

## Full Length Research Paper

## Effectiveness assessment of mouthwashes formulated from the essential oils of some beninese medicinal plants against oral germs

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Faced with the increasing growth of microorganism's resistance to conventional antibiotics, the search of new bioactive molecules having pharmaceutical interest is more than ever committed. This study aimed to evaluate *in vitro* the effectiveness of seven mouthwashes formulated from essential oils of *Ocimum gratissimum*, *Ocimum basilicum*, *Cymbopogon citratus*, *Clausena anisata*, *Lippia multiflora*, *Eugenia caryophyllata* or *Mentha piperita* against five oral germs (*Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 29213, *Proteus mirabilis* ATCC 24974, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* IP 4872). The antimicrobial power of different mouthwashes was evaluated through the determination of their minimum inhibitory and bactericidal concentrations by microdilution method. All mouthwashes had an inhibitory and bactericidal effect against the studied germs. Their minimum inhibitory and bactericidal concentration varied according to the type of germ (from 0.125 to 1 µg/ml). The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were the most effective against all germs. They were followed by mouthwashes formulated from essential oils of *O. gratissimum* and *C. anisata*.

**Key words:** Medicinal plants, essential oils, antimicrobial activity, minimum inhibitory concentration, minimum bactericidal concentration, Mouthwash.

### INTRODUCTION

The oral diseases appear among the most common diseases in the world because of their prevalence and their high impact. These diseases represent the third

worldwide scourge after cardiovascular and cancer disease (Ameziane, 2014). Indeed, the dental caries and periodontal diseases induce the progressive destruction

of oral tissues, which may have the repercussions on their functions (phonation, mastication and swallowing) (Wheater et al., 2004) and on aesthetics and relationship skills of patients. These oral diseases are caused by some microorganisms (fungi and bacteria) of oral cavity and dental plaque. Indeed, a large number of microorganisms coexist in the very complex oral environment (Peluchonneau, 2011). These microorganisms found in this medium, require nutrients for metabolism from food debris, desquamated cells and some saliva constituents.

There are a large variety of antibiotics and antiseptics against these dental germs. However, the conventional treatment of these diseases is extremely costly. In many low income countries, if the treatment of dental caries was available, the cost of treatment of a child exceeds the total health care budget spent on them (Yee et al., 2002). This situation forces the people to resort to traditional medicine. According to the World Health Organization, approximately 65 to 80% of developing countries population uses the medicinal plants for their health needs because of difficulty to access to modern medicine (Sekoussounon, 2012). These plants are used in decoction, maceration, infused or essential oils forms.

Thus, thanks to new extraction techniques, identification and characterization of organic molecules, over 25 to 50% of prescribed drugs today have for active constituents, the bioactive molecules of medicinal plants (Akibou, 2014). In Africa and Benin in particular, the exploration of active constituents from plants for therapeutic use focuses more and more the researcher's attention in recent years. In addition, the essential oils extracted from medicinal plants are easy to use and often effective (Quenum et al., 2003). Recent studies have shown that *Cymbopogon citratus*, *Clausena anisata*, *Ocimum gratissimum*, *Ocimum basilicum*, *Lippia multiflora*, *Eugenia caryophyllata* and *Mentha piperita* have developed a very strong antimicrobial activity against many oral bacteria (Bonou et al., 2016a, b, c). The chemical compositions of essential oils extracted from the precedent medicinal plants are shown in Table 1. In this work, seven mouthwashes based on previous essential oils were formulated and evaluated for effectiveness against five oral germs.

## MATERIALS AND METHODS

### Plant

The plant material consisted of the following medicinal plants: *O. gratissimum* (Lamiaceae), *O. basilicum* L. (Lamiaceae), *C. citratus* DC. Stapf (Poaceae), *C. anisata* W. (Rutaceae), *L. multiflora* M. (Verbenaceae), *E. caryophyllata* (Myrtaceae) and *M. piperita* L. (Lamiaceae). The plants were collected from southern Benin (West

Africa) by Bonou et al. (2016). These plants are commonly used to treat the oral diseases by the population of studied area (Benin, West Africa). The essential oils used in this study were extracted from these plants using hydrodistillation method (Bonou et al., 2016a).

### Microorganisms

Four bacterial strains including two Gram positive bacteria (*Micrococcus luteus* ATCC 10240 and *Staphylococcus aureus* ATCC 29213) and two Gram negative bacteria (*Proteus mirabilis* ATCC 24974 and *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* IP 4872) were used in this study. They were obtained from the Laboratory of Biology and Molecular Typing in Microbiology (University of Abomey, Benin) and National Quality Control Laboratory (Ministry of Health, Benin). They are kept in Muller Hinton broth supplemented with glycerol (10%) at -20°C.

### Formulation of mouthwashes

The mouthwashes were formulated using conventional components. Hundred millimeters (100 mm) of mouthwash solution contained the essential oil (0.2 ml), saccharin (0.5 g), glycerol (0.4 g), sodium hydroxide (0.4 g), methyl salicylate (0.12 g), tween 60 (4 g), alcohol 96°C (4 ml) and sterile distilled water (100 ml). Each essential oil was used to formulate one mouthwash. The different mouthwashes are presented in Table 2.

### Assessment of effectiveness of mouthwashes

The effectiveness of mouthwashes was evaluated by the determination of their minimum inhibitory and bactericidal concentrations against the microorganisms (*M. luteus* ATCC 10240, *S. aureus* ATCC 29213, *P. mirabilis* ATCC 24974, *P. aeruginosa* ATCC 27853 and *C. albicans* IP 4872) through the microdilution method using microplate of 12 wells (Yayi-Ladekan et al., 2011).

### Determination of minimum inhibitory concentration of mouthwashes

The minimum inhibitory concentration is the lowest concentration for which there is no visible growth of studied microorganism. The determination of minimum inhibitory concentration of different mouthwashes was performed by microdilution method.

### Preparation of microbial inoculum

The previous cultures of strains were centrifuged at 10,000 rpm for 10 min. The microbial pellets obtained were washed and suspended in Mueller Hinton broth (for bacteria) or Sabouraud (for yeast). The concentrations of these suspensions were adjusted to 10<sup>8</sup> CFU/ml (OD 1 at 600 nm) using a spectrophotometer (BIOMATE 3S, Thermo Scientific). The different suspensions were diluted to 10<sup>6</sup> CFU/ml (bacteria) or 10<sup>7</sup> CFU/ml (*C. albicans*).

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**Table 1.** Chemical composition of essential oils extracted from some medicinal plants.

Plant species	Family	Compounds	Total (%)
<i>Ocimum gratissimum</i>	Lamiaceae	Thymol (30.62%), para-cymene (25.25%), gamma-terpinolene (24.24%), alpha-thujene (7.60%) and myrcene (6.56%).	94.27
<i>Ocimum basilicum</i>	Lamiaceae	Estragole (85.92%), trans-alpha-bergamotene (3.71%), para-méthoxy-cinnamaldehyde (2.03%) and 1,8-cineole (2.01%).	93.67
<i>Cymbopogon citratus</i>	Poaceae	Myrcene (11.48%), neral (33.53%), geranial (43.10%), geraniol (5.58%) and geranyl acetate (4.47%).	98.16
<i>Clausena anisata</i>	Rutaceae	Estragole (97.10%).	97.10
<i>Lippia multiflora</i>	Verbenaceae	Linalol (45,10%), 1,8-cineole (25,40%), Myrtenol (10,40%), $\alpha$ -terpineol (8,20%) and $\alpha$ -pinene (5,90%).	95,00
<i>Eugenia caryophyllata</i>	Myrtaceae	Eugenol (85.90%), eugenyl acetate (10.20%) and beta-caryophyllene (1.98%).	98.08
<i>Mentha piperita</i>	Lamiaceae	Menthone (38.50%), menthol (52.09 %), menthyl acetate (3.21%) and 1,8-cinéole (2.87%).	96.67

Bonou et al. (2016a, b, c)

**Table 2.** The different mouthwashes.

No	Code	Correspondence
1	Og	Mouthwash formulated from <i>Ocimum gratissimum</i>
2	Ob	Mouthwash formulated from <i>Ocimum basilicum</i>
3	Cc	Mouthwash formulated from <i>Cymbopogon citratus</i>
4	Ca	Mouthwash formulated from <i>Clausena anisata</i>
5	Lm	Mouthwash formulated from <i>Lippia multiflora</i>
6	Ec	Mouthwash formulated from <i>Eugenia caryophyllata</i>
7	Mp	Mouthwash formulated from <i>Mentha piperita</i>

**Preparation of microplate**

Nine hundred and fifty (950) microliters of Mueller Hinton broth or Sabouraud broth were distributed into each plate well. Fifty (50) microliters of the mouthwash were added to each well of first column and 950  $\mu$ l to the wells of third column of microplate. Serial dilutions from the third column were performed well by well and line by line (series of half dilutions). All wells except those in first column were inoculated with 50  $\mu$ l of a bacterial inoculum. Two control wells were performed.

The first control well contained the bath medium (950  $\mu$ l) and mouthwash (50  $\mu$ l) while the second control well contained the bath medium (950  $\mu$ l) and microbial suspension (50  $\mu$ l). Each line of microplate was used for one mouthwash. The microplate was covered and

incubated at 37°C during 24 h (bacteria) or at 27°C during 48 h (*C. albicans*). The reading of result was made by comparing control wells and test wells.

**Determination of minimum bactericidal concentration of mouthwashes**

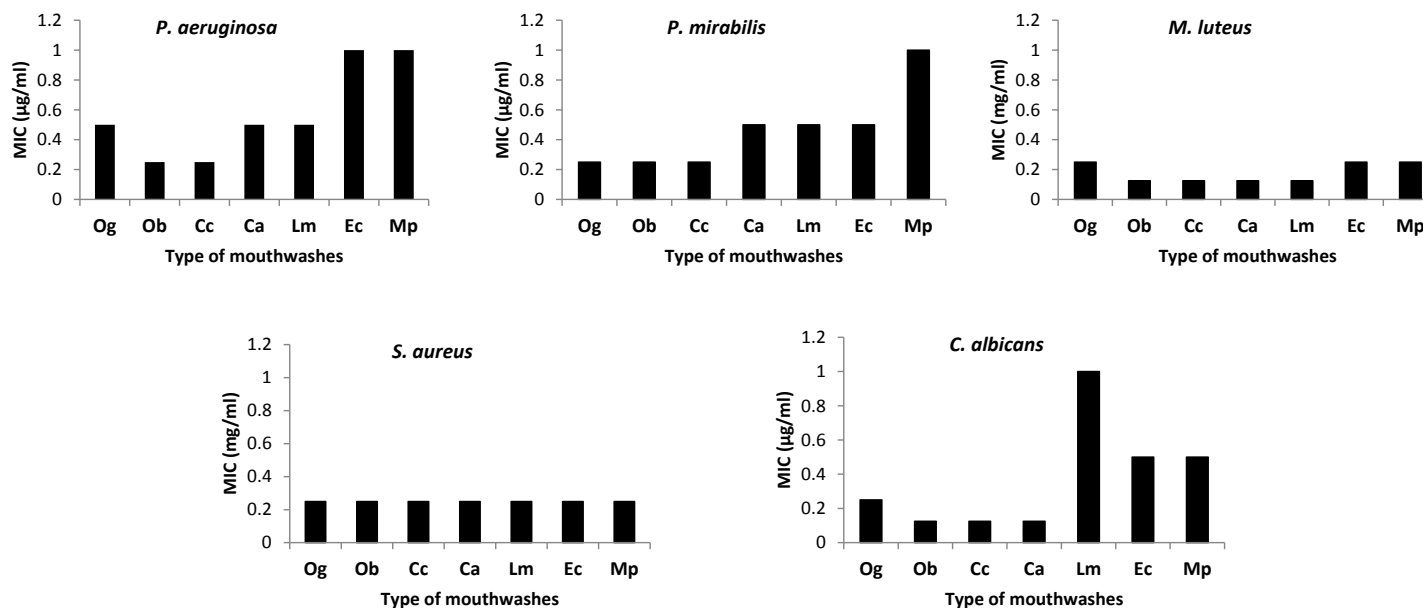
The minimum bactericidal concentration of tested mouthwashes was determined by sub culturing the content of all wells onto a Mueller Hinton (bacteria) or Sabouraud (yeast) agar and incubated at 37°C during 24 h for bacteria and 48 h for yeast. The lowest dilution of mouthwash that no visible microorganism growth on solid medium was taken as minimum bactericidal concentration (Farshori et al., 2013).

**Data processing**

The software Microsoft Office Excel 2010 was used to process the data specially to calculate the average values of minimum inhibitory and bactericidal concentration of each mouthwash according the different oral germs and to generated the figures.

**Preparation of microplate**

Nine hundred and fifty (950) microliters of Mueller Hinton broth or Sabouraud broth were distributed into each plate well. Fifty (50) microliters of the mouthwash were added to each well of first column and 950  $\mu$ l to the wells of third column of microplate. Serial dilutions from the third column



**Figure 1.** Minimum Inhibitory Concentration (MIC) of different mouthwashes against some reference strains. *P. aeruginosa* = *Pseudomonas aeruginosa*; *P. Mirabilis* = *Proteus mirabilis* ; *M. Luteus* = *Micrococcus luteus*; *S. Aureus* = *Staphylococcus aureus*; *C. Albicans* = *Candida albicans*. Og = Mouthwash formulated from *Ocimum gratissimum*; Ob = Mouthwash formulated from *Ocimum basilicum*; Cc = Mouthwash formulated from *Cymbopogon citratus*; Ca = Mouthwash formulated from *Clausena anisata*; Lm = Mouthwash formulated from *Lippia multiflora*; Ec = Mouthwash formulated from *Eugenia caryophyllata* and Mp = Mouthwash formulated from *Mentha piperita*.

microplate was covered and were performed well by well and line by line (series of half dilutions). All wells except those in first column were inoculated with 50 µl of a bacterial inoculum. Two control wells were performed. The first control well contained the bath medium (950 µl) and mouthwash (50 µl) while the second control well contained the bath medium (950 µl) and microbial suspension (50 µl). Each line of microplate was used for one mouthwash. The incubated at 37°C during 24 h (bacteria) or at 27°C during 48 h (*C. albicans*). The reading of result was made by comparing control wells and test wells.

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#### Data processing

The software Microsoft Office Excel 2010 was used to process the data specially to calculate the average values of minimum inhibitory and bactericidal concentration of each mouthwash according the different oral germs and to generated the figures.

## RESULTS AND DISCUSSION

### Inhibitory effect of different mouthwashes against germs growth

The antimicrobial activity of mouthwashes formulated from essential oils of *O. gratissimum*, *O. basilicum*, *C. citratus*, *C. anisata*, *L. multiflora*, *E. caryophyllata* and *M. piperita* tested *in vitro* against two Gram positive bacteria (*M. luteus*, *S. aureus*), two Gram negative bacteria (*P. aeruginosa*, *Proteus mirabilis*) and one yeast (*C. albicans*) revealed interesting results. Indeed, all mouthwashes have developed antimicrobial

activity against all germs (Figure 1).

The minimum inhibitory concentrations of the mouthwashes are varied according the type of germ and ranged from 0.125 µg/ml to 1 µg/ml. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were most effective against *P. aeruginosa* with a minimum inhibitory concentration of 0.25 µg/ml. In contrary, the mouthwashes formulated from *E. caryophyllata* and *M. piperita* were the least efficient (1 µg/ml). The mouthwashes based of essential oils of *O. gratissimum* (0.25 µg/ml), *O. basilicum* (0.25 µg/ml) and *C. citratus* (0.25 µg/ml) were most effective against *P. mirabilis* contrary to the mouthwashes formulated from *M. piperita* (1 µg/ml). Against *M. luteus*, the mouthwashes were more effective. Indeed, the minimum inhibitory concentration of mouthwashes formulated from *O. basilicum*, *C. citratus*, *C. anisata* and *Lippia multiflora* was 0.125 µg/ml while the minimum inhibitory concentration of mouthwashes based *O. gratissimum*, *E. caryophyllata* and *M. piperita* was 0.25 µg/ml. All mouthwashes were active against *Staphylococcus aureus* with a same minimum inhibitory concentration (0.25 µg/ml). This result is in agreement with those obtained by Baba-Moussa et al (2012) and who had shown a high inhibitory activity of essential oil of *Lippia multiflora* rich in 1,8-cineole against *Staphylococcus aureus*. Similarly, Oussalah (2007) has shown that 1,8-cineole is very active against *Staphylococcus aureus*. The effectiveness of mouthwashes formulated from *O. basilicum*, *C. citratus* and *C. anisata* were also very remarkable against the yeast strain *C. albicans* with a minimum inhibitory concentration of 0.125 µg/ml. Only the mouthwash of *L. multiflora* had a maximal inhibitory concentration against *C. albicans* (1 µg/ml).

Globally, the mouthwashes were more effective against Gram positive bacteria (*M. luteus* and *S. aureus*) than Gram negative bacteria (*P. aeruginosa* and *P. mirabilis*). Indeed, the architectural organization of cell wall of Gram positive bacteria is less complex than that of Gram negative bacteria. This complexity lies in the fact that the external membrane of Gram negative bacteria is hydrophilic and can block the penetration of hydrophobic compounds in the cell membrane (Ouattara et al., 2008). This structural difference disposes the Gram-positive bacteria more sensitive to essential oils (Kalemba and Kunicka, 2003). The sensitivity of Gram positive bacteria against the essential oils compounds was observed by other authors (Remmal et al., 1995; Deena, 2000, Kalemba and Kunicka 2003).

*M. luteus* used in this study was the strain more sensitive to all mouthwashes while *P. aeruginosa* strain was more resistant to these mouthwashes. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were the most effective of all studied micro-organisms. This effectiveness of different mouthwashes is in agreement with effectiveness of essential oils which they derived (Bonou et al., 2016a, c).

### Bactericidal profiles of different mouthwashes

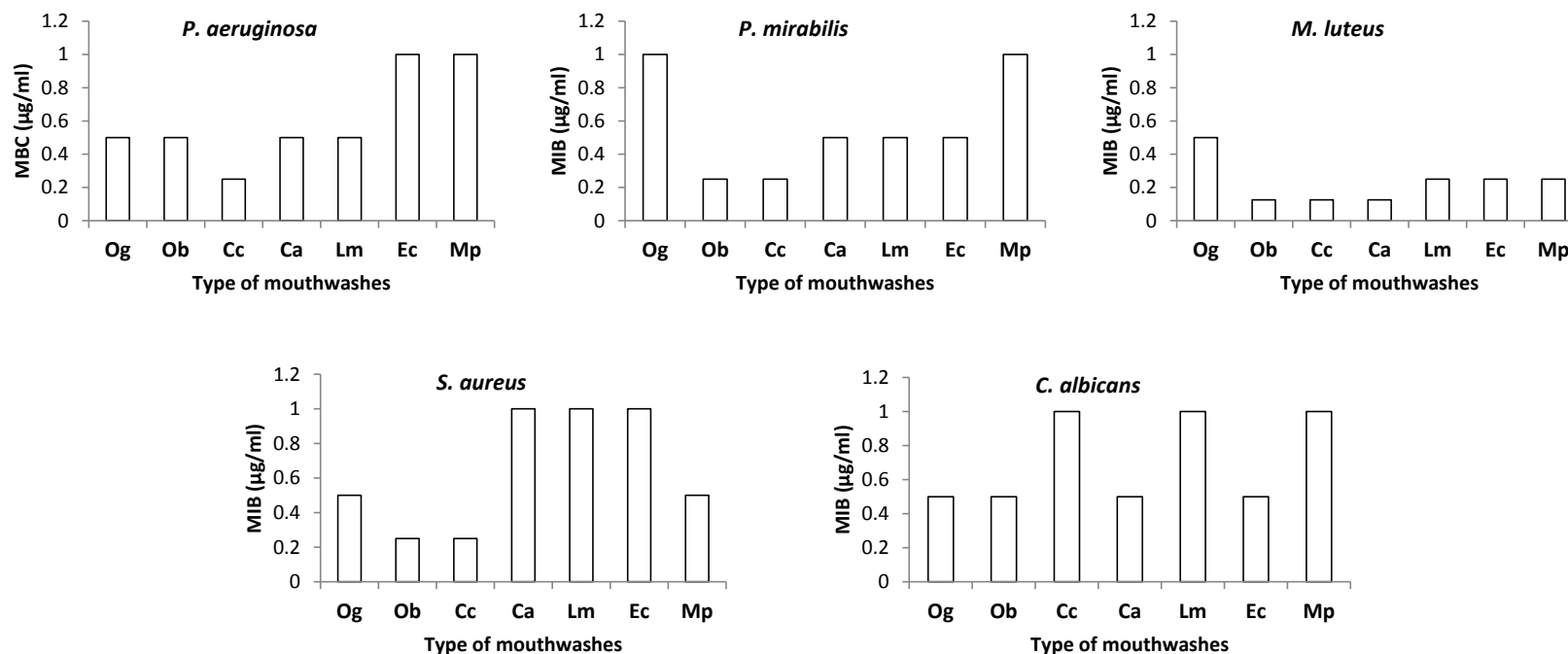
Figure 2 shows the bactericidal profiles of studied mouthwashes against the five reference germs. The minimum bactericidal concentrations have varied both according to the type of mouthwash and the type of germ. It was varied from 0.125 to 1 µg/ml. With few exceptions, the effectiveness tendency observed with minimum bactericidal concentrations was the same for minimum inhibitory concentrations. We conclude that the most inhibitory mouthwashes are the most bactericidal. So the mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* are most bactericidal against all germs. Note that all mouthwashes have a bactericidal effect against the germs. These results confirm the uses of these medicinal plants by Beninese population to treat several diseases including oral diseases. Girard (2010) had reported the use of *C. anisata*, *L. multiflora*, *E. caryophyllata* and *M. piperita* in the treatment of oral diseases.

### Relationship between antimicrobial activity of mouthwashes and chemical composition of essential oils

In the present study, all mouthwashes have shown inhibitory and bactericidal effects against all studied germs. The effectiveness of the mouthwashes has varied according to the mouthwashes and germs. The antimicrobial activity of each mouthwash has proven to be similar to the activity of the components of the essential oils used to formulate the mouthwashes. These differences could be explained by the fact that the essential oils used to formulate these mouthwashes not have the same chemical composition. Thus, the major components of essential oils used to formulate the two most powerful mouthwashes (Ob and Cc) are estragole (85.92%) for *O. basilicum* and neral (33.53%) and geranial (43.10%) for *C. citratus* (Table 1).

Dongmo et al (2002) found that the major compounds of most active essential oils extracted from citrus were neral and geranial. Two other mouthwashes (Og and Ca) were very active against the germs. The major compounds of essential oils used to formulate these previous mouthwashes are thymol (30.62%), para-cymene (25.25%) and gamma-terpinolene (24.24%) for *O. gratissimum* and estragole (97.10%) for *C. anisata*. Eugenol (85.90%) is the major chemical compounds of *E. caryophyllata*.

The importance of thymol in antibacterial activity of essential oil of *O. gratissimum* was shown by Oussou et al. (2010). Indeed, testing the split essential oil of *O. gratissimum* against some enterobacteria: its was seen by the authors that the mainly fraction containing oxygenated compounds principally thymol, was more active than other fractions. Indeed, thymol (family of



**Figure 2.** Minimum Bactericidal Concentration (MBC) of different mouthwashes against some reference strains. *P. aeruginosa* = *Pseudomonas aeruginosa*; *P. Mirabilis* = *Proteus mirabilis*; *M. Luteus* = *Micrococcus luteus*; *S. Aureus* = *Staphylococcus aureus*; *C. Albicans* = *Candida albicans*. Og = Mouthwash formulated from *Ocimum gratissimum*; Ob = Mouthwash formulated from *Ocimum basilicum*; Cc = Mouthwash formulated from *Cymbopogon citratus*; Ca = Mouthwash formulated from *Clausena anisata*; Lm = Mouthwash formulated from *Lippia multiflora*; Ec = Mouthwash formulated from *Eugenia caryophyllata* and Mp = Mouthwash formulated from *Mentha piperita*.

monoterpene phenols) is an aromatic compound particularly present in thyme. It uses to formulate several drugs through its antibacterial, antiseptic and antifungal properties. The thymol is found to treat mouth ulcers, throat irritations and insect bites. Eugenol among to family of propenylphenols, it is very commonly used for its antiseptic and analgesic properties and used to formulate the mouthwashes and gingival pastes against oral diseases. It can also be found in ointments used to decongest the airways in case

of cold or bronchitis. The estragole (methyl chavicol) belong to phenol methyl ether family is used in pharmacology for their intestinal anti-spasmodic, antibacterial and antifungal properties. Geranial has calming virtues (nervous system and muscles) and sedative properties.

On the other hand, it has antiseptic and antiviral potentials. Inducer of the glutathione-S-transferase enzyme, geranial induce apoptosis and inhibit the promotion of skin cancers (Kapur et al., 2016).

## Conclusion

The formulated mouthwashes were effective against all oral tested microorganisms. The antimicrobial activity of each mouthwash stems from the constituents of the plant essence of which it is composed. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were most bactericide against the germs. They are followed in order of effectiveness to the mouthwashes formulated from essential oils

of *O. gratissimum* and *C. anisata*.

### Conflict of interests

The authors have not declared any conflict of interests.

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