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Variation in chemical composition of *Eucalyptus globulus* essential oil under phenological stages and evidence synergism with antimicrobial standards

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ABSTRACT

Bacterial resistance to antibiotics is a serious public health problem which is due to the immoderate and often inappropriate prescription of antibiotics. As a result, the exploitation of new bioactive molecules with limited or no side effects from natural sources and their adoption as a therapeutic alternative to synthetic molecules have become priorities. It is in this perspective that our study was undertaken with the objective was to investigate the phenological stage effect on the yield and chemical composition of essential oils extracted from Tunisian Eucalyptus (*Eucalyptus globulus* L.) aerial parts. The synergitic effect of this essential oil with conventional antimicrobials was also evaluated against nine pathogenic bacteria and *Candida albicans* ATCC 10231. The cytotoxicity of these combinations was tested on Vero cells. Gas chromatography–mass spectrometry analysis showed two different chemotypes depending on the growth stage which were characterized as 1,8-cineole (13.23%) at vegetative stage and p-cymene at full flowering (32.19%) and fructification (37.82%) stages. A predominance of monoterpene hydrocarbons (72.84%) during the fructification stage was detected with p-cymene (12.58%–37.82%) and α-pinene (10.41%–13.39%) as the determinants of this class. In relation to the phenological stage, Eucalyptus essential oil showed potent capacity to reduce stable free radical DPPH (IC_{50} up to 740 µg/mL) and moderate ability to reduce the concentration of iron ions (EC_{50} and IC_{50} up to 2 and 12 mg/mL, respectively). *Eucalyptus globulus* essential oil was active against the different bacterial strains especially during the vegetative and full flowering stages (MIC = 2 mg/mL) against *Bacillus cereus* ATCC 14579 and *Enterococcus faecalis* ATCC 29212. *Candida albicans* ATCC 10231 was more sensitive than bacteria during the fructification stage. The combination of Eucalyptus essential oil with ampicillin revealed a partial synergy effect toward Methicillin-resistant *Staphylococcus aureus* (FICI of 0.53). Nevertheless, an addition effect was recorded under the combination of amphotericin B and Eucalyptus essential oil. The cytototoxity activity showed that these associations enhanced cell viability and made Eucalyptus essential oil non toxic toward Vero cells. The results showed that the precious Eucalyptus oil in association with antimicrobial agents could be not only a promising alternative but also a potential molecular complex which is hardly recognizable by microorganisms.

1. Introduction

Since its discovery until today, antibiotic has led to great advances in therapeutics and contributed to the rise of modern medicine. In addition, the introduction and clinical use of the first classes of antibiotics have considerably reduced the mortality attributable to previously

Abbreviations: Vero, verda reno; MEM, Eagle minimum essential medium; ROS, Reactive Oxygen Species; MRSA, Methicillin-resistant *Staphylococcus aureus*; BHT, butylated hydroxytoluene; TBHQ, tertbutylhydroquinone; EO, essential oil; ST1, vegetative stage; ST2, full flowering stage; ST3, fructification stage; GC, Gas gaz chromatography; HP, Hewlett–Packard; MS, mass spectrometry; Co, co-chromatography; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IC_{50}, the concentration of 50% of inhibition; BHA, Butylated hydroxyanisole; UV–vis, ultraviolet–visible; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; EC_{50}, efficient concentration; rpm, rounds per minute; CFU, Colony forming unit; MH, Mueller hinton plate; WB, winge broth; IZ, inhibition zone; MIC, minimal inhibitory concentration; FICI, fractional inhibitory concentration index; RPMI, roswell park memorial institute medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; CC_{50}, 50% cytotoxicity concentration; SI, selectivity index; ANOVA, analysis of variance; ppm, parts per million (mg kg^{-1}); w, weight

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incurable diseases. Therefore, the effectiveness of antibiotic therapy in controlling and limiting the spread of pathogens has given optimism that all infectious diseases can be eradicated (Desalegn, 2014). The different antimicrobials used in therapeutic medicine can be classified by chemical family and action mode. The targets described until now are the membrane, nucleic acid and ribosomes of microorganisms (Singh and Barrett, 2006). Among the most antimicrobials commonly used, we can mention; ampicillin and amphotericin B. Ampicillin, belonging to β-Lactam antibiotics, is broad-spectrum penicillin that is often effective against *Escherichia coli* and *Proteus* spp. Amphotericin B has significant efficacy and a broad spectrum of action against fungal infections.

In spite of the proven track record of these antimicrobial standards, ampicillin and amphotericin B were known for their side effets and toxicity including diarrhoea, a macular rash, a stomach pain, nausea, vomiting, rigors, fever, hypertension or hypotension, and hypoxia (Maggs, 2008; Laniado-Laborín and Cabrales-Vargas, 2009). In addition, bacteria have developed several strategies leading to the decrease of drug intracellular concentration leading to its inefficiency. These microorganisms are able to modify the membrane permeability as well as the active efflux of antibiotics towards the extracellular medium. Of the same, there is a risk related to the ability of these resistant bacteria to transmit the resistance factor to pathogenic descendants (Desalegn, 2014). In another hand, Candida (*Candida albicans*) yeasts are responsible for severe and invasive infections that spread through the body causing a serious damage and 40% of patient mortality (Kettani et al., 2006). Likewise, the antifungal molecules available at the present time do not obey to the criteria of ideal antifungal agents given the limited range of molecules used and yeast emerging. Thus, this antimicrobial resistance phenomenon has greatly accelerated and spread in animal and human microbial flora following the massive use and inappropriate of antibiotics (Levy and Marshall, 2004).

This imbalance increases the risk of therapeutic failure and the emergence of side effects of antibiotics such as nephrotoxicity, ototoxicity and tendinopathy (Kalghatgi et al., 2013). In addition, these synthetic drugs are responsible for ROS (Reactive Oxygen Species) generation and mitochondrial dysfunction in mammalian cells leading to oxidative damage of DNA (Kalghatgi et al., 2013). Hence, face to this global threat, new strategies must be deployed at the global, national, and local levels to search new antimicrobial substances from other sources including plants. In this context, researchers have adopted two lines of strategies either to develop new molecules which are not yet concerned by resistance to antibiotic therapy or to find products capable, by combination effect, to restore the sensitivity to existing antibiotics in order to enhance its therapeutic usefulness (Zainol et al., 2017; Chouhan et al., 2017).

In the search for bioactive molecules, the exploration of natural resources appears as a promising alternative. In fact,due to its biodiversity, these molecules constitute a large reserve of active substances especially essential oil (EO). The complexity of its chemical constituents makes it extremely difficult for bacterial germs to develop resistance. These molecules protect the human cells from damage caused by free radicals that would be involved in the development of cardiovascular diseases, certain cancers, and other diseases related to aging (Willcox et al., 2004). The use of natural antioxidant molecules, unlike synthetics, seems to be very favored by consumers in industry to prevent food oxidative deterioration. Indeed, the use of antioxidant synthetic materials such as butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ) was suspected to carcinogenic side effects (Lanigan and Yamarik, 2002; Gharavi et al., 2007).

Tunisia is one of the richest Mediterranean countries in plant genetic resources of these natural antioxidants given the diversity of its bioclimatic stages. The genus Eucalyptus from the Myrtaceae family is part of the wide range of spontaneous medicinal and aromatic plants that characterize the Tunisian flora. *Eucalyptus globulus* (*E. globulus*) is the main producer species of EO where its industrial value is due to numerous medicinal effects such as febrifuge, vermifuge, sedative, and antidiabetic (Raho and Benali, 2012). The presence of secretory apparatus of EOs makes Myrtaceae a particularly important family in the world of aromatherapy.

Despite the number of researches focusing on the antimicrobial activity of *E. globulus* in the world, there is no study taking into consideration the combination effect on the cytotoxicity of *E. globulus* against healthy cells until now. Thus, it is within this context that the current study attempts to evaluate the antioxidant, antimicrobial, and cytotoxicity assays of *E. globulus* EOs with regards to physiological aspects. In a second part, a synergy between the EOs and conventional antimicrobials was investigated.

2. Materials and methods

2.1. Cells and microorganisms

Eight bacterial strains: *Staphylococcus aureus* ATCC 6816, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* CIP 104727, *Salmonella enteridis* DMB 560 and one yeast; *Candida albicans* ATCC 10231 strains were provided from the microorganism collection from the laboratory of Bioactive Substances, CBBC, Tunisia. These strains have been maintained by subculture on nutrient agar favorable to their growth and thereafter subcultured on conservation medium until use.

African green monkey kidney normal cell lines (Vero; ATCC no. CCL81) were kindly provided by the laboratory of clinical virology, Pasteur institute of Tunis, Tunisia.

2.2. Plant material

Eucalyptus globulus aerial parts were harvested randomly (three times) from Takelsa (Cap-Bon) (latitude 36°46′60.0″(N), longitude 10°37′60.41″(E)and altitude 168 m) at vegetative (ST1, 02 February 2017), full flowering (ST2, 30 March 2017) and fructification (ST3, 30 May 2017) stages. The plants were identified by Professor A. Smaoui (Biotechnology Center in Borj Cedria Technopole, Tunisia) according to the Tunisian flora (Pottier-Alapetite, 1979). The voucher specimen was numbered and kept in the research laboratory for further reference. After that, the samples were freeze-dried, ground to fine powder by an electric mill and conserved in a dessicator at room temperature (~25 °C) in darkness for further use.

2.3. Essential oil extraction

Dried aerial parts (100 g) were placed in a round-bottomed flask and 1000 mL of distilled water was added. All was subjected to hydrodistillation by Clevenger apparatus for 90 min in accordance with European Pharmacopoeia method (Council of Europe, 1997). This time was fixed after a kinetic survey during 30, 60, 90 and 120 min. The Steam pressure was fixed at 3 bars and steam flow rate was 0.89 kg/min. When the boiling water begun and we got the first drop of EO, we regulate the temperature to a point where there was controlled boiling. EO extractions were carried out in triplicate for each *E. globulus* stage. Yield percentage was calculated as volume (mL) of EO per 100 g of dried aerial parts.

2.4. Gas chromatography analysis

The analysis of EO volatile compounds by gas chromatography (GC) was carried out on a Hewlett–Packard (HP) 6890 GC (Palo Alto, CA, USA) equipped with a flame ionization detector and an electronic pressure control injector. A polar polyethylene glycol HP Innowax and a 5% diphenyl, 95% dimethylpolysiloxane apolar HP-5 capillary columns (30 m × 0.25 mm, 0.25 mm film thickness; HP) were used. The flow of