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Phytochemical composition, antibacterial activity against sore throat pathogens and toxicological evaluation of *Cymbopogon citratus* essential oil from Benin

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Abstract

Introduction: Sore throat is a common reason for people to present for medical care. Essential oils can constitute a good alternative to classic drugs provided that their safety of use is guaranteed. The aim of this study was to evaluate the phytochemical composition, the antibacterial properties against bacteria implicated in sore throat, the mutagenicity, the cytotoxicity and the pulmonary toxicity of essential oil of *Cymbopogon citratus* (EOCC).

Material and methods: EOCC composition was determined by GC-MS. Direct antimicrobial activity was tested by determination of Minimal Inhibitory Concentration (MIC). Mutagenic potential was assessed by the Ames test using *Salmonella typhimurium* strains TA98 and TA100. Cytotoxicity was evaluated *in vitro* against human non-cancer fibroblast cell line (WI38) by MTT assay and pulmonary toxicity was assessed by administration of a single dose of 0.125 and 0.5% of EOCC with bronchoalveolar lavage after 24h.

Results: Citral (83.29%) was detected as major compound. The results confirmed the antimicrobial activity of EOCC against *Streptococcus* and *Haemophilus* strains (MIC from 0.25 to 0.5% v/v). Also, EOCC is not mutagenic on *Salmonella typhimurium* strains at the highest concentration tested (200 µg/plate) and had a low cytotoxicity (IC₅₀: 49.39 ± 9.65 µg/mL). Single doses of 0.125 or 0.5% v/v did not induce inflammation, cell recruitment or cytotoxicity in lungs 24h after administration, suggesting safety at these concentrations. This is the first report of pulmonary toxicity of EOCC by this technique.

Conclusion: Taking together, these results provide a scientific basis for the use of EOCC as a treatment for sore throat.

Keywords: Essential oil, *Cymbopogon citratus*, sore throat, antibacterial properties, toxicity

Introduction

Sore throat is one of the major reason for consultation in general medicine [1] and it has been proved that at least 25% of population have 2-3 sore throat episodes per year [2]. It's manifested by tingling and burning sensation associated with other symptoms (fever, pain, cough etc). Sore throat may have a non-infective or infective origin and in most cases, the origin is viral [3]. When the origin is bacterial, the most involved bacteria is *Streptococcus pyogenes*, called β-hemolytic group A streptococcus (GAS). *Haemophilus influenzae* and *Streptococcus pneumoniae* were also involved [4]. Analgesics, anti-inflammatory and local anesthetics are often advised to treat sore throat. Bacterial infections were treated with antiseptic like chlorhexidine because of its broad spectrum of action and/or with antibiotics. Medicinal plants and their extracts are an inexhaustible source of new bioactive molecules and are used by people in developing countries for their health care [5]. Among the alternative therapeutic arsenal, essential oils (EO) represent a good candidate to treat sore throat. The volatile and antibacterial characteristics of EO are particularly interesting for upper (sinus, rhino-pharyngeal junction) and lower (tracheobronchial level) respiratory tract by inhalation and direct contact between EO and pathogenic germs. Indeed, some authors report the effectiveness of essential oils against bacteria in involved tonsillopharyngitis and sore throat [6]. For example, nowadays, essential oils of oregano, peppermint or tea-tree are proposed orally (2 drops on a support, 3 times a day) [7] and advised to treat tonsillopharyngitis. In this context, EO can represent a good alternative to classic drugs for treatment.

Cymbopogon species belong to the Poaceae family and are used as medicinal drugs in many countries. *Cymbopogon citratus* (CC) commonly known as lemongrass is cultivated in tropical and subtropical region of Asia, South America and Africa [8]. CC was used like antiseptic, analgesic, antipyretic, diuretic and sedative [9].

Many studies have reported antimicrobial and anti-inflammatory activity of essential oil of CC (EOCC) ^[10-12]. However, the safety use of plants and their derivatives must not be neglected and must be checked before human consumption. Therefore, the toxicological characterization of EOCC is a crucial step to validate its use like medicine.

Our goal being to propose EOCC like phytomedicine spray in the treatment of sore throat, the aims of this present study were (i) to determine the phytochemical composition of EOCC, (ii) to evaluate the potential antibacterial activity against bacteria involved in sore throat, and (iii) to do *in vitro* and *in vivo* toxicological characterization of EOCC.

Material and methods

Plant material

Collection and extraction of essential oil

CC was harvested in area of Cotonou (Benin). Crops were identified by Herbarium National du Bénin (Université Abomey-Calavi) where a voucher specimen was deposited under number AA6635/HNB. Essential oil was obtained by hydrodistillation of *C.citratus* air-dried leaves using Clevenger apparatus and the yield was calculated. Essential oil was kept at 4 °C.

Phytochemical analysis

EOCC was analyzed by a gas chromatograph (Trace GC 2000 series Thermo Quest, Rodano, Italy) interfaced with a Trace MS (Thermo Quest) operating in the impact electronic mode at 70eV. The compound separation was carried out by a CP-WAX52CB column (25 m x 0.25 mm; 0.2µm film thickness; Agilent Palo Alto, CA, USA). Carrier gas: helium in constant flow mode (1.3mL/min) and the oven temperature program was: 5min at 45 °C, 45-250 °C (3 °C/min) and 5min at 250 °C. EOCC was diluted at 1% in TBME and 1µL was injected at 230 °C for the front inlet in split-less mode. Mass spectra of the resulting peaks were analyzed and term-to-term compared with the NIST / EPA / NIH 98 library. The similarity score must be greater than 700.

Bacterial and Culture conditions

Streptococcus pyogenes I-CNHU-HKM, *Haemophilus influenzae* XIV-CNHU-HKM and *Streptococcus pneumoniae* XXIII-CNHU-HKM were used in this study (Collection of Laboratoire de Microbiologie du CNHU). These strains were isolated from patients having suffered from episode of pharyngitis. Minimal Inhibitory Concentration (MIC) was determined by broth dilution method in Brain Heart Broth (BHB, HiMedia Laboratories Pvt. Ltd, India). All strains were conserved in BHB containing 20% v/v of glycerol at - 80 °C. Strains were cultivated on Brain Heart Agar supplemented with 5% of sheep blood and grown overnight at 37 °C in the CO₂ incubator.

Determination of Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration of EOCC was established by microbroth dilution in 96 well plates ^[13]. To enhance the solubility of EOCC in BHB, tween 80 was added. EOCC solution was made to the highest concentration (1% v/v) with tween 80 (1% v/v) and was serially diluted in 50µL of sterile BHB. 50µL of bacterial inoculum (10⁶cfu/ml) was then added to each well. Positive and negative growth controls were performed for each plate. The plates were incubated at 37 °C and 5% of CO₂ for 16h-20h. The MIC was the lowest concentration where the growth of bacteria was inhibited.

This interpretation was facilitated by adding 30µL of resazurin solution (0.02% v/v) which switch from blue to pink when bacteria grow.

Bacterial reverse gene mutation test (Ames test)

Emulsions of EOCC (with Tween 80) at concentrations of 200, 50, 20, 5 and 2µg/plate were incubated with *Salmonella typhimurium* TA98 and TA100 according to OECD test guideline 471 (OECD, 1997), with slight modifications as described by Mortelmans *et al.* (2000) ^[14]. The mutagenicity test was made with and without S9 metabolism mix (prepared from lyophilized rat liver S9 mixed with nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system – both from Moltox). Positive, negative and solvent control plates were also tested in parallel with the test substance plates. A solution of tween 80 (20µg/plate) was used as negative control. Sodium azide (2µg/plate) and 2-aminoanthracene (1µg/plate) were used as positive control respectively for test without S9 mix and test with S9 mix. The background lawn and the number of revertant colonies were determined and scored after 48h of incubation. All experiment was triplicate. A substance is considered to be mutagenic when the number of colonies obtained for the test substance / number of colonies obtained for the negative control is greater than 2 (N>2) and a dose-dependent effect is observed.

Determination of cytotoxicity

The cytotoxicity of EOCC sample on WI38 cells were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltetrazolium bromide] (Sigma-Aldrich, St Louis MO, USA) test ^[15] with slight modifications ^[16]. Cells (20 × 10³ cells/mL) were plated in 180µL of DMEM medium/well and incubated for 24h. 20µL of EOCC solutions at different concentrations (1–0.008 mg/mL) were added to each well and plates were incubated for 72h. Cytotoxicity of tween 80 which was used to enhance solubility of EO in DMEM medium was not cytotoxic at the highest concentration at 0.1mg/mL. Camptothecin (Sigma-Aldrich, St Louis MO, USA) was used as a positive control. After 72h, the DMEM medium was carefully removed and 100µL of MTT solution was added to each well for 45min incubation time. 100µL of DMSO was then added to each well after rejection of MTT solution to dissolve formazan and the optical density (OD) measured at 570 nm with a reference wavelength at 620 nm using a spectrophotometer (SpectraMax-Molecular Devices, Berkshire, UK). All assays were done in triplicates. The IC₅₀ values were obtained with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Acute pulmonary toxicity test

Pulmonary toxicity of EOCC was performed by oropharyngeal aspiration. This research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH publication No. 85- 23, revised 2010). All procedures were performed in accordance with the ethical standards of the institution (Université catholique de Louvain, Comité d'Ethique pour l'Expérimentation Animale, Secteur des Sciences de la Santé, Brussels, Belgium (No LA1230312).

15 female rats wistars were purchased from Janvier Labs (St Bert erin, France). They were divided in three groups of five animals, kept with sterile rodent feed and water and housed in positive pressure air-conditioned units (25 °C, 50% relative humidity) on 12h light/dark cycle. Emulsions of EOCC were prepared at 2% v/v (2% of EOCC and 0.2% v/v of solution of

lecithin in sterile water) and different concentrations of emulsion were prepared by serial dilution in sterile water. After anesthesia with a mix of Nimatek (Eurovet, Bladel, The Netherlands) and Rompun (Bayer, Kiel, Germany), a single dose (300 μ L) of different concentrations (0.125 and 0.5% v/v) of emulsion of EOCC was directly administrated by oropharyngeal aspiration. A group control received solution of lecithin (0.2% v/v of solution of lecithin in sterile water). Rats were sacrificed 24 hours after administration. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and infusing the lungs with 10ml of NaCl 0.9%. BAL was centrifuged 10 min at 4 °C (240 g). Cell-free supernatant (BALF) was used for biochemical measurements. After resuspension in PBS, total BAL cells were counted in Turch (crystal violet 1%, acetic acid 3%). Total proteins and lactate dehydrogenase (LDH) activity were assayed on BALF as described by Arras *et al.* (2001) [17].

Table 1: MIC of EOCC against clinical isolates of *streptococcus pneumoniae*, *streptococcus pyogenes* and *haemophilus influenzae*

Strains	<i>Streptococcus pyogenes</i> I CNHU-HKM	<i>Streptococcus pneumoniae</i> XXIII-CNHU-HKM	<i>Haemophilus influenzae</i> XIV-CNHU-HKM
MIC (in % v/v)	0,25	0,5	0,5

Phytochemical analysis

The chemical composition of EOCC was analyzed by gas chromatography (GS) interfaced with spectrometry (MS) to identify some active compounds. As shown in table 2, 94.91%

Statistics

Graphs and statistical analyses were performed with GraphPad Prism 5.0 and/or Microsoft excel 2016. All results are expressed as mean \pm standard error on the mean (SEM). Data from male and female animals were analyzed separately for sub-acute toxicity. Differences between control and treated groups were evaluated using one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison. Statistical significance was considered at $p < 0.05$.

Results

Antibacterial activity

EOCC show good antibacterial activity against clinical strains implicated in human tonsillitis (see table 1). The best activity was reported for *Streptococcus pyogenes* with MIC of 0.25% v/v.

of composition of EOCC was determined. Geranial (47.80%), neral (35.49%) and geraniol (4.38%) were detected as main components of EOCC.

Table 2: Percentage composition of EOCC obtained by hydrodistillation

Compounds	RT	RSI	Relative Composition (%)
(Z)- α -ocimene	2.60	916	0.31
(E)- α -ocimene	2.64	905	0.37
6-methyl-5-Hepten-2-one	4.09	932	2.71
Linalool	11.71	900	1.68
Neral	17.35	914	35.49
Geranial	20.03	943	47.80
Geraniol	26.05	925	4.38
Total identified 94.91%			

Note: RT: Retention Index; RSI: matching coefficient (>800 compared to the NIST database)

In vitro toxicity of EOCC

Cytotoxicity of EOCC was evaluated on mammalian cells (WI38) with an IC₅₀ value of 49.39 \pm 9.65 μ g/mL. Positive control (camptothecin) showed an IC₅₀ value of 0.04 \pm 0.001 μ g/mL.

The results of mutagenicity assay are depicted in Table 3 which shows that EOCC was non mutagenic either for TA 98 and TA100 strains both with and without S9 activation.

Table 3: Results of Ames test for EOCC with *Salmonella thyphimurium* TA98 and TA100

Strain	TA98					
	Without S9 Mix			With S9 Mix		
Concentration (μ g/plaque)	Mean	SD ^d	N ^a	Mean	SD ^d	N
200	22,34	4,62	1,24	25,67	5,04	0,89
50	21,67	6,43	1,20	27,34	4,51	0,95
20	23,67	4,2	1,31	22,67	4,2	0,79
5	24,34	2,9	1,35	18,67	1,53	0,65
2	15,34	2,52	0,85	24	3,61	0,82
Positif control ^b	385,34	16,17	21,40	326,67	66,50	11,27
Vehicle control ^c	18	1	1	29	9,17	1
Spontaneous colonies	22	4,36	1,23	26,34	7,6	0,91
Strain	TA100					
	Without S9 Mix			With S9 Mix		
Concentration (μ g/plaque)	Mean	SD ^d	N ^a	Mean	SD ^d	N
200	82,34	4,05	0,56	163,67	1,15	0,9
50	154,34	6,36	1,05	182	19,08	1
20	142	6,56	0,97	168	8,72	0,93
5	149,67	8,74	1,02	162,67	10,97	0,9
2	156	6,56	1,06	172,34	15,54	0,95

Positif control ^b	1378.67	48.23	9.34	736	117.85	4.04
Vehicle control ^c	147.67	6.35	1	182.34	6.43	1
Spontaneous colonies	157.34	8.15	1.07	173.64	11.60	0.96

Note: ^a N: number of colonies obtained for the test substance / number of colonies obtained for the vehicle control

^b Positive control: 2µg/plate of sodium azide (TA100) and 0.2 µg/plate of 4-nitroquinoline-n-oxide (TA98) for test without S9 mix and 1µg/plate of 2-aminoanthracene for test with S9

^c Vehicle control: Solution of Tween 80 (20µg/plate)

^d SD: Standard deviation

In vivo toxicity of EOCC

The results of pulmonary test are shown in figure 4. A single dose of EOCC at 0.125 and 0.5% v/v with determination of

parameters after 24 hours do not affected the levels of LDH activity, total proteins and total BAL cells.

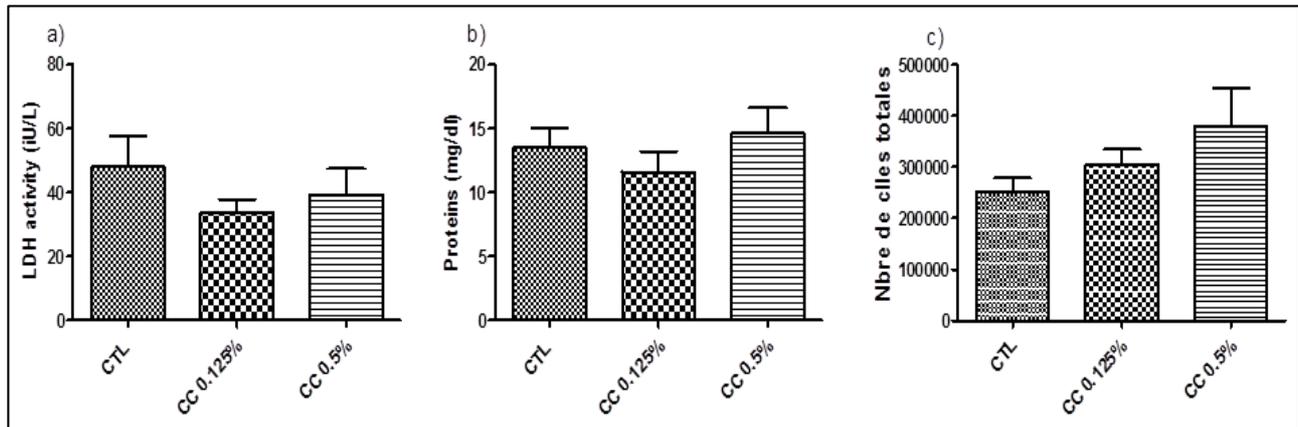


Fig 1: Effect of single administration of 300µL of emulsion of EOCC at 0.125% v/v (CC 0.125%) or 0.5% v/v (CC 0.5%) by oropharyngeal aspiration on LDH activity (a), total proteins activity (b) and total cells (c) measured in BAL (Broncho Alveolar Lavage) after 24h; Each condition was compared to control (CTL). $p > 0.05$ for all experiments. One-way ANOVA followed by a Dunnett's multiple comparison, $n = 5$, means \pm SEM)

Discussions

Traditional medicine constitutes a good alternative to classic or synthetic drugs to treat bacterial infections but scientific investigations about their antibacterial activity and their toxicological characterization are needed to validate their uses in medicine. Essential oils are known to contain a mixture of 20-60 compounds with different concentrations [18] and have been used a long time ago as natural therapies. In this study, we focused on antimicrobial activity of EOCC and its pulmonary toxicity. We showed that EOCC was efficient against all strain with the best activity against *Streptococcus pyogenes* (0.25% v/v). Indeed several authors reported the antimicrobial activity of essential oils [19-21]. Inouye *et al.* (2001) demonstrated the effectiveness of essential oils against respiratory tract pathogens [22]. For example, lavender and oregano essential oils are known to inhibit *Streptococcus pyogenes* and *Staphylococcus aureus* [7]. As well, Sfeir *et al.* (2013) showed that EOCC was active against *Streptococcus pyogenes* with MIC of 0.91% v/v [6]. This activity was linked with aromatic compounds containing in EOCC and known to have some biological activities. Citral (neral and geranial) was found as major compound of EOCC. This result was in accordance with literature. Kpoviessi *et al.* (2014) also report that citral was the major compound of EOCC originating of Benin [23]. This compound is well documented to have antibacterial properties [24, 25]. The main mechanism of antibacterial effect of EOs is alteration of cell wall and cytoplasmic membrane because of theirs lipophilic characters [26]. This effect causes permeabilization of membrane followed by cell lysis [18].

The mutagenicity of emulsion of EOCC was assessed at five different concentrations (200, 50, 20, 5 and 2µg/plate). No mutagenic effects were recorded at the tested concentrations. Indeed, most essential oils do not exhibit mutagenic effect [27].

Having toxicological data on medicinal plants and theirs extracts was very important to safe uses of these medicine. Concerning the oral toxicity of EOCC, its DL_{50} of acute oral toxicity is more than 2000mg/kg and its No Observed Adverse Effect Level (NOAEL) is about 71.4mg/kg/day [28]. But the knowledge of deleterious effect of EOCC on lung is crucial for its eventual use like spray for sore throat. Oropharyngeal aspiration is a good technique that reproduces the same effects on lungs like inhalation test [29]. Increased of different parameters (LDH activity, total protein and total cell number) have been used as an indicator of inflammation of lungs. From analysis that emerges out of these results, there is no significant increase in the various parameters compared to the control suggesting that these concentrations did not induce inflammation. To the best of our knowledge, this is the first report of pulmonary toxicity of EOCC by this technique. These studies confirm the relative safety of EOCC at these concentrations for eventual use in phytomedicine spray.

Conclusion

Our study shows that EOCC was active against pathogens implicated in tonsillopharyngitis. This EO, already used in traditional medicine, could also be used in treatment of pharyngitis and sore throat. The Ames test reveal that EOCC was non-mutagenic and the acute pulmonary test suggest an absence of toxicity of EOCC at concentrations tested. This is the first report of acute pulmonary toxicity of EOCC. All these data contribute to valorization of traditional medicines. Nevertheless, further toxicity tests are necessary to confirm his safety.

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