International Journal of Biosciences (IJB)
ISSN: 2220-6655 (Print) 2222-5234 (Online)
Vol. 2, No. 11, p. 90-100, 2012
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Antimicrobial activities of essential oil extracted from leaves of *Ocimum gratissimum* L. against pathogenic and adulterated microorganisms associated to tomato in Benin

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Received: 30 October 2012
Revised: 18 November 2012
Accepted: 19 November 2012

Key words: essential oil, *Ocimum gratissimum*, chemical composition, antimicrobial activities.

Abstract

The present work has studied the chemical composition of the essential oil (EO) extracted from the fresh leaves of *Ocimum gratissimum* and tested its efficacy against pathogenic and adulterated microorganisms associated to tomato in the perspective of its preservation. The chemical composition of this essential oil analyzed by GC and GC/MS has revealed the presence of thymol (26.9%), γ-terpinene (20.0%) and p-cymene (17.6%) as major components. The Minimal Inhibitory Concentrations (MICs) of EO determinated by microdilution method against *E. coli* and *S. aureus* isolated from fresh tomato and their homologue strains collection ATCC varied between 0.27±0.04 mg/mL and 8.70±1.45 mg/mL. *E. coli* ATCC 25922 seems to be the most sensible strain against EO of *Ocimum gratissimum* with MIC value equal to 0.27±0.04 mg/mL whereas *S. aureus* the most resistant one with the highest MIC (8.70±1.45 mg/mL). The Minimal Fungicidal Concentrations (MFCs) determined from this essential oil against pathogenic fungi isolated from tomato were respectively for *Fusarium oxysporum, Fusarium graminearum, Fusarium poae* and *Aspergillus niger ;* 200±0.00 ppm, 400±0.00 ppm, 800±0.00 ppm and 1600±0.00 ppm, the last one which exhibited the highest resistance. According to these different activities, EO of *Ocimum gratissimum* can be used as a good biopreservative of tomato in the perspective of reducing post harvest losses and consequently its relative availability for our population.

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Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most important vegetable crops cultivated all over the world for its fleshy fruits. It is recognized today as one of the important commercial and dietary vegetable Crops (Bauer *et al.,* 2004). Tomato is an important source of vitamins A and C and antioxidants such as lycopene which is considered as a preventive agent against coronary heart disease and cancers (Clinton, 1998; Okolie and Sanni, 2012). However, a proportion of this vegetable is rendered unsalable on farms and in markets due to physical, chemical and microbiological defects (Amadioha and Uchendu, 2003). Microorganisms have been reported to cause extensive, deterioration of fruits and vegetables. Some of these micro-organisms cause rotting, discoloration or fermentation of the fruits which affect their preservation. Fruits rot caused primarily by microorganisms (fungi and bacteria) does not only constitute a major challenge to food security but also to human health in the event of toxin production by the microbes. Fungi are able to utilize the nutrients of the fruit vegetables and may cause deterioration and decay (Mensah and Owusu, 2012). A better control measures to prevent spoilage of tomatoes is necessary to avoid its contamination by mycoflora particularly and minimize public health hazards. The use of synthetic antimicrobial to control tomato spoilage moulds and pathogen bacteria has been discouraged due to their carcinogenicity, teratogenicity, high and acute residual toxicity, and long-term degradation (Barkat and Bouguerra, 2012). One of the major problems in relation with the use of these chemicals is the development of resistance. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products. Alternative natural additives are therefore needed in order, to guarantee food safety in preserved (Sessou *et al.,* 2012a). In the same way, consumers seeking a more natural food encouraged the research, the development and the application of new natural products having antimicrobial activities. Aromatic plants are traditionally employed for seasoning and prolongation of shelf life of food (Wang and Huang, 2010). The majority of their properties are due to the essential oils produced by their secondary metabolism (Rashid *et al.,* 2010). Essential oils (EOs) as antimicrobial agents are recognized as safe natural substances to their users and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms (Burt, 2004). Among the aromatic plants, *Ocimum gratissimum* is used as a food spice and in traditional medicine against pains such as urinary tract infections and respiratory diseases, diarrhea, bronchitis, liver disease and dysentery, cardiovascular disease, HIV_1 infections (Sessou *et al.*, 2012b). Several authors have showed strong antimicrobial activities of the essential oil of this plant (Saliu *et al.*, 2011; Nwigni *et al.*, 2009) but its efficacy on tomato flora was weaker studied in the literature data. The efficacy of this essential oil on fungi (*Aspergillus niger, Fusarium oxysporum, Fusarium graminearium* and *Fusarium poae*) and on bacteria (*Escherichia coli, Staphylococcus aureus*) isolated from tomato must be verified in order to measure its potential biopreservative for the valorization of this product. Therefore, this study was initiated to emphasize this EO extracted from fresh leaves of *O. gratissimum* efficacy on some microorganisms isolated from fresh tomato and confirm its role of biopreservative on this important fruit.

Material and methods

Collect and identification of plant material

Leaves of *Ocimum gratissimum* were collected at Abomey-Calavi. The botanical material was identified by Prof. Akoegninou and a voucher specimen was deposited in the Herbarium of the Botanic Garden of Department of Vegetal Biology (University of Abomey-Calavi).

Microorganisms

Antimicrobial tests were conducted in LERCA, Polytechnic School of Abomey-Calavi (LERCA/EPAC/UAC) using Gram negative bacteria *E. coli;* Gram positive bacteria *S. aureus*; and the

fungi *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium graminearum*, and *Fusarium poae*. All microorganisms were isolated from fresh fruit of tomato. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as references strains. The bacteria were identified with API System (Apparatus and Identification Procedures La Balme-les-Grottes Cedex 2 France) and fungi, with the key of Samson *et al.* (1995).

Extraction of essential oil

The essential oil was extracted from fresh leaves (150 g) by hydrodistillation during 3 h, using a Clevenger apparatus, in LERCA/Polytechnic School of Abomey-Calavi, University of Abomey-Calavi (LERCA/EPAC/UAC). Oil recovered was dried over anhydrous sodium sulphate and stored at + 4°C until it was used.

Analysis of essential oil

The obtained essential oil was packed in an amber vial and freeze storage. A sample of this oil was diluted in dichloromethane (1 mg/ml) and was subjected to analysis by gas chromatography coupled to flame ionization detector (GC-FID) and by gas chromatography coupled to mass spectrometry (GC-MS). Analysis parameters by GC-FID were: column DB-5 (25 m × 0.25 mm × 0.25 µm), temperature programming from 60 to 240°C, with increase of 3°C min-1, using hydrogen and synthetic air as carrier gases, with a flow rate of 1.0 ml/min. The identification of chemical components was carried out by GC and GC quadruple mass spectrometry (SM).

Biological assay

Antibacterial tests:

Minimum inhibitory concentration (MIC)-broth microdilution method

To determine the MIC, broth microdilution method proposed by Bajpai et al. (2008a) and reported by Yèhouénou et al. (2010a, b) were used. The microdilutions in 96 well plates were used with MHB (Mueller Hinton Broth) and 0.02 g/L phenol red. EO and MHB constitute the negative control. The positive one is the bacteria strain added with MHB. The microplates were incubated at 37 ± 1°C for 24 h, covered with a parafilm paper.

Minimum bactericidal concentration (MBC)

MBC were appreciated by the method proposed by Oussou et al. (2004) reported by Kpadonou *et al.* (2012). To determine the MBC, each microliter-plate well content 50 µl in which no color change occurred, the mixture of EO and the strain was isolated on sterile MHA (Mueller Hinton Agar) poured in Petri dishes. These plates were incubated at 37°C for 24 h. The MBC is the lowest concentration of essential oil which 99.9% of the microorganisms were killed. The tests were carried out in triplicate.

Antifungal assay:

Preparation of the culture medium

11.5 g agar of yeast extract (Yeast extract AGAR) and 10 g of anhydrous glucose were mixed with 500 ml of distilled water for the preparation of culture medium. After sterilization and addition of oxytetracycline (0.1%) 5 ml, this medium was cast in limp of Petri dish 9 cm in diameter at a rate of 17 ml.

Determination of minimum inhibitory concentration (MIC)

Antifungal assay was performed by the agar medium assay (de Billerbeck *et al.,* 2001; Koudoro *et al.,* 2011). Agar medium with different concentrations of essential oil (100, 200, 400, 800, 1600 ppm) were prepared by adding appropriate quantity of essential oil to mixed medium, followed by manual rotation of Erlenmeyer to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri-dishes (9 cm). Each Petri-dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of an *A. niger, F. Osysporum, F. graminearum, F. poae* colony grown on the agar medium for 48 h. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25°C for 7 days and the colony diameter was recorded each day. The antifungal activity was assessed by measuring the

radial growth of *A. niger*, *F. oxysporum, F. graminearum* and *F. poae* daily after 24 h of incubation at least until the 7th day. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the seventh one at, least after 7 days (Khallil, 2001) cited by Koudoro *et al.* (2011). The antifungal activity was evaluated by the percentage of mycelial growth reduction (I) of each concentration of the extract for 7 days of incubation and was calculated by subtracting the radial growth of the fungus with the extracts (d) from that of the control (dc), and latter divided by the radial growth of the control (dc), according to the equation: $I = [1 - (d/dc)] \times 100$ (Chang *et al.,* 2000).

I: index antifungal; d: diameter of growth of Petri dish treated out of essential oil; dc: diameter of growth of the control (witness) [Petri dish without essential oil].

Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of essential oil in which the growth of the fungus added with the essential oil was reduced. All tests were performed in triplicate.

Test of determination of the fungistatic or fungicidal activity

With the experimental concentrations where neither growth nor germination was observed, the fungistatic or fungicidal activity was tested. This test consisted in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth is always inhibited, the fungicidal activity of the natural extracts was confirmed, and in the contrary case, it's spoken about fungistatic activity.

Statistical analysis

Data from three independent replicate trials were subjected to statistical analysis using Statistica version 6.0. Differences between means were tested using Z-test.

Results and discussion

Essential oil composition

Essential oil was obtained by steam distillation for about 3 h each with a yield of 0.6%. GC and GC–MS analyses of essential oil enabled the identification of many volatile components (Table1). In the volatile extract different groups of terpenoid compounds were present but hydrogenated monoterpenes are dominant (61.3%). Thymol (26.9%), γ-terpinene (20.0%), p-cymene (17.6%) were the major components of *Ocimum gratissimum* oil. The main chemotype is the thymol - γ-terpinene. This chemotype is similar to that identified by Yayi (1998) in Benin, which has for chemotype γ-terpinene (37.4%) - thymol (19.7%), in different proportions. Vasconcelos et al. (1999) highlighted in Brazil the main chemotype of this oil as eugenol-1-8 cineole just like Madeira *et al.* (2005) in the same country which observed from the same analyzed essential oil the prevalence of the 1-8-cinéole (39.03%) and eugenol (35.5%); Akojobi *et al.* (2004) in the volatile extracts of this plant especially in the sheets noted the presence of the thymol (32-65%) and eugenol. Cavalcanti *et al.* (2004) isolated in essential oil from *Ocimum gratissimum* from Brazil eugenol (43.7%), 1-8 cineole (32.7%), (Z) - Ocimene (6.2%), trans-caryophyllene (4.1%). Oussou *et al.* (2004) identified in the essential oil extracted from the sheets *of Ocimum gratissimum* of Ivory Coast the chemotype thymol-p-cymene; Lemos *et al.* (2005) isolated from the essential oil of the sheets of *Ocimum gratissimum* of Brazil eugenol (57.82%) and (Z)-α-bisabolene (17.7%). In Kenya, Matassyoh (2007) highlighted in essential oils of the various varieties of this plant eugenol (68.81%), methyl-eugenol (13.21%) like main compounds; Oussou *et al.* (2010) identified in the essential oil of *Ocimum gratissimum* of Ivory coast, the thymol (34.6%), the p-cymene (25.2%), α- selinene (6.8%), the myrcene (5.4%), (E)-β-caryophyllene (4.9%) and α- thujene (4.5%);Saliu *et al.* (2011) highlighted in the essential oil of *Ocimum gratissimum* of Nigeria eugenol

(61.9%) and the cis-ocimene (8.2%); all these results differ from our results. This difference can be explained by the specificity of pedological structure on which this different plant had been collected harvesting localities and the climatic conditions.

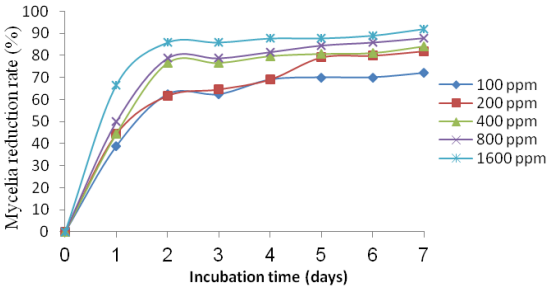

Fig. 1. Action of the essential oil *Ocimum gratissimum* with various concentrations on the mycelial growth of *Aspergillus niger*.

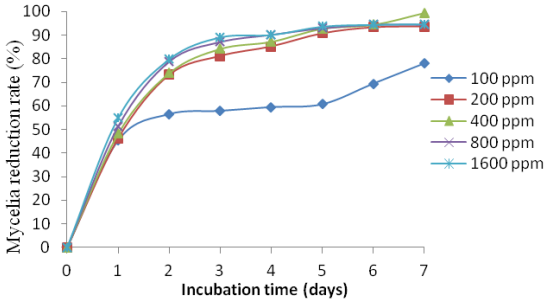

Fig. 2. Action of the essential oil *Ocimum gratissimum* with various concentrations on the mycelial growth of *Fusarium oxysporum*.

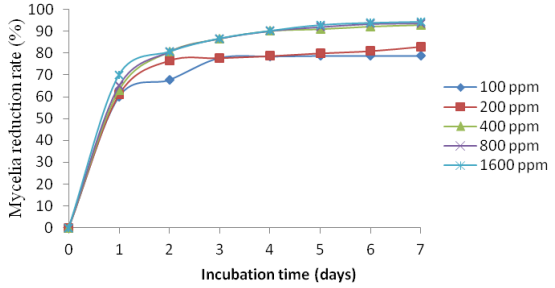

Fig. 3. Action of the essential oil *Ocimum gratissimum* with various concentrations on the mycelial growth of *Fusarium graminearum*.

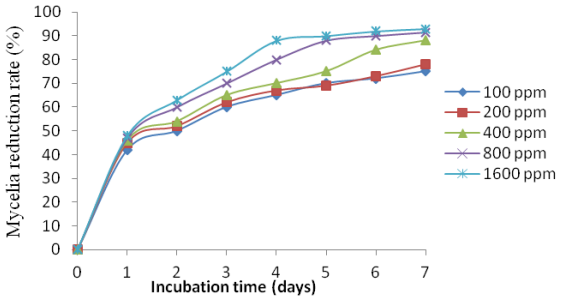

Fig. 4. Action of the essential oil *Ocimum gratissimum* with various concentrations on the mycelial growth of *Fusarium poae*.

Antimicrobial tests

Antibacterial activity

The table 2 showed that, the MICs vary from 0.27±0.04 mg/mL to 8.70±1.45 mg/mL. The MIC obtained from the stock of *Escherichia coli* ATCC 25922 (0.27±0.04 mg/mL) is sixteenth of that of the stock of *E coli* isolated from tomato and that calculated for *Staphylococcus aureus* ATCC 25923 (8.70±1.45 mg/mL) is the double of the same stock isolated from the fresh tomato (04.35±0.72 mg/mL). The fundamental remarks which are done through these different MIC are on the one hand the weak activity of EO on *Staphylococcus aureus* ATCC (25923) and on the other hand the strong sensitivity of *Escherichia coli* ATCC 25922 against this EO. The stocks isolated from tomato seem fairly sensitive to oil compared to stock of *E coli* strain reference. Essential oil tested has an antibiotical capacity against the stocks of *E coli* ATCC 25922, *E coli* and *S. aureus* isolated from tomato. It should however be noted that the extract has only one bacteriostatical action on *S. aureus* ATCC 25923. The essential oil was bactericidal for *E. coli* and *S. aureus*. The stocks isolated from tomato are sensitive to the essential oil of *Ocimum gratissimum*. This antibacterial action might be due to the different constituents of the essential oil of this plant such as: terpinen-4-ol and terpineol (Carson et al., 2006). Almost all the proportions of these components were relatively low in this oil; possible synergistic and antagonistic effects of compounds in the oil should be taken into consideration.The variation of the antimicrobial activity could be correlated to chemical composition variability (Burt, 2004; Lahlou, 2004).

Table 1. Chemical composition of essential oil of *Ocimum gratissimum* leaves.

Compounds	Kovacs Index	%
α-thujene	992	8.2
α-pinene	934	1.2
camphene	948	0.3
sabinene	969	-
β-pinene	974	0.7
myrcene	985	6.4
α-phellendrene	997	0.5
α-terpinene	1011	4.2
p-cymene	1018	17.6
limonene	1024	2.5
1,8-cineole	1025	2.1
(E)-β-ocimene	1041	0.3
γ-terpinene	1054	20.0
trans-oxyde de linalol	1067	-
cis-oxyde de linalol	1071	-
p-cymenene	1077	2.2
terpinolene	1085	0.1
linalol	1091	0.2
borneol	1160	0.2
butanoate de methyle	1172	-
terpinen-4-ol	1180	1.2
p-cymen-8-ol	1185	0.2
α-terpineol	1188	0.1
thymol méthylether	1235	0.3
thymol	1281	26.9
carvacrol	1288	0.7
α-copaene	1370	0.1
β-caryophyllene	1417	1.2
trans-α-bergamotene	1429	0.1
α-humulene	1449	0.2
germacrene D	1478	0.1
β-selinene	1485	0.4
α-selinene	1592	0.2
δ-cadinene	1515	0.1
oxyde de caryophyllene	1611	0.2
Hydrogenated monoterpenes		62.3
Oxygenated monoterpenes		31.9
Hydrogenated sesquiterpenes		2.4
Oxygenated sesquiterpenes		0.2
Aromatics compounds		2.2
Total		99.0

Table 2. Minimum inhibitory concentration and minimum (MIC) bactericidal concentration (MBC) of the essential oil extracted from the leaves of *Ocimum gratissimum* by micro dilution method.

Bacterial strains	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
Escherichia coli (from tomato)	4.35±0.72b	8.70±1.45b	2.00
Escherichia coli ATCC 25922	0.27±0.04c	0.54±0.09c	2.00
Staphylococcus aureus (from tomato)	4.35±0.72b	17.40±2.9a	4.00
Staphylococcus aureus ATCC 25923	8.70±1.45a	nd	nd

nd: not determined. Data in the column followed by different letters are significantly different ($p < 0.05$). The values are means of three repetitions ± standard deviation

Table 3. Minimum inhibitory concentration and fungicidal of the essential oil of *Ocimum gratissimum* against the strains obtained from tomato.

Fungal strains	MIC (ppm)	MFC (ppm)	MFC/MIC
Aspergillus niger	400±0.00a	1600±0.00a	4.00
Fusarium graminearum	200±0.00b	400±0.00c	2.00
Fusarium oxysporum	100±0.00c	200±0.00d	2.00
Fusarium poae	400±0.00a	800±0.00b	2.00

Data in the column followed by different letters are significantly different ($p < 0.05$). The values are means of three repetitions ± standard deviation

Cosentino *et al.* (1999) and Lattaoui and Tantaoui-Elaraki (1994) showed that carvacrol and thymol possess high activity against bacteria whereas Lis-Balchin *et al.* (1998), Carson *et al.* (1995), Chalchat *et al.* (1995), Lattaoui and Tantaoui-Elaraki (1994) showed that terpinene exerted weak antimicrobial activity. It has been shown that thymol and its precursors, p-cymene and terpinene, have strong antimicrobial activities. Based on previous studies, the antimicrobial role of thymol may induce through the alteration of the plasma membrane permeability. Its alteration leads to leakage of intracellular material The third major compound, p-cymene, identified in *O. gratissimum* oil is hydrophobic molecule and causes swelling of the cytoplasmic membrane (Burt, 2004). Using p-cymene alone did not show any effective antibacterial activity (Juven *et al.*, 1994; Dorman and Deans, 2000). Some studies have shown that the use of the whole of EO detain more antibacterial activity. It shows the important synergistic effect of minor components of EO (Burt, 2004; Karami-Osboo *et al.*, 2010).

Antifungal activity

The minimal inhibitory concentrations (MIC) and the minimal fungicidal concentration of essential oil are showed on figures 1 to 4 which expressed evolution in percentage of the mycelia reduction rate of the stocks of moulds in contact with various concentrations of moulds according to the incubation time. The mycelia reduction rates of the moulds increase in functions not only of concentration of essential oil of *Ocimum gratissimum* but also according to the time of incubation. For *Aspergillus niger*, the mycelia reduction rates are lower than 90% with all the concentrations of oil tested until the sixth day of the experimentation. Concerning the stock of *Fusarium oxysporum*, a mycelial reduction rate equal to 90.77% is obtained at the 5th day of incubation to the concentration of 200±0.00 ppm. At the 4th day and at the concentration of 400±0.00 ppm, the stock of *Fusarium graminearum* underwent a mycelia reduction of 90.16% while this rate is obtained for the stock of *Fusarium poae* after six days of incubation to the concentration of 800±0.00 ppm of

essential oil. Basing itself on the theory brought back by Mohammedi (2006) which noticed that essential oil presents a fungicidal activity on the stock of mould tested when its mycelial reduction rate is at least 90%, we can say that the essential oil of *Ocimum gratissimum* presents a fungicidal activity on the stocks of *Aspergillus niger*, *Fusarium graminearum*, *F oxysporum* and *F poae* respectively with the concentrations of 1600±0.00 ppm, 400±0.00 ppm, 200±0.00 ppm and 800±0.00 ppm. After transplantation of the mycelia discs not having pushed in the essential oil boxes containing on again culture medium without oil, the minimal inhibitory concentrations of and minimal fungicidal concentration of the EO of *Ocimum gratissimum* given on the moulds isolated from tomato were summarized in Table 3.

Table 3 shows that the minimal inhibitory concentrations of inhibition of the essential oil of *Ocimum gratissimum* on the stocks of moulds tested vary from 100±0.00 to 400±0.00 ppm while the fungicidal minimal concentrations of the same oil on the same stocks vary from 200±0.00 to 1600±0.00 ppm. *Fusarium oxysporum* seems to be the stock most sensitive to this essential oil while the stock more resistant to the essential oil of *Ocimum gratissimum* is *A. niger*. By drawing up report/ratio MFC/MIC, we note that essential oil tested presents a fungicidal capacity on all the stocks investigated. It has consequently fungicidal activity on the stocks tested with the concentrations indicated above by the theory brought back by Mohammedi (2006). As Dubey *et al.*(2000), Lemos *et al.* (2005) and Faria *et al.* (2006), this research showed that the essential oil of *Ocimum gratissimum* detains interesting antifungal activities. The bioactive concentrations of essential oil on the above mentioned stocks do not exceed 1600 ppm looking to its fungicidal activity. According to its antibiotical capacity, Oussou *et al.* (2004, 2010), Kpadonou *et al.* (2012) showed the similarity of our observations on the essential oil of *Ocimum gratissimum* against the stock of *Escherichia coli*. This antimicrobial activity displayed by the essential oil of *Ocimum gratissimum* is in relation with its composition in phenolic compounds in particular the thymol which has gathering free hydroxyl molecules which are also at the origin of its antifungal capacity. Murray *et al.* (2003) sometimes evoked the mechanism of action of the fungicidal induced by the essential oil of *Ocimum gratissimum*. According to these authors, the major sterol (the ergosterol) found in the fungi cellular membrane is hydrolyzed in contact with these phenolic compounds and thus occasioning a reduction of the mycelia growth, giving of this fact on essential oil its fungicidal property. The same phenomenon is observed according to Dormans and Deans (2000) which evoked the association of these phenolic groupings with proteins in particular of the thymol which is hydrophobic and for this reason disturbs the external membrane of negative Gram slackening the lipopolysaccharides and increases the permeability of the cytoplasmic membrane of *Escherichia coli*, which justifies the antibiotical capacity determined against *E coli*.

Conclusion

The present study enabled us to determine the chemical composition of EO extracted from the fresh leaves of *Ocimum gratissimum* and to evaluate its antimicrobial properties. EO extracted from the leaves of this plant is mainly made up of thymol, γ-terpinene and p-cymene. It has an antibiotical capacity on the stocks of *E. coli* tested and *S. aureus* isolated from tomato, a bacteriostatic activity on the stock of *S. aureus* ATCC 25923 and a fungicidal activity against the tree fusaria (*Fusarium graminearum, F oxysporum and F poae*) and fungistatic activity against *Aspergillus niger* strain. The strong antimicrobial activity of this EO is certainly in relation with its chemical composition more varied in monoterpenes and hydrogenated and oxygenated sesquiterpenes. It can, in general, constitute of this fact a natural antimicrobial substance against pathogenic and spoilage microorganisms (fungi and spoilage microorganisms isolated from our foodstuffs and tomato in particular).

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