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ORIGINAL PAPER

Anticariogenic and cytotoxic activity of clove essential oil (*Eugenia caryophyllata*) against a large number of oral pathogens

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Abstract The occurrence of dental caries is mainly associated with oral pathogens, especially cariogenic bacteria. Numerous studies have validated the traditional use of medicinal plants by investigating the biological activity of essential oils. The *Eugenia caryophyllata* (clove) essential oil was tested in vitro against a large number of oral pathogens (114 streptococci and 46 yeast strains) using a disc diffusion method. The cytotoxicity assay of *Eugenia caryophyllata* essential oil on cancer cells (HT29, A549, Hep2, raw 264.7) and normal cells (MRC-5) was determined by the ability of the cells to metabolically reduce MTT to a formazan dye. Our results revealed that *Eugenia* essential oil possessed an excellent antibacterial activity against oral streptococci including the cariogenic bacteria as well as an excellent antifungal activity. Furthermore, the *Eugenia caryophyllata* essential oil showed significant cytotoxic effects against all studied cancer cell lines as judged by IC50 and its value ranges from 15.75 to 200 μg/ml. In conclusion, it is clear that clove oil shows powerful antibacterial and antifungal activity. The cytotoxic activity of the essential oil was dependent on the tested cell lines.

Keywords *Eugenia caryophyllata* · Clove essential oil · Antimicrobial activity · Antifungal activity · Cytotoxicity · *Streptococcus* spp. · *Candida* spp.

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Introduction

Dental caries is a multifactorial disease, which is characterized by a local destruction of the tooth. Among microorganisms, both yeast and bacteria are associated with dental caries (Zaremba et al. 2006). While yeasts have a role in the caries process on root surfaces, some bacteria correlate to caries on any dental surface. *Streptococcus* spp. has been implicated as primary causative agent of dental caries (Hamada et al. 1984; Oztan et al. 2006). Especially, *Streptococcus mutans* is known as the main cariogenic oral bacteria (Loesche 1986). The cariogenic bacterial species have the capacity to rapidly metabolize fermentable carbohydrates to acids, especially at low pH, and to grow under acidic conditions. Many attempts have been made to eliminate *S. mutans* from the oral microflora. Antibiotics have been proved to be very effective in preventing dental caries (Jarvinen et al. 1993). However, excessive use of these chemicals can result in disarrangements of the oral and intestinal flora (Chen et al. 1989).

The use of natural essential oils as functional antibacterial agents is increasing in medicine and dentistry (Matsumura et al. 2000). Essential oil mouthwashes may kill oral microorganisms by inhibiting their enzymatic activity and breaking down their cell walls (Ouhayoun 2003). Essential oils also inhibit co-aggregation between early colonizers and late colonizers, e.g., Gram-negative anaerobic periodontopathogens (Ouhayoun 2003).

The clove plant (*Eugenia caryophyllata*, Merr., Myrtaceae), native to tropical Asia, is a source of essential oils widely used in both medicine and cosmetics. The healthy use of natural products rich in bioactive substances has promoted the growing interest of the pharmaceutical, food, and cosmetic industries. Many studies have reported that

clove essential oil displays antimicrobial (Kalemba and Kunicka 2003), antifungal (Chami et al. 2005; Mari, Mari et al. 2003), and anticarcinogenic activities (Zheng et al. 1992). Many antimicrobial compounds against oral bacteria associated with dental caries have been isolated from *Eugenia caryophyllata* (Cai and Wu 1996). It has been found to be effective against a large number of bacteria: *Echerichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Campylobacter jejuni*, *Salmonella enteritidis*, and *Staphylococcus aureus* (Cressy et al. 2003; Friedman et al. 2002). In addition, the composition, by GC /MS analysis, as well as the antimicrobial activity against a large number of multi-resistant *Staphylococcus epidermidis* have been studied (Chaieb et al. 2007). However, little is known about the activity of clove essential oil against streptococci.

The aims of the present study was to further investigate in vitro antibacterial and antifungal properties of clove essential oil, against a large number of oral streptococci and fungi, as well as its cytotoxic properties on different cell lines in order to find an alternative to current synthetic antibacterial and antifungal drugs.

Material and methods

Plant material and essential oil isolation

Eugenia caryophyllata essential oil used in this study were isolated by hydrodistillation and its chemical composition was determined by Gas Chromatography-Mass Spectrometry analyses in our laboratory as previously published (Chaieb et al. 2007). The obtained oil was collected and dried over anhydrous sodium sulfate and stored at 4°C.

Antibacterial activity of the clove essential oil

The oral streptococci (n=104) used in this study were obtained from patients suffering from dental caries (Monastir, Tunisia), cultured on Columbia blood agar plates (supplemented with 5% sheep blood) and identified with 20 Strep strips (bioMérieux, France) according to the manufacturer's recommendations. The results were readed with microbiological mini-Api automate (bioMérieux).

The antibacterial activity of clove essential oil was tested against 104 oral bacterial strains associated with dental caries using the agar disk diffusion assay (Bagamboula et al. 2001; Erdemoglu et al. 2003). All streptococci strains were first grown on blood agar plate at 37°C for 18–24 h under anaerobic conditions. Several colonies were transferred into API suspension medium (bioMérieux) and adjusted to 0.5 McFarland turbidity standards with a densimat (bioMérieux). The inoculate (1 ml) of the respective bacteria was streaked on blood agar plates at 37°C and then dried. A sterile filter disc, diameter 6 mm (Whatman paper No. 3) was placed in the plate. Three microliters of the essential oil were dropped on each paper disc (3 mg/disc). The treated Petri dishes were placed at 4°C for 1–2 h to allow a good diffusion of the essential oil without bacterial growth and then incubated at 37°C for 18–24 h under anaerobic conditions. A standard disc of erythromycin (15 μg) was used as positive control. An unfilled paper disc was used as negative control. The inhibitory effect of the essential oil against each test strain was determined by measuring the diameter zones (in millimeters) around the discs. Each experiment was carried out in triplicate

Screening for antifungal activity

The human pathogenic yeasts (n=46) used in this study were also isolated from patients suffering from dental caries. These strains were grown on Sabouraud chloramphenicol agar plates and identified with Api ID 32 C strips (bioMérieux) according to the manufacturer's recommendations.

For screening the antifungal activity of clove essential oil, the agar-disc diffusion method was used as previously described (Cox et al. 2000). All *Candida* strains were first grown on Sabouraud chloramphenicol agar plate at 30°C for 18–24 h. Several colonies were transferred into API suspension medium (bioMérieux) and adjusted to 2 McFarland turbidity standards with a densimat (bioMérieux). The inoculate (1 ml) of the respective yeast was streaked on to Sabouraud chloramphenicol agar plates at 30°C using a sterile swab and then dried. A sterile filter disc, diameter 6 mm (Whatman paper No. 3) was placed in the plate. Three microliters of the essential oil were dropped on each paper disc (3 mg/disc). The treated Petri dishes were placed at 4°C for 1–2 h to allow a good diffusion of the essential oil without fungal growth and then incubated at 37°C for 18–24 h. The antifungal activity was evaluated by measuring the diameter of the growth inhibition zone (in millimeters) around the discs. A standard disc of amphotericin B (100 μg) was used as positive control. An unfilled paper disc was used as negative control. Each experiment was carried out in triplicate

Cells culture

The murine leukemia macrophages raw cell line (raw 264.7), the human colon adenocarcinoma cell line (HT-29), the human epidermoid cancer cell line (Hep-2), the human lung adenocarcinoma epithelial cell line (A549), and the human fibroblast-like foetal lung cell line (MRC-5) were maintained in Dulbecco's modified Eagle's medium

(DMEM; Biowest) containing 10% foetal Bovin serum (FBS), 1% L-glutamine, and 1% (v/v) penicillin–streptomycin (Biowest). The cells were sub-cultured after trypsination once or twice per week in a 1:5 split ratio. The cell lines were maintained as monolayers (10^4 cells/cm^2) in 75 cm^2 cell culture flasks at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cytotoxicity assay

The essential oil was screened for cytotoxic activity expressed as cell viability, assessed on confluent cell cultures. Cells (10^4 cells/well) were cultured in 96-well multidishes and treated with medium containing the essential oil at concentrations ranging from 12.5 to 800 μg/ml dissolved in DMSO. The final concentration of DMSO in the test medium and controls was 1%. Each concentration was tested in quadruplicate together with the control and repeated twice in separate experiments.

After incubation for 24 h, the medium in each well was removed and the cytotoxic effect was measured with the MTT colorimetric assay (Mosmann 1983). To determine the cell viability, 20 μl of MTT (5 mg/ml) was added to each well and cells were incubated for additional 4 h. The supernatant was then removed and the insoluble formazan product was dissolved in acidified isopropanol. Then, the optical density (OD) of 96-well culture plates was measured using an enzyme-linked immunosorbent assay (ELISA) reader (D.E. E.D Reader) at 578 nm. The OD of formazan formed in untreated control cells was taken as 100% of viability.

Results and discussion

Natural products have been recently investigated more thoroughly as promising agents for the prevention of oral diseases, especially dental caries (Pai et al. 2004). *Eugenia* is one of the largest genera of the Myrtaceae family and comprises around 350 native species. Several studies have reported the antimicrobial properties of *Eugenia caryophyllata* essential oil (Chaieb et al. 2007; Kalemba and Kunicka 2003).

Streptococcus mutans is the main etiological agents of caries disease. We selected it and also included other important oral streptococci and oral candida to be evaluated on the present study to display the antimicrobial and antifungal activity as well as the cytotoxic properties of *Eugenia caryophyllata* essential oil.

Table 1 Antibacterial activity of *Eugenia caryophyllata* essential oil against oral bacteria

Strains	Numbers of tested strains (%)	Means of clear zone (mm) ± SD	
		Eugenia essential oil (3 mg)	Eryt (15 μg)
Streptococcus mitis	15 (14.42)	15.43 ± 1.93	14.67
Enterococcus faecalis	13 (12.50)	10.35 ± 1.251	9.77
Streptococcus constellatus	12 (11.54)	17.21 ± 3.8	11.5
Streptococcus oralis	10 (9.62)	15.45 ± 2.75	15.8
Streptococcus salivarius	9 (8.65)	12.78 ± 1.41	11.44
Streptococcus pyogenes	6 (5.77)	12.25 ± 1.29	12.67
Streptococcus mutans	5 (4.81)	16.1 ± 2.4	11.2
Enterococcus faecium	5 (4.81)	10.5 ± 1.273	10.2
Gemella morbillorum	5 (4.81)	14.1 ± 1.84	10.4
Streptococcus anginosus	4 (3.85)	12.75 ± 1.41	12.25
Gemella haemolysans	4 (3.85)	11.88 ± 1.23	11
Streptococcus uberis	3 (2.88)	11.33 ± 1.41	10.33
Aerococcus viridans	2 (1.92)	12 ± 0.7	10.5
Streptococcus bovis	2 (1.92)	12.25 ± 1.06	12.5
Streptococcus sanguis	2 (1.92)	11.75 ± 1.061	12
Lactococcus lactis ssp. *cremoris*	2 (1.92)	11.75 ± 0.35	12.5
Lactococcus lactis ssp. *lactis*	2 (1.92)	12.25 ± 1.06	12.5
Leuconostoc spp.	1 (0.96)	13	12
Enterococcus avium	1 (0.96)	11 ± 1.41	11
Streptococcus equinus	1 (0.96)	9	8
Total	104 (100)		

Eryt Erythromycin, *SD* standard deviation

Table 2 Antifungal activity of *Eugenia caryophyllata* essential oil against oral yeast

Strains	Numbers of tested strains (%)	Mean of clear zone (mm) ±SD	
		Eugenia essential oil (3 mg)	AmpB (100 μg)
Candida albicans	41 (89.13)	18.4 ± 0.5	10.07 ± 0.172
Candida guilliermondii	2 (4.35)	20.5 ± 0.707	11 ± 0.707
Candida glabrata	1 (2.17)	14.5 ± 0.707	9.5 ± 0.707
Candida tropicalis	1 (2.17)	19	9 ± 1.4
Geotricum capitatum	1 (2.17)	24.5 ± 2.12	8
Total	46 (100)		

AmpB Amphoterecin B, *SD* standard deviation

This study showed that clove essential oil is very effective at a very low concentration (3 mg) against a large number of oral bacteria and yeast associated with dental caries generally showing a clear zone of inhibition upper, similar or larger compared to erythromycin (15 μg).

The highest level of activity (inhibition zone >15 mm) was observed against *Streptococcus constellatus, Streptococcus mutans, Streptococcus oralis*, and *Streptococcus mitis* (Table 1). All tested strains demonstrated a significant degree of sensitivity to the clove essential oil using the disk diffusion method as evidenced by the low SD values of inhibition zones.

The tested oil was also active against other oral pathogens (mean diameter of inhibition zone ranging from 10 to 14 mm). In addition, the essential oil (3 mg) showed a mean of clear zone larger than the Erytromycin (15 μg). This results confirm previous studies reporting that clove essential oil exhibited antibacterial activity against some periodontal pathogens including *Streptococcus mutans* (Cai and Wu 1996), foodborne Gram positive bacteria (*Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis*, and *Listeria monocytogenes*) and Gram-negative bacteria (*E. coli, Yersinia enterocolitica, Salmonella choleraesuis*, and *P. aeruginosa* (Lopez et al. 2005). The antibacterial activity was also shown against a large number of methicillin-resistant *S. epidermidis* and *S. aureus* (Chaieb et al. 2007). The inhibitory activity of clove is due to the presence of several constituents, mainly phenyl-propanoides such as carvacrol, thymol, eugenol and cinnamaldehyde as previously published by our laboratory (Chaieb et al. 2007). The antibacterial activity of the essential oil may be associated with the high eugenol content, which has been tested previously and was found to have a significant antibiotic activity (Chaieb et al. 2007; Zheng et al. 1992). Eugenol is also used widely as an analgesic and antiseptic in clinical dentistry (Maralhas et al. 2006). Its antibacterial activity against cariogenic bacteria including *Streptococcus mutans* and *Streptococcus salivarius* has been reported (Rasheed and Haider 1998). A relationship between the inhibitory effect and the presence of eugenol was found (Ouattara et al. 1997). Another study reported the antimicrobial activity of the main constituents of the clove essential oil such as thymol, carvacrol, cinnamaldehyde and eugenol alone or combined on oral bacteria (Didry et al. 1994). The compounds showed a potential inhibitory effect on tested microorganisms and certain combinations revealed a synergistic effect. The four compounds can be used alone or combined, as eugenol and thymol, eugenol and carvacrol, and thymol and carvacrol, during the treatment of oral infectious diseases.

Concerning the antifungal activity, the high value of the mean of diameter inhibition growth was attributed at *Geotricum capitatum* (24.5±2.12 mm), *Candida guilliermondii* (20.5±0.707), *Candida tropicalis*, in comparison with the effect of amphetorecin B (100 μg) as presented in Table 2. In addition, the tested oil showed a significant effect against *Candida albicans* (18.4±0.5 mm).

This study demonstrated that clove essential oil exhibited antifungal activity against a large number of human pathogenic yeasts. In agreement with our results, the antifungal activity of clove oil has been reported by many investigators (Arina and Iqbal 2002; Gayoso et al. 2005;

Table 3 Cytotoxic activity of *Eugenia caryophyllata* essential oil against normal and cancer cells

Cell lines	IC50 ± SD (μg/ml)
Hep-2	500±10.2
A549	112±3.1
MRC-5	15.75±1.6
HT29	30±2.6
Raw264.7	18.8±2.4

IC50 Inhibitory concentration of 50% of cells viability, *SD* standard deviation

Giordani et al. 2004; Park et al. 2007; Pawar and Thaker 2006).

Eugenol was shown to be effective against *Candida albicans* and *Trichophyton mentagrophytes* (Tampieri et al. 2005). The analysis of eugenol's structure suggests that the activity may depend on the presence of both an aromatic ring and the free phenol hydroxyl group. The main antifungal action appears to be exerted on the cellular membrane, probably in association with the lipophilic features of the components present in the oil (Cox et al. 2000).

Cell viability, determined by the ability of the cells to metabolically reduce MTT to a formazan dye, was performed after 24 h exposure to essential oil at different concentrations ranging from 12.5 to 800 μg/ml. A dose-dependent inhibitory effect on all cell lines tested was observed. The IC50 values of the oil are summarized in Table 3. *Eugenia caryophyllata* essential oil showed different degrees of cytotoxicity on tested cell lines as shown by IC50, and its value ranges from 15.75 to 500 μg/ml. The highest cytotoxicity was observed against the non-cancer human fibroblasts (MRC-5) with an IC50 value of 15.75±2.4 μg/ml.

Among the tested cancer cell lines, the murine leukemia macrophages raw (264.7) cells were the most vulnerable to *Eugenia caryophyllata* essential oil, with an IC50 value of 18.8±2.4 μg/ml followed by Human colon adenocarcinoma cells (HT-29) with an IC50 value of 30.0±2.6 μg/ml, whereas,the human epidermoid cancer cells (Hep-2) exhibited the lowest sensitivity to the essential oil with an IC50 value of 500±10.2 μg/ml followed by the human lung adenocarcinoma epithelial cells (A549) with an IC50 value of 112±3.1.

This study reports the cytotoxicity of *Eugenia* essential oil in cancer cell lines supporting previous studies on its anticarcinogenic effect (Zheng et al. 1992) and antimutagenic potential of clove essential oil (Miyazawa and Hisama 2001). The cytotoxicity is likely due to the high concentrations of phenolic compounds, particularly eugenol. As described previously (Chaieb et al. 2007), our sample of *E. caryophyllata* essential oil consisted mainly of eugenol. The cytotoxic effects of eugenol have been previously described in different cellular models, especially in tumor cell lines. Comparative evaluation of its components' cytotoxicity (generally recognized as safe) showed that this type of oil and its major component eugenol (which constitutes 78% of the oil) were highly cytotoxic against human fibroblasts and endothelial cells even at low concentrations. Eugenol has also displayed an excellent cytotoxic action in a dose-dependent manner in malignant cells (human hepatoma cells HepG2 and human colon cells Caco-2) (Yoo et al. 2005) and also in non-malignant human fibroblasts VH10, and has been reported to show anticarcinogenic activities (Zheng et al. 1992).

In conclusion, our results confirmed the potential in vitro antimicrobial and cytotoxic properties of *Eugenia caryophyllata* essential oil. It is a powerful and easily available source of natural compounds with low toxicity and high efficacy for therapeutic uses. Its broad spectrum of biological activity indicates the importance of further studies related to its application in infectious diseases and anticancer treatments.

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