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In vitro cytotoxic activity of *Cymbopogon citratus* L. and *Cymbopogon nardus* L. essential oils from Togo

Koffi Koba¹, Komla Sanda¹, Catherine Guyon², Christine Raynaud³, Jean Pierre Chaumont² and Laurence Nicod²

¹Unité de Recherche sur les Agroressources et la Santé Environnementale, Ecole Supérieure d'Agronomie, Université de Lomé, BP. 20131, Lomé Togo; ²Equipe des Sciences Séparatives, Biologiques et Pharmaceutiques, IFR 133, Faculté de Médecine et de Pharmacie, Université de Franche-Comté, Place Saint-Jacques, 25030, Besançon, Cedex France; ³Laboratoire de Chimie Agro-Industrielle, Arômes et Métrologie Sensorielle, UMR 1010, INP-ENSIACET, 118, route de Narbonne, 31077 Toulouse cedex, France.

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Abstract

The leaf essential oils of *Cymbopogon citratus* L. and *Cymbopogon nardus* L. (Poaceae) from Togo were steam-distilled, analyzed for percentage composition and investigated *in vitro* for their potential cytotoxic activity on human epidermic cell line HaCat. The percentage composition showed that the main constituents of essential oils samples were respectively geranial (45.2%), neral (32.4%) and myrcène (10.2%) for *C. citratus* essential oil and citronellal (35.5%), geraniol (27.9%) and citronellol (10.7%) for that of *C. nardus*. The *in vitro* cytotoxicity bioassays on human epidermic cell line HaCaT revealed that the toxicity of the essential oil from *C. citratus* (IC₅₀: 150 µL.mL⁻¹) was higher than that of the essential oil from *C. nardus* (IC₅₀: 450 µL.mL⁻¹). Pure commercial neral, geranial, and citronellal standards showed respectively the following IC₅₀ values: 100, 250 and 300 µL.mL⁻¹. Conversely, pure citronellol standard appeared almost non-toxic (IC₅₀>1000 µL.mL⁻¹), proving the major role played in synergy by neral and geranial in the overall toxicity showed by the *citratus* oil sample tested in this work.

Introduction

During the last decades, chemists and biologists have been intensively investigating tropical and subtropical plants species with potential medicinal properties in order to assess the feasibility of developing natural, sustainable, and affordable drugs and cosmetics (Aké Assi and Guinko, 1991; Iwu, 2000; Orafidiya et al., 2001).

Cymbopogon citratus L. and *Cymbopogon nardus* L. essential oils, apart from their broad use in food or drinks, are widely involved in perfumery, body care products and soap manufacture. Also important is their pharmaceutical usage, which still remains under exploited especially in West Africa where these aromatic herbs are widely distributed.

Recently, particular attention has been given by researchers to the use of essential oils from tropical origin as active ingredients in various pharmaceutical formulations against some skin troubles (Orafidiya et al., 2001; Orafidiya et al., 2002) like human mycosis. Indeed, naturally occurring molecules of essential oils are more and more considered as valid additives to conventional antibiotherapies (Chaumont et al., 2001; Chaumont, 2003; Koba, 2003).

While intensive work is done to record the antimicrobial potential of plant essential oils and their other biological applications, some important works have been reported in the relevant literature regarding their possible cytotoxicity on human cutaneous cell lines (Foray et al., 1999; Hayes et al., 1999; Koba et al., 2007) and leukemia cells (Dubey et al., 1997a).



The objective of this paper was to investigate chemical composition and the *in vitro* cytotoxic potential of *C. citratus* and *C. nardus* essential oils. The assumption was made that these essential oils, commonly used or proposed for use in soap and body care and in different formulations to treat skin troubles like acnes and tinea in West Africa (Orafidiya et al., 2001; Orafidiya et al., 2002) may be toxic to the human epidermic cell line HaCaT. Hence the cytotoxicity evaluation of these volatile essential oils undertaken in this work constitutes one of the important steps prior to their possible exhaustive assessment for pharmaceutical use against superficial skin mycosis.

Materials and Methods

Plant material and volatile oils isolation

Aerial parts (leaves) of the tested plants were harvested in April 2005, from the experimental field of the *Unité de Recherche sur les Agroressources et la Santé Environnementale* at the Université de Lomé, Togo.

Plant material was identified by Prof. Akpagana at the Université de Lomé, where voucher specimens were stored in the herbarium respectively under references N°247 for *C. citratus* and 268 (K) for *C. nardus*.

A sample (50 g) of air-dried plant material was extracted by the hydrodistillation technique during 2 hours in a modified Clevenger-type apparatus (Craveiro et al., 1976). The extracted essential oils were stored in hermetically sealed dark glass flasks with rubber lids, covered with aluminium foil to protect the contents from light and kept under refrigeration at 4°C until use without any prior purification.

Essential oils analyses

Gas chromatographic analysis was carried out on a Varian 3300 type gas chromatograph equipped with FID detector. An apolar capillary column DB-5 (30 m x 0.25 mm i.d.; film thickness 0.25 µm) and on a polar column supelcowax 10 with the same characteristics as above mentioned were used. DB-5 column operating conditions were as follows: from 50°C (5 min), 50°C to 250°C at the rate of 2°C/min and Supelcowax 10 from 50°C (5 min), 50 to 200°C at 2°C/min. The injector and detector temperatures were 250 and 300°C respectively. The carrier gas was helium at a flow rate of 1.50 mL/min. Samples (0.2 µL) of undiluted essential oil were injected manually.

The GC/MS analysis was carried out on a Hewlett Packard 5890 SERIES II chromatograph, coupled with a mass spectrometer of the Hewlett Packard 5971 SERIES type operating in the EI mode at 70 eV. The capillary column type was DB5-MS (30 m x 0.25 mm i.d.; film thickness 0.25 µm). The amount of sample injected and

GC/MS parameters were the same as above.

Identification of components

The components of oils samples were identified by their retention time, retention indices relative to C₈-C₂₄n-alkanes, computer matching with Willet 275.L library and as well as by comparison of their mass spectra with the authentic samples or with data already available in the literature (Kondjoyan and Berdagué, 1996; Adams, 2001).

The percentage of composition of the identified compounds was computed from the GC peak area without any correction factor and was calculated relatively.

Chemicals

Dubelcco's Modified Eagle's Minimum Essential Medium (DMEM), fetal calf serum (FCS), trypsin (0.25%) were from D. Dutscher (Brumath, France). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), polyoxyethylene 20 sorbitan monoleate (Tween 80®), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Phosphate buffered saline (PBS) without calcium and magnesium was purchased from VWR International (Cergy-Pontoise, France). Pure neral, geranial, citronellal and geraniol from commercial origin were purchased from Sigma Chemical Co. (St. Louis, USA).

HaCaT cell culture

HaCaT, an immortalized human keratinocyte line was a generous gift from Nathalie Gault (Commissariat à l'Énergie Atomique, Bruyère Le Châtel, France) (Boukamp et al., 1988; Gault et al., 2002). Cells were routinely grown in Costar plastic flasks in monolayer cultures in DMEM medium supplemented with 10% (v/v) FCS, and 5 M of HEPES + 80 mg.L⁻¹ of gentamicin. They were grown in a humidified atmosphere of 5% CO₂ in air. The medium was routinely renewed 2, 4 and 6 days after passage and when confluence was reached; cells were trypsinized and split for subcultures (seeding density 3,500 cells/cm² in a 75 cm² flask) or used for cytotoxicity assays. Cells were used for experiments within 10 passages to ensure cell line stability. All the experiments were carried out at 37°C.

Cytotoxicity assay

HaCaT cells were seeded at a density of 6 x 10⁴ cells per well in 100 µL culture medium containing 10% FCS on 96 multi well culture plates and incubated overnight for adherence. The next day, the medium was removed and cells were incubated in FCS-free medium containing increasing concentrations (from 50 to 1,000

$\mu\text{L}\cdot\text{mL}^{-1}$) of *C. citratus*, *C. nardus* essential oils or their major components from commercial origin (Pure neral, geranial, citronellal and geraniol) solubilized in 1/10 Tween 80® in culture medium (with <0.1% Tween 80® an ethanol vehicle). Each experiment was carried out in triplicate.

Determination of cell viability

After the exposure period, the reaction medium was removed and the adhering cells washed with PBS. One hundred microliter of MTT solution (0.5 $\text{g}\cdot\text{L}^{-1}$ in medium) was added to each culture well. After incubating for 4 hours at 37°C, the MTT reaction medium was removed and formazan-blue was solubilized in 100 μL DMSO. This assay is based on the reduction of yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble formazan-blue product. Only viable cells with active mitochondria reduce significant amounts of MTT (Mosmann, 1983) and formazan-blue formation absorbance was recorded in an EL_{X800} UV, universal microplate reader spectrophotometer at 570 nm. Values of absorbance were converted into percentage of residual viability. Usually, inhibition concentration 50% (IC₅₀) is chosen as the best biological marker of cytotoxicity.

The inhibition (I) of the essential oils dilutions in percent was calculated as follows:

$$I = (A_0 - A_1 / A_0) \times 100$$

A_0 is the absorbance of the control reaction (containing all reagents except the tested compounds), and A_1 the absorbance with the tested substances (crude essential oils or pure commercial component).

The IC₅₀ value represented the concentration of the tested compounds or essential oils that caused 50% cells inhibition.

Results and Discussion

The light yellow essential oil of *C. citratus* and *C. nardus* were obtained in yields of 1.6 and 1.3% respectively, based on dried extracted material. The percentage composition of the studied oil samples are listed in Table I.

Nineteen compounds were identified in the *C. citratus* oil sample representing 99.8% of the detected compounds that included geranial (45.2%), neral (32.4%) and myrcene (10.6%) as major components. This oil consisted of two monoterpene hydrocarbon (10.6%) and nine oxygenated monoterpenes (86.4%). This sample appeared similar to previous reports on oil chemotypes from Togo (Koumaglo et al., 1996; Koba et al., 2003) but it differed from that previously described in Cuba (Pino and Rosado, 2000) with geranial (52.3%),

Table I

Chemical composition of <i>C. citratus</i> and <i>C. nardus</i> essential oils from Togo			
Compounds	Retention indices (RI)	Peak area [%] of essential oils	
		<i>C. citratus</i>	<i>C. nardus</i>
<i>Monoterpene hydrocarbons</i>		10.6	1.9
Myrcene	990	10.2	1.4
Limonene	1036	0.4	0.5
<i>Oxygenated monoterpenes</i>		86.4	79.0
Methyl-5 epten-2one	986	0.4	
Sabinene hydrate cis	1076	0.3	
Citronellal	1153	0.2	35.5
α -Terpineol	1207	0.9	0.3
Citronellol	1226	0.3	10.7
Neral	1238	32.4	0.4
Geraniol	1253	5.5	27.9
Geranial	1267	45.2	0.7
Geranyl acetate	1381	1.2	3.5
<i>Sesquiterpene hydrocarbons</i>		1.8	12.3
β -Elemene	1388	1.4	5.1
α -Farnesene	1504	0.3	0.2
Bicyclogermacrene	1502	1.2	0.2
Germacrene D	1520	0.2	3.3
δ -Cadinene	1543		3.2
<i>Oxygenated sesquiterpenes</i>		1.4	2.7
Elemol	1550	0.6	0.1
β -Eudesmol	1651	0.2	0.2
Citronellyl tiglate	1658	0.1	2.1
γ -Eudesmol	1784	0.5	0.3
Total identified		99.8	95.9

Peak area percentage is based on apolar DB-5 column, and values represent average of three determinations; Retention index on apolar DB-5 column

cis-pinocarveol (20.2%), neral (9.8%) and 1, 2-epoxide (3.6%).

Eighteen compounds were identified in the essential oil of *C. nardus* representing 95.9% of detected constituents including citronellal (35.5%), geraniol (27.9%) and citronellol (10.7%) as major components. This oil consisted of two monoterpene hydrocarbons (1.9%),

Table II

***In vitro* viability of human skin cell line HaCat exposed to *C. citratus* and *C. nardus* essential oils and their four major constituents**

Concentrations ($\mu\text{L}/\text{mL}$)	Cell viability (%)					
	Essential oils		Major constituents of essential oils			
	<i>C. citratus</i>	<i>C. nardus</i>	Neral	Geranial	Citronellal	Geraniol
Control (0)	100.0 \pm 0.0	100.0 \pm 00.0	100.0 \pm 00.0	100.0 \pm 00.0	100.0 \pm 00.0	100.0 \pm 00.0
25	96.0 \pm 1.7	97.3 \pm 1.2	85.7 \pm 1.2	95.7 \pm 1.2	95.7 \pm 1.2	98.7 \pm 0.6
50	85.7 \pm 1.2	93.0 \pm 1.0	75.0 \pm 00.0	85.0 \pm 00.0	85.0 \pm 00.0	104.0 \pm 1.7
75	72.0 \pm 2.0	85.3 \pm 0.6	64.0 \pm 1.0	76.0 \pm 1.0	76.0 \pm 1.0	108.7 \pm 1.2
100	63.0 \pm 1.0	80.7 \pm 1.2	50.7 \pm 0.6	66.0 \pm 1.0	66.0 \pm 1.0	110.7 \pm 1.2
150	50.7 \pm 0.6	77.0 \pm 1.0	47.3 \pm 0.6	58.0 \pm 00.0	58.0 \pm 00.0	114.3 \pm 1.2
200	45.0 \pm 1.0	68.3 \pm 1.2	44.7 \pm 0.6	53.7 \pm 1.2	53.7 \pm 1.2	116.0 \pm 1.7
250	40.3 \pm 0.6	64.0 \pm 1.7	40.3 \pm 0.6	50.3 \pm 0.6	52.7 \pm 0.6	119.3 \pm 1.2
300	38.7 \pm 0.6	57.7 \pm 0.6	38.7 \pm 0.6	47.3 \pm 2.1	50.3 \pm 1.5	125.3 \pm 0.6
350	30.3 \pm 0.6	54.7 \pm 0.6	30.3 \pm 0.6	45.3 \pm 0.6	45.7 \pm 0.6	129.3 \pm 1.2
400	25.7 \pm 0.6	52.3 \pm 0.6	25.7 \pm 0.6	36.0 \pm 1.7	36.7 \pm 1.5	134.3 \pm 0.6
450	21.0 \pm 1.0	50.0 \pm 1.0	21.0 \pm 1.0	31.0 \pm 1.0	31.7 \pm 0.6	137.7 \pm 0.6
500	19.3 \pm 1.2	45.7 \pm 0.6	19.3 \pm 1.2	24.7 \pm 0.6	25.3 \pm 0.6	140.3 \pm 0.6
600	16.7 \pm 1.5	42.3 \pm 0.6	16.7 \pm 1.5	21.7 \pm 1.5	22.0 \pm 1.0	142.7 \pm 0.6
700	11.7 \pm 1.5	38.0 \pm 1.0	11.7 \pm 1.5	15.7 \pm 1.2	16.7 \pm 0.6	144.7 \pm 0.6
800	10.7 \pm 1.2	35.3 \pm 0.6	10.7 \pm 1.2	15.3 \pm 0.6	15.3 \pm 0.6	147.3 \pm 1.2
900	5.7 \pm 0.6	31.3 \pm 1.2	5.7 \pm 0.6	10.7 \pm 1.2	11.7 \pm 1.5	150.3 \pm 0.6
1000	4.7 \pm 0.6	26.3 \pm 1.5	4.7 \pm 0.6	9.3 \pm 1.2	10.7 \pm 1.2	154.3 \pm 1.2

Viability expressed as mean \pm SD, n= 3

seven oxygenated monoterpenes (79.0%), five sesquiterpene hydrocarbons (12.3%) and four oxygenated sesquiterpenes (2.6%). This composition was similar to previous reports on *C. nardus* oils from Togo (Koumaglo et al., 1996; Koba et al., 2003), but differed from those described in Thailand (Nakahara et al., 2003) with geraniol (35.7%), trans-citral (22.7%), cis-citral (14.2%) and geranyl acetate (9.7%) as major components, in India by Mahalwal and Ali (2003) with citronellal (29.7%), geraniol (24.2%), γ -terpineol (9.7%) and cis-sabinene hydrate (3.8%) and different from those described in Bangladesh (Dugo et al., 1998).

The sample of *C. citratus* essential oil was notably richer in citral (neral and geranial upper than 77%) than that of *C. nardus* (with only a total of 1.1% in citral).

The percentage of HaCaT cell viability and the IC₅₀ values recorded for both tested essential oil samples and their four major constituents from commercial origin are shown in Table II.

At lower concentrations in the range from 25 to 100 $\mu\text{L}\cdot\text{mL}^{-1}$, none of the six tested substances showed any cytotoxicity. Conversely, both tested essential oils induced significantly increased cell cytotoxicity, at higher concentrations ranging from 100 to 1,000 $\mu\text{L}\cdot\text{mL}^{-1}$.

Two types of profiles were observed: (i) tested *citratus* and *nardus* oils and pure neral, geranial and citronellal standards showed cytotoxicity towards HaCaT with following respective IC₅₀ values (150, 450, 100, 250 and 300 $\mu\text{L}\cdot\text{mL}^{-1}$) shown in Table II; (ii) pure geraniol standard did not show any cytotoxicity up to 1,000 $\mu\text{L}\cdot\text{mL}^{-1}$ with cell viability up to 154%.

These experimental results demonstrated that, of the four major constituents of both tested essential oils, neral, geranial and citronellal were the most toxic for HaCaT cell line. Consequently, the cytotoxic effect of the essential oils of *C. citratus* and *C. nardus* found in this study was undoubtedly due to citral (neral and geranial), for the *citratus* oil, and to citronellal for that of *nardus*. Previous works only assumed what we experimentally established to a large extent in this investigation, but unfortunately authors did not provide any numerical data on the toxicity of the essential oils rich in citral which they used in high concentrations on human skin (Franchomme et al., 1996; Baudoux, 2002; Nakamura et al., 2003). Citral *in vitro* cytotoxicity was established on leukemia cells (Dubey et al., 1997b). Apart from its inherent toxicity, some authors have reported that citral, a mixture of both geranial and neral stereoisomers, had a significant

ability to suppress oxidative stress possibly through induction of the endogenous anti-oxidant glutathione system, providing a new insight into skin cancer (Nakamura et al., 2003). Besides, geraniol, as one of the main constituents of the *nardus* sample tested here, did not show any cytotoxicity. But it could probably expressed its potential as a cytoprotector or as an anti-oxidant in subsequent oxidative stress assays.

Conclusion

Our findings clearly showed that essential oils of *C. citratus* and *C. nardus*, with a percentage composition identical to our chemotypes, when used in appropriate doses, could be quite suitable as active components in pharmaceutical formulations for skin treatment and its damages repairing.

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Author Info

Koffi Koba (Principal contact)
e-mail: danielkkoba@yahoo.fr