5% rate were added. Thus, surfactants which allow making soluble the essential oils

into the water were created. The whole was homogenized with vortex. In result, 20 mg/ml concentration was obtained.

Preparation of the microbial suspension

After isolation, a colony of bacteria was transplanted into 10 ml of sterile distilled water contained in a test tube. From stock solution was carried out a series of dilution with sterile distilled water. Each dilution obtained was cultured on Mueller Hinton agar or Sabouraud. After 24 or 48 hours, the number of Colony Forming Unit was counted. The dilution that gives a concentration of 10^6 cells/ml of bacteria and 10^7 germs of *Candida albicans* was chosen.

Preparation of the microplate

The microplate method was used to visual assessment of the growth of microorganisms in 24 wells plate. In each well, 950 μ l of Mueller Hinton broth was distributed. Then was added:

- 50 μ l of the essential oil stock solution to 20 mg/ml in each of the wells of the first column
- 950 μ l in each of the wells of the third column at a rate of one oil per row.

Then successive well-to-well dilutions were performed starting from the third column, row by row, so as to have a series of dilution at rate 2. The solution is homogenized at each mixture. We cultured all the wells except those in the first column with 50 μ l of an inoculum of 10^6 germs/ml. We have thus made two control wells namely: a culture medium control well plus essential oil (950 μ l + 50 μ l); a culture medium control well plus microbial suspension (950 μ l + 50 μ l). The microplate was covered and incubated at 37°C for 24 hours. At the end of time the comparison of the control wells and the tested wells was done. For *Candida albicans*, the cultivation was done on Sabouraud broth at 27°C for 48 hours.

Calculation of the MBC

This assessment was performed by together with the calculation of the MIC. Once the MIC known, the wells or the microorganism were inhibited, starting from the wells used in the calculation of the MIC and graduate towards the higher concentrations, they are removed and transplanted on Mueller Hinton agar or Sabouraud. We then made a control agar. We culture it using a platinum loop by streaks. In case of any visible growth, the proliferation of the germs is compared with that of the control agar. The CMB is then the lowest concentration for which there is at most 0.01% of surviving microorganisms.

Data processing

An input mask was designed with the software Epi-data version 3.1. It inserts the various species of the germs, the different type of Tween, the different type of essential oils

collected, and the different concentrations obtained in each test. Then we proceeded to the typing of the collected data from the input mask. At the end, the analysis was performed using the EPI-INFO software (version 7.1.5) and SPSS statistics (Version 21). The Tables and Figures have been processed through Microsoft Excel software (version 2016).

We used a method of quantitative analysis by comparing different concentrations obtained and illustrate them with trendlines.

RESULTS

The study revealed that the plants contain a lot of compounds as shown in Table 1.

Table 1. Main chemical components of four essential oils studied

Essential oils	Main compounds	Total (%)
<i>Cymbopogon citratus</i>	Myrcene 11.48%, Neral 33.53%, Geranial 43.10%, Geraniol 5.58%, Geranyl acetate 4.47%	98.16
<i>Clausena anisata</i>	Estragole 97.10%	97.10
<i>Citrus aurantifolia</i>	Para cymene 15.25%, limonene 61.40%, alpha terpineol 7.12%, alpha pinene 2.96%, beta pinene 9.20%	95.93
<i>Eucalyptus camaldulensis</i>	Alpha Pinene 12, 45%, beta pinene 3.05%, 1, 8-cineole 61.601%, limonene 9.740%, para- cymene 8.340%	95.18

Data analysis of the Figures 1 to 5 shows in general, that any essential oil when mixed with Tween, the curve of the MIC decreases from the Tween 20 to Tween 40 and Tween 40 to Tween 60. From Tween 60 the curve remains constant to Tween 80. This reflects the values of the MIC and decrease from Tween 20 to Tween 60 but become constant from Tween 60 to Tween 80 beside the five bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Candida albicans*). Table 2 showed the effects of tweens on different germs.

Antimicrobial power of the essential oils

The Table 3 shows the ratio CMB/MIC of each essential oil on the studied germs. This report has enabled us to determine the bactericidal of the oils. When the ratio is less

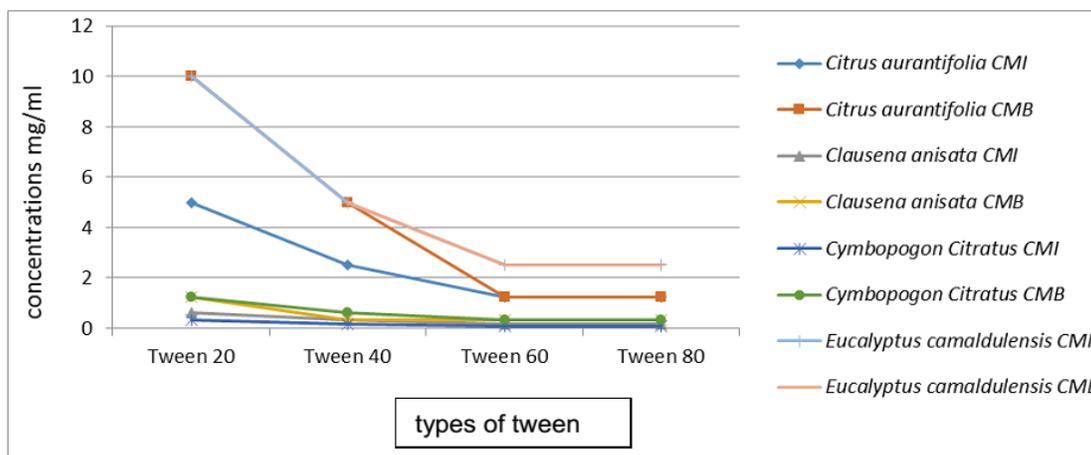


Figure 1: Evolution of CMI essential oils according to the different types of tween at 5% rate on *Pseudomonas aeruginosa*

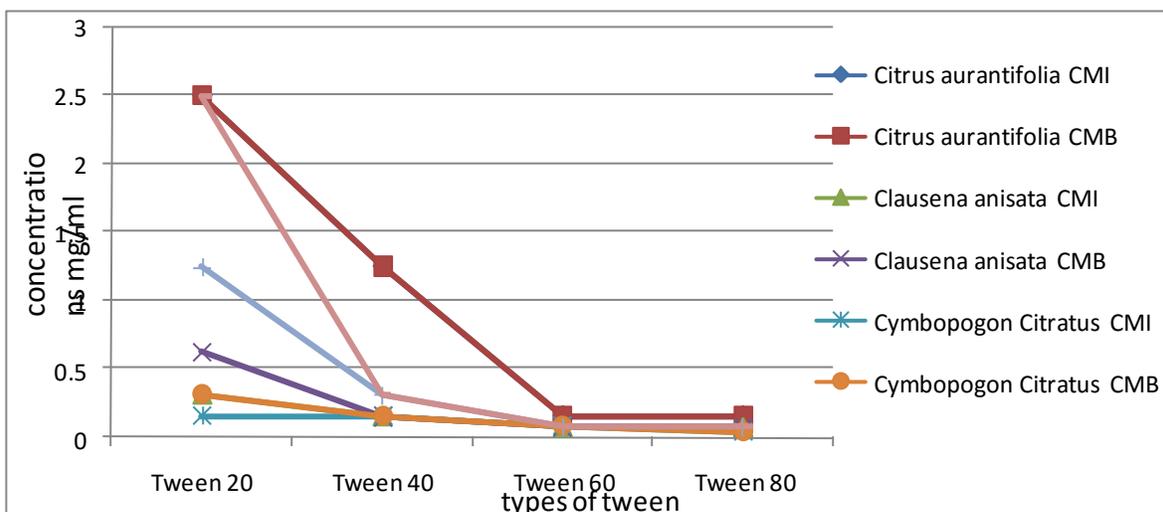


Figure 2: Evolution of CMI essential oils according to the different types of Tween at 5% rate on *Proteus mirabilis*

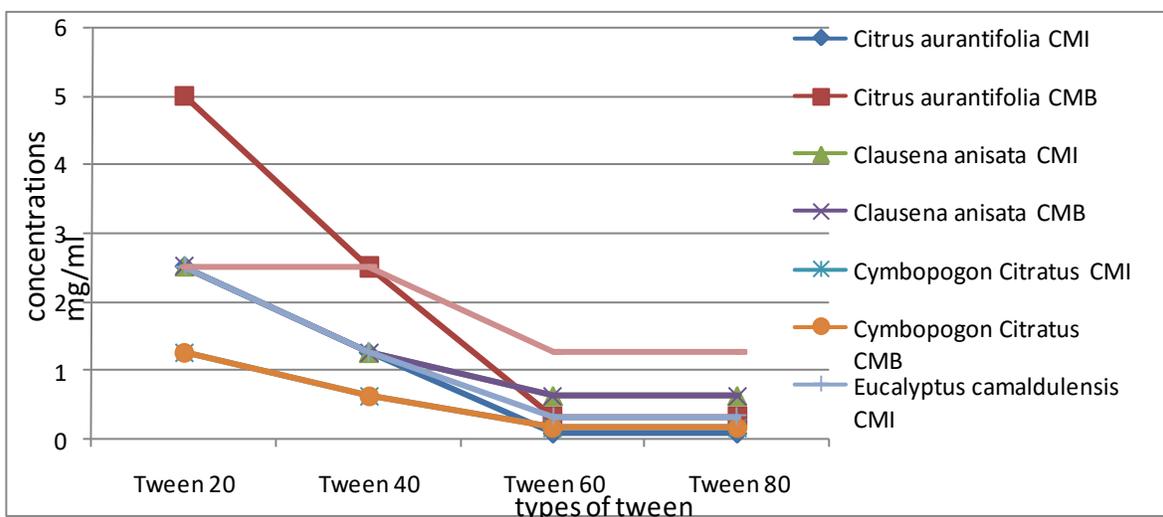


Figure 3: Development of CMI essential oils for the different types of tween at 5% rate on *Micrococcus luteus*

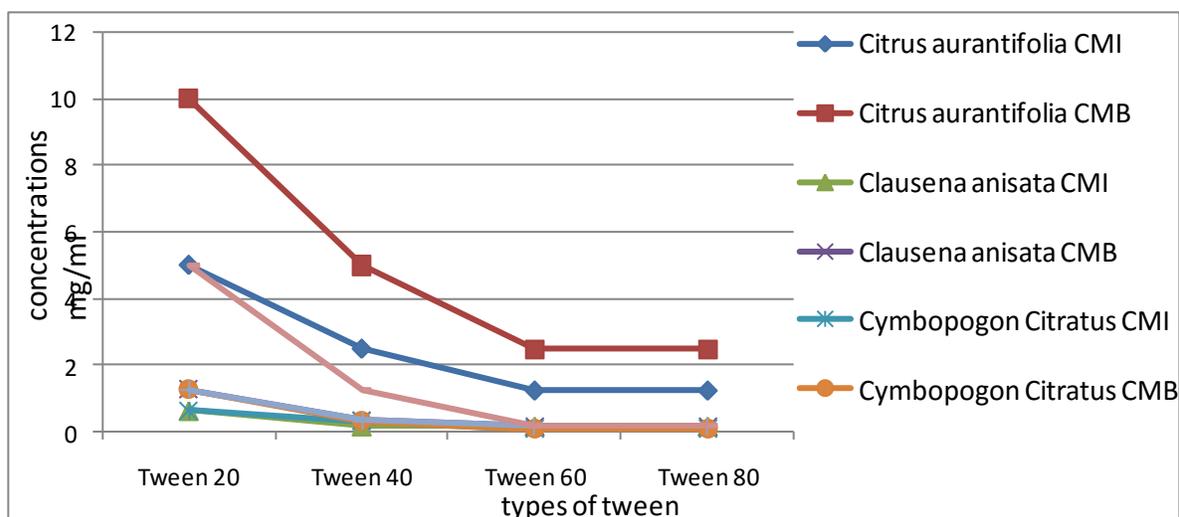


Figure 4: Evolution of the MIC of the essential oils according to the different types of tween at 5% rate on *Staphylococcus aureus*

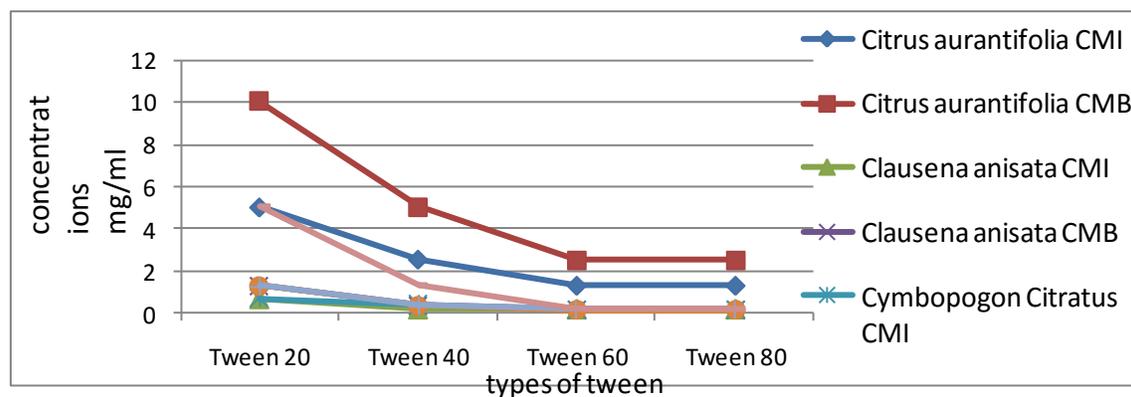


Figure 5: Evolution of the MIC of the essential oils according to the different types of Tween at 5% rate on *Candida albicans*

than or equal to 4, the oil is bactericidal; when it is higher than 4, the oil is bacteriostatic.

DISCUSSION

The antimicrobial activity of essential oils of *Cymbopogon citratus*, *Clausena anisata*, *Citrus aurantifolia* and *Eucalyptus camaldulensis* was tested *in vitro* on two gram positive bacteria (*Micrococcus luteus*, *Staphylococcus aureus*), two gram-negative bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis*) and the yeast *Candida albicans*.

Among the tested bacteria, Gram negative bacteria have proved more resistant to the products tested than the gram positive bacteria. *Pseudomonas aeruginosa* has proved the most resistant bacteria. This *Pseudomonas aeruginosa* resistance has been demonstrated by several authors (Remmal et al., 1993; Sivropoulou et al., 1995; Chouitah

et al., 2011).

The resistance of the gram is attributed to their hydrophilic outer membrane that can block the penetration of hydrophobic compounds in the target cell membrane (Ouattara et al., 2008). The MICs obtained with the Tweens 20 and 40 at 5% range from 0.156 to 5 mg / ml, whereas the use of Tween 60 in the same conditions allows to have MICs ranging from 0.078 to 2.5 mg / ml beside our germs. These results show that the performance of these essences depend on the type of Tween used. Previous works have shown that the nature and the concentration of an emulsifying agent can influence the antimicrobial activity of an essential oil (Bencheqroun et al., 2012). The result of the Tweens 20 and 40 at 5% shows a very high CMI.

This would be due to their amphiphilic structure which does not allow them to lay well at the interface in the hydrophilic phase and the hydrophobic phase of the essential oils. On the other hand, the amphiphilic structure

Table 2. Effect of the different tweens at 5% on germs

Type of tween	Germs				
	<i>Pseudomonas aeruginosa</i>	<i>Proteus mirabilis</i>	<i>Micrococcus luteus</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
Tween20	ND	ND	ND	ND	ND
Tween40	ND	ND	ND	ND	ND
Tween60	ND	ND	ND	ND	ND
Tween80	ND	ND	1/2	ND	ND

ND: Not Determined

Table 3. Results of the ratio CMB/MIC obtained with Tween 60

Essential oils	CMB/CMI				
	<i>Pseudomonas aeruginosa</i>	<i>Proteus mirabilis</i>	<i>Micrococcus luteus</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
<i>Citrus aurantifolia</i>	1	1	1	4	2
<i>Clausena anisata</i>	2	4	4	1	1
<i>Cymbopogon citratus</i>	4	2	1	1	1
<i>Eucalyptus camaldulensis</i>	1	1	1	4	1

of the Tweens 60 and 80 enables them to properly lay at the interface of the hydrophilic phase and the hydrophobic phase, thus forming molecular films (Nogarede et al., 1983), which allows a good dispersion of the essences in liquid media and a good spread in agar media (Nogarede et al., 1983; Lens-Lisbon, 1988; Bouchikhi, 1994; Wan et al., 1998).

Moreover, the results in Table 2 show that Tween 80 inhibits microbial activity of *Micrococcus luteus* with a 1/2 dilution. Although it presents good results, the use of this Tween could have a synergistic action with some essential oils thus distorting the final results of the evaluation of the antimicrobial activity of the essential oils. From these observations the Tween 60 has the best characteristics. In addition, the results obtained with the Tween 60 differs from those of Kpavode (2005) which showed that the essential oil *Clausena anisata* has no effect on the germs namely *Staphylococcus aureus* and *Candida albicans*. They also show a difference toward the essential oil of *Cymbopogon citratus* which yielded 0,078mg / ml of MICs for 5 mg / ml. These differences could be explained by the fact that the essential oils tested did not have the same chemical composition and the same type of Tween.

To better compare the biological activities of our essential oils, we indicated the main components of each of them. Essential oils have no chemical similarity. Some are rich in 1,8-cineole (*Eucalyptus camaldulensis*), others in estragole (*Clausena anisata*), and in geraniol (*Cymbopogon citratus*), limonene (*Citrus aurantifolia*).

In this study, the essential oil of *Cymbopogon citratus* rich in neral and geraniol proved the most effective. Also, Dongmo et al. (2002) found that the most active essential oils extracted from citrus were rich in neral and geraniol. The essential oil of *Eucalyptus camaldulensis* rich in 1,8-cineole gave 0.312mg /ml of MIC with Tween 60 on

Staphylococcus aureus. This result obtained by Baba-Moussa et al. (2012). However; Lens-Lisbon and his colleagues publishes in the 1988 year showed that 1, 8-cineole and para-cymene have MICs higher than 1 mg / ml on *Staphylococcus aureus*. This would be due to the type of Tween used. In our study, the essential oil of *Eucalyptus camaldulensis* on the same germ with Tweens 20 and 40 MIC gave respectively 2.5 and 1.25 mg / ml of MIC which are higher than 1mg / ml. These values are identical to those obtained by Gbenou in 1999.

In 2001, S. Inouye and his colleagues showed that 1,8-cineole is active on *Candida albicans*. Similarly, Oussalah et al. (2007) demonstrated that 1,8-cineole (61.60%) with the majority component of *Eucalyptus camaldulens* is very active in *Staphylococcus aureus* (Knobloch, 1989; Deena, 2000). The estragole (97.10%) was the only major component worked out at the level of the essential oil *Clausena anisata* which was effective on all germs tested contrary to the results of Kpavode which found no activity despite a content of 83.19% of estragole. This assessment confirmed by studies of Oussalah et al. (2007) who have demonstrated that essential oils more than sixty different plant whose major component may be more than 85%.

In contrast, the essential oils *Cymbopogon citratus*, *Citrus aurantifolia* and *Eucalyptus camaldulensis* consist of several active compounds. This difference of chemical compounds helped the show differences in antimicrobial activities.

CONCLUSION

The results obtained in this study show that the evaluation of the antimicrobial activity of the essential oils can be influenced by the type of surfactant used. Among the four types studied, Tween 60 has the best characteristics and

the Tween 80 although showing good results seems to have a synergistic action with the essential oils. The essential oils of *Cymbopogon citratus* and *Clauseana anisata* revealed the most effective on the tested germs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper

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