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CHEMICAL STUDY AND ANTIMICROBIAL ACTIVITIES OF VOLATILE EXTRACTS FROM FRESH LEAVES OF *CRASSOCEPHALUM RUBENS* (JUSS & JACK) S. MOORE AGAINST FOOD-BORNE PATHOGENS

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Abstract: The present work has studied the chemical composition of the essential oil (EO) extracted from the fresh leaves of *Crassocephalum rubens* and tested its efficacy against some pathogenic microorganisms isolated from common foods largely consumed, which had been incriminated as responsible of food infections and toxi-infections. The chemical composition of the essential oil extracted from the fresh leaves of *Crassocephalum rubens*, has revealed the presence of limonene (48.8%), myrcene (30.7%), E-(β)-ocimene (7.4%) and α-thujene (4.6%) as the main components. In addition some oxygenated components had been identified. They are linalool, terpinen-4-ol, p-cymen-8-ol, E-nerolidiol and E-bisabol-11-ol. The Minimal Inhibitory Concentration (MIC) determined on some bacteria Gram+ (*Staphylococcus aureus*, *Streptococcus faecalis*), bacteria Gram- (*Escherichia coli, Salmonella typhi*) and against a pathogenic yeast (*Candida albicans*) vary from 0.54 mg/mL to 4.38 mg/mL. The two Gram+ are more sensitive to the EO activity while the two Gram- less sensitive and *Candida albicans* relatively resistant to the EO activity.

Keywords: *Essential Oil (EO), food infections and toxi-infections, pathogenic microorganisms, Minimal Inhibitory Concentration (MIC).*

INTRODUCTION

In spite of modern innovations in slaughter hygiene and food production techniques, food diseases stand in an increasing important public health issue [1]. According to the same information source, 30% of industrialized countries people are submitted to a food-borne disease each year and particularly in 2000, two million people died from diarrheal disease over the world [2].

Microbial activity is a primary mode of deterioration of foods [3] and many microorganisms associated with these affections after contributed for the loss of food quality and safety.

Escherichia coli, enterohemorragic (0:157 H: 7) is responsible of affections manifested by a simple diarrhea or bloody one associated to an hemolytic and uremic syndrome, nephritic insufficiency occurring in particular to the young child aged 6 months to 5 years [4].

Staphylococcus aureus is mainly responsible of post operative wound infections, endocarditis, osteomyelitis and food poisoning [5].

Salmonella typhi is responsible of typhoid and paratyphoid fevers to the adults and some of its serovars like *S. typhimurium* and *S enteritidis* caused gastro-enteritis to the small child.

Listeria monocytogenes is mainly associated to diseases as the encephalitis, listeriosis, which has been recognized to be one of the emerging zoonotic diseases during the last two decades [6]. It had also been implicated in the immunodepression of the pregnant woman and the new-born.

Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors had been largely used as post harvest treatments of the foods, but unfortunately the uncontrolled concentrations applied increase the risk of toxic residues in the products [7]. Due to the increasing of the negative effects of these chemical synthetic products on human and animal health, the importance of alternative natural products to control pathogenic microorganisms is urgently needed [8].

Essential oils (EO) as antimicrobial agents are recognized as safe natural substances to their user and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms [9, 10].

The fresh leaves of *Crasocephalum rubens* constitute a potential source of essential oil in Benin. This aromatic plant collected on the ferruginous soil of Savalou has previously been botanically described by the National Herbarium of Abomey-Calavi University [11]. Belonging to the family of *Asteraceae* or *Compositae*, its fresh leaves are consumed as vegetable legume in soup and used as medical treatment of some affections, in particular against stomacal inflammations, liver disfunctioning, ocular pains, ears pain and breast cancer [12]. In order to promote it as an antimicrobial essential oil supplier, the present work aims to study the chemical composition of essential oil of *Crasocephalum rubens* fresh leaves to investigate on its antimicrobial properties and to test it against some food borne pathogenic microorganisms.

MATERIALS AND METHODS

Collection of *Crassocephalum rubens* leaves and their essential oil

Fresh leaves of the plant were collected in Savalou area, at Ouèssè and Kpakpassa, in West-center of Republic of Benin (246 kilometers away from Cotonou). They were hydrodistilled for about 3 hours, using a Clevenger apparatus. Oil recovered was dried over anhydrous sodium sulfate and stored at +4 °C until it was used.

Identification of chemical components of *Crasssocephalum rubens* essential oil

The essential oil of *Crassocephalum rubens* was analyzed with Gas Chromatography (GC) using an apparatus Deloi DI with flame ionization detector and a column DB5 (L: 25 m, diameter: 0.25 mm and d_i: 0.25 µm). The identification of chemical components was carried out by GC and GC quadruple mass spectrometry (SM) [13].

Tested microorganisms

They have been detected and isolated, for some, from largely consumed food borne like meat soup, refreshed drink called "Adoyo" in fon language, and from cooked maize flour sold on Cotonou and Abomey Calavi roads. The microorganisms were detected, purified and identified in the laboratory of food safety and water quality of the Ministry of Health Republic of Benin in Cotonou.
The strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, maintained in culture collection and the five one isolated from previous foods (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Streptococcus faecalis* and *Candida albicans*) had been supplied by the laboratory of food safety and water quality of Ministry of Health after being identified with API System (Apparatus and Identification Procedures La Balme-les-Grottes Cedex 2 France).

Determination of Minimal Inhibitory Concentration

The method used is the one reported by Bajpai *et al.* [14] using the microplates with 96 wells and Muller Hinton Broth (MHB) added with phenol red 0.02 g/L. A negative control made of a mixing of tested essential oil and the medium MHB and a positive control carried out with a mixing of tested microorganism and the MHB without the oil standardize the method. Serial dilutions have been made well by well, beginning by the first well to the twelfth one; the remaining aliquot is rejected at the end. The cultured microplates are incubated at 37 ± 1 °C for 24 hours, covered with a parafilm paper.

Effect of essential oil on viable counts of bacteria

The two strains ATCC *Staphylococcus aureus* and *Escherichia coli* have been chosen and used on the basis of their sensibility to the essential oil, considered their MIC. Each tube which well be used for the viable counts contains the bacteria suspension (10^7 CFU/mL) of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 in the medium MHB. They are inoculated with the MIC of the essential oil approximately 10 mL and kept at 37 °C [15]. Samples for viable cell counts were taken out at 0, 20, 40, 80, 100 and 120 minutes time intervals. Enumeration of viable counts in Muller Hinton Agar (MHA) plates was monitored as followings: 0.1 mL sample of each treatment was diluted into buffer peptone water there by diluting it 10-fold and spread on the surface of MHA. The colonies were counted after 24 hours of incubation at 37 °C. The controls (the two strains of *Staphylococcus aureus* and *Escherichia coli* ATCC) were inoculated without essential oil for each bacteria strain under the same experimental condition as mentioned above [3].

RESULTS

Yields in essential oil

Yields in essential oil of *Crassocephalum rubens* vary from 0.13% to 0.21% with arithmetic medium average equal to 0.16%.

Chemical composition of essential oil of *Crassocephalum rubens*

The chemical composition of essential oil extracted from fresh leaves of *Crassocephalum rubens* is consigned in Table 1.
The oil contains limonene (48.8%), myrcene (30.7%), E-(β)-ocimene (7.4%) α-thujene (4.6%), β-caryophyllene (1.3%), *para*-mentha-2,4(8) diene (1.0%). In all, 94.5% of components are hydrogenated monoterpens, 0.4% oxygenated monoterpens, 2.6% of hydrogenated sesquiterpens, 0.8% of oxygenated sesquiterpens and 0.1% of other components.

Minimal Inhibitory Concentration (MIC)

The MIC values determined for essential oil of *Crassocephalum rubens* are reported in Table 2.
The MIC varies from 0.54 mg/mL to 4.38 mg/mL. The MIC determined for *Escherichia coli* ATCC 25922 (2.18 mg/mL) is the double of the isolated one from meat soup and the MIC calculated for *Staphylococcus aureus* ATCC 25923 is the half of the same bacterium isolated from cooked maize flour (0.54 mg/mL – 1.09 mg/mL). The MIC determined for *Candida albicans* is high (4.38 mg/mL) while MICs calculated for *Streptococcus faecalis* and *Salmonella typhi* are respectively 1.05 mg/mL and 2.19 mg/mL. The fundamental observations mentioned throughout these different MICs are on one part, the low activity of essential oil of *Crassocephalum rubens* against *Candida albicans*, the disproportion of MICs against isolated bacteria from foods and their homologues strains collection ATCC and on the second part, the high sensibility of Gram+ bacteria against the essential oil versus the Gram- which are less sensible.

Table 1. *Chemical composition of essential oils extracted from fresh leaves of Crassocephalum rubens*

Components identified	KI	Area
		KD
		Organe
		F (%)
α-thujene	**930**	**4.6**
sabinene	969	0.4
β-pinene	974	0.2
myrcene	**988**	**30.7**
α-phellandrene	1002	0.1
para-cymene	1022	0.6
limonene	**1030**	**48.8**
δ-3-carene	1033	0.3
E-β-ocimene	**1044**	**7.4**
γ-terpinene	1055	0.3
para-mentha-2,4(8)-diene	1082	1.0
3-methyl-2-(2-methylbutenyl)-furane	1087	0.1
linalol	1095	0.2
terpinen-4-ol	1178	0.1
naphtalene	1181	0.1
para-cymen-8-ol	1183	0.1
β-elemene	1386	0.2
cyperene	1401	0.1
β-caryophyllene	1418	1.3
trans-α-bergamotene	1429	0.1
E-β-farnesene	1447	0.3
α-humulene	1454	0.2
ar-curcumene	1477	0.1
α-selinene	1494	0.1
(Z)-α-bisabolene	1503	0.2
E-nerolidol	1554	0.1
caryophyllene oxide	1579	0.4
E-bisabol-11-ol	1665	0.3
Hydrogenated monoterpens	**94.5**	
Oxygenated monoterpens	**0.4**	
Hydrogenated Sesquiterpens	**2.6**	
Oxygenated Sesquiterpens	**0.8**	
Other components	**0.1**	
TOTAL	**98.4**	

KD = Kpakpassa-Djidja; F (%) = leave

***Table 2**. MIC values for essential oil of fresh leaves of Crassocephalum rubens*

Germs tested	MIC (mg/mL)
Escherichia coli	1.09
Escherichia coli ATCC	2.18
Staphylococcus aureus	1.09
Staphylococcus aureus ATCC	0.54
Salmonella typhi	2.19
Streptococcus faecalis β-hemolysante	1.05
Candida albicans	4.38

Effect of *Crassocephalum rubens* essential oil on viable counts of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923

Based on the MICs determined for *Crassocephalum rubens* essential oil, a quantitative study was carried out to measure its effect on the viable count of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. This study has showed the efficacy at the viable counts reduction of the two microorganisms at the respective MICs. At 60 minutes of exposure, *E coli* relatively sensible, has been reduced over 50% of its quantum compared to *Staphylococcus aureus* which had registered its reduction on the range of 70% of its count. From 100 minutes time of exposure, *Staphylococcus aureus* was quickly reduced over 95% while *Escherichia coli* stand over 85% of its count.

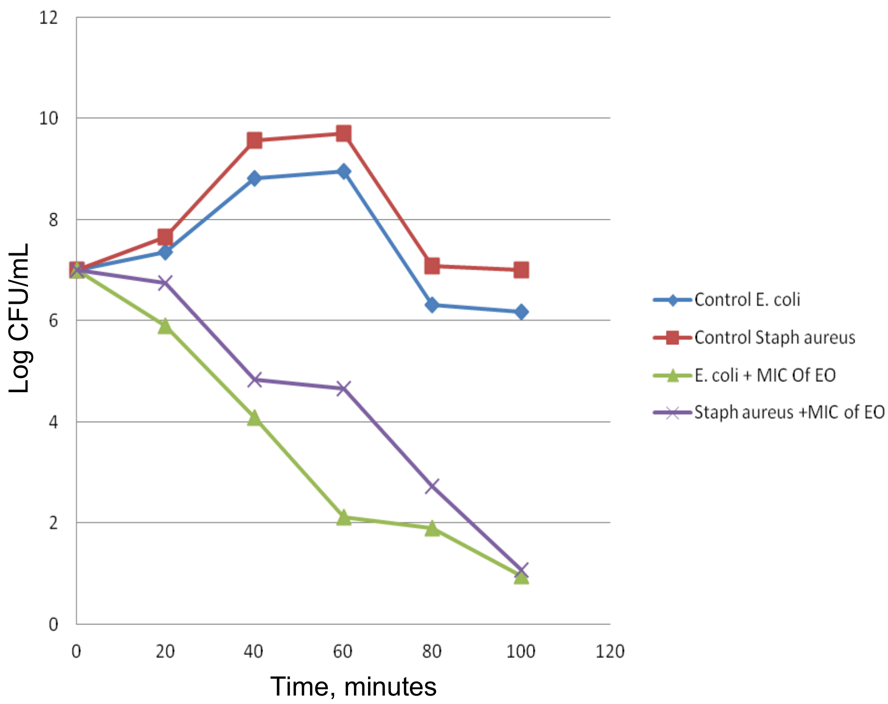

***Figure 1.** Effect of Crassocephalum rubens essential oil (MIC) on the viability of E. coli ATTC and S. aureus ATTC*

DISCUSSION

In a recent past, the use of volatile extracts in foods systems as preservation was quite known some preservators made with essential oil were rarely available and few information had been reported on that subject [16]. Nevertheless, these ten years were marked by a manifest interest for essential oil insertion in the foods and many scientific works had emphasized the antimicrobial activities detained by these latters, but also had reported their multiple applications inside foods as antimicrobials [9, 17 – 19]. Some studies [14, 15, 20] had showed the relations between chemical compositions of these natural substances and their biological activities.

According to our works, essential oil extracted from leaves of *Crassocephalum rubens* contains twenty eight components among 94.5% of hydrogenated monoterpens were identified and the chemotype noted was limonene-myrcene with 48.8% of limonene and 30.7% of myrcene. The other main of this essential oil are E-(β)-ocimene (7.4%) and α-thujene (4.6%). This chemical composition also revealed 0.4% oxygenated monoterpens (linalol, terpinen-4-ol and *p*-cymen-8-ol), 2.6% of hydrogenated sesquiterpens and 0.8% of oxygenated sesquiterpens. These results are analogue to the one published by Lemmens [21] and Grubber and Denton [12] only that these latters had identified in more, α-copaene in the chemotype they had previously described.

Generally the essential oils possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds [17, 22] and their actions are generally considered to be the disturbance of the cytoplasmic membrane, disruption of the proton motive force electron flow, active transport and coagulation of cell contents [23 – 25]. Nonetheless the chemical structure of essential oil components influences the precise action mode and its antimicrobial activity. The limonene (1-methyl-4(methylethenyl)-cyclohexene) possessing an alkyl group is more active than *p*-cymene for example and confirm observations of Dorman and Deans [22] revealing enzymes disfunctionment like the ATPases localized under the cytoplasmic membrane. *Crassocephalum rubens* essential oil containing a high percentage of limonene (48.8%) seems to active on Gram+ and Gram- bacteria, in particular the presence of some oxygenated components as *p*-cymen-8-ol, linalol and terpinen-4-ol which act by a conjugated effect of major components (limonene, myrcene, E-(β)-ocimene, α-thujene) and minor one which are the oxygenated monoterpens and sesquiterpens. Lipophilic hydrocarbons molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction alternatively; direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible [2]. Our main chemotype identified for *Crassocephalum rubens* essential oil is constituted of 94.5% of hydrogenated lipophilic molecules.

These chemical compounds are very active on the bacteria while this activity is not relevant on the unicellular yeast *Candida albicans*, but the big number of isolated components could explain its strong antimicrobial activity on the bacteria and relatively on *Candida albicans.*

The MICs determined against Gram- (*E coli* and *Salmonella typhi*) vary from 1.09 mg/mL to 2.19 mg/mL, against Gram+ (*Streptococcus faecalis* and *Staphylococcus aureus*) from 0.54 mg/mL to 1.09 mg/mL and against *Candida albicans* the MIC is 4.38 mg/mL. The sensibility of Gram+ seems to be more relevant than for the Gram- to the *Crassocephalum rubens* essential oil activity many studies had reported the

antimicrobial properties detained by plants volatile extracts and the main role of terpenic compounds throughout this antimicrobial activity [26]. The mechanisms of these compounds action and their effects on cellular membrane and cell wall are known. Their influence on the cell permeability, interfering on the membranous and cytoplasmic functions (electron flow, nucleic acids synthesis, protein synthesis coagulation of cell contents activity on proton motive force and on ATP production could be at the origin of this antimicrobial character [2, 14, 15]. The results of viability count done over the two strain tested *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 had shown that coliform less sensible at the beginning, was reduced up to 70% of its number at the 60th minute while the coccus Gram+ at the same time was reduced up to 50% of its quantum. And it's after 80 minutes *Staphylococcus aureus* was reduced to 75% of its inhibition and at the 100th minute, the two microorganisms were at 95% of their inhibition in the same proportion.
Thereby, the sensibilities are relative and specific according to the fact they are Gram+ or Gram-, essentially in the relation with the chemical composition of the bacteria cell wall. The Gram- bacteria which the cell wall is constituted of lipopolysaccharides, hydrophobic structure, didn't allow essential oil accumulation in their cell wall, the essential oil hydrophobic also one. For this, the Gram+ bacteria, with a cell wall exclusively composed of peptidoglycane and which didn't content lipopolysaccharides, seemed to be more sensible to the effect of volatile extract than the Gram- [14, 15].

CONCLUSION

The present study permitted the determination of chemical composition of essential oil extracted from fresh leaves of *Crassocephalum rubens* (Juss & Jack) S. Moore collected in Benin and to investigate on its antimicrobial activities. The essential oil extracted from leaves of *Crassocephalum rubens* revealed as major components, the limonene (48.8%), the myrcene (30.7%), the E-(β)-ocimene (7.4%) and α-thujene (4.6%). This oil contains in more 0.4% of oxygenated monoterpens, 2.6% of hydrogenated sesquiterpens and 0.8% of oxygenated sesquiterpens. The strong antimicrobial activity of this essential oil was certainly due to its various composition in hydrogenated monoterpens and sesquiterpens. This justifies the present activity on the bacteria Gram+ and Gram- and relatively less activity on the unicellular yeast and can constitute a natural antimicrobial against largely consumed food-borne pathogens in Benin.

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