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Croton lechleri Müll. Arg. (Euphorbiaceae) stem bark essential oil as possible mutagen-protective food ingredient against heterocyclic amines from cooked food

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ABSTRACT

The Amazonian *Croton lechleri* stem bark essential oil was tested for its anti-mutagenic potential by performing the Ames test against heterocyclic amines (HCAs), in continuing research on applicative functional profile of this phytocomplex as food ingredient (Rossi et al., 2011). *Salmonella typhimurium* strain TA98 was used with and without metabolic activation (S9 mix). The anti-mutagenic properties was assayed with the following HCAs: 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). All HCAs with S9 induced mutagenicity at 10^{-10} mol/plate. Without S9, IQ and MeIQ showed mutagenicity at 10^{-8} mol/plate, MeIQx and Glu-P-1 at 10^{-5} mol/plate, while Glu-P-2 was inactive. In presence of HACs (10^{-9} mol/plate), *C. lechleri* essential oil was tested for mutagen-protective properties (concentration range: 0.01–0.10 mg/plate) taking the Highest Uneffective Dose (HUD) as threshold reference. With S9 mix, *C. lechleri* essential oil displayed a significant reduction of revertants at 0.05 mg/plate, from 21% to 34%. The essential oil showed mutagen-protective efficacy against IQ and MeIQ tested as direct mutagens (10^{-7} mol/plate), with a revertants percentage reduction of 39% and 40%, respectively. No anti-mutagen capacity was noted for MeIQx and Glu-P-1 (10^{-5} mol/plate). Since HACs are known as possible colon and liver cancer inducers, *C. lechleri* essential oil was tested for its cytotoxicity and anti-proliferative capacity against LoVo and HepG2 cancer cell lines showing IC_{50} of 74.95 ± 0.05 μg/ml (LoVo) and 82.28 ± 0.03 μg/ml (HepG2), displaying a promising role of this essential oil as a functional food ingredient with interesting mutagen preventing properties.

1. Introduction

Plants are constantly monitored in the search for new therapeutic agents to treat disorders and diseases based on ethnobotanical, ethnopharmacological, chemosystematic and ecological information. In spite of the literature which emphasises biological applications of phytocomplexes and/or bioactive pure molecules, many natural derivatives remain largely untapped (Bezerra et al., 2009). However, this research contributes to the chemical and biological knowledge of those plants which are rarely or never studied but which may potentially contribute to improving the efficacy and safety of pharmaceuticals and health products. Among these studies, those concerning Amazonian plants are particularly interesting since the Amazonian basin is one of the most important biodiversity hotspots, where the ecological conditions and high density and diversity of species per unit area drive the plant secondary metabolism to biosynthetic pathways which are particularly rich in different chemical structures (Hopkins, 2007).

In recent years the research on plant extracts has been performed through two main binaries, i.e., chemical characterization and biological activity, the latter diversified in a plethora of biocapacities from those which are antibiotic to those which are anti-cancerogenic, taking into account efficacy and safety aspects. Particularly interesting as plant derivatives are the essential oils, as traditional herbal products are employed in many different applications (cosmetics, foods, pharmaceuticals, agriculture, etc.). As with many other plant derived products, they are investigated also for their capacity to prevent cancer onset as anti-mutagenic tools. One of the most important way through which humans come in contact with carcinogens is diet. In fact, the human diet contains a large number and variety of mutagens and carcinogens, and many of them have an action mechanism involving the generation of mostly oxygen radicals. Heterocyclic amines (HACs), produced mainly from household cooking of food rich in proteins, are known as possible human carcinogens as asserted by epidemiological and

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risk assessment studies (Saito, Sakai, & Nagase, 2006). The carcinogenic risk induced by these compounds is due not only to the level of exposure depending on the diet, but also to the fact that they interfere with uptake and biotransformations of dietary factors. In fact, the research into the aetiology of neoplasia has focused its attention on the complex mixture of chemical entities characterising the diet which can inhibit or modulate the onset and development of cancer. Moreover, somatic mutations are recognised not only as an initiating event in the development of sporadic cancers, but also as key steps in the development of atherosclerosis and a large number of chronic diseases in humans such as diabetes and neurodegenerative diseases (Berić, Nikoliv, Stanojević, Vuković-Gačić, & Knežević-Vukčević, 2008; Ferguson, 2010). Even if the best approach to decrease the incidence of cancer and related diseases is to avoid contact with carcinogens and mutagens, exposure to such compounds is however unavoidable in a large number of cases (Saito et al., 2006). There is abundant evidence that chemical mutagens and carcinogens can be inhibited by a plethora of naturally occurring minor constituents of plant origin, including spices, fruits and beverages, vegetables, etc. Therefore, chemical characterization of phytocomplexes, fractions and pure compounds and investigation into their bioactivities as tools to prevent mutagenesis and/or carcinogenesis are of great and increasing interest (Shishu, Singla, & Kaur, 2003). For example, there are many papers which report the mutagenic and antimutagenic potential of essential oils as phytocomplex and of terpenes (mono-, sesqui- and diterpenes), either isolated pure compounds against common environmental mutagens, or processed foods containing them, by mainly performing the Ames test employing *Salmonella typhimurium* strains (Aydin, Başaran, & Başaran, 2005; Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Berić et al., 2008; Ipek et al., 2005; Saito et al., 2006; Sghaier et al., 2010; Vuković-Gačić, Nikčević, Berić-Bjedov, Knežević-Vukčević, & Simić, 2006).

The anti-mutagenic properties of the essential oils may be due to different factors, e.g., (i) the inhibition of mutagen penetration into the cells, (ii) activation of cell antioxidant enzymes, (iii) mutagen neutralisation by direct scavenging activity or inactivation of radicals produced by mutagens, (iv) inhibition of metabolic conversion of pro-mutagens into mutagens by microsomal enzyme pools, (v) activation of enzymatic detoxification of mutagens, (vi) involvement interference with DNA repair systems, (vii) general and unspecified hepatoprotective activity (Bakkali et al., 2008; Edris, 2007). Moreover, this kind of growing interest in the use of essential oils needs however to assess their cytotoxic and genotoxic potential, identify possible toxic/mutagenic components, and try to display an almost complete profile of risks and benefits of employing these herbal derivatives as health promoters (Bakkali et al., 2008, and references therein).

Among the genus *Croton*, *C. cajucara* was investigated for its mutagenicity and antimutagenicity, in particular methanolic bark extracts of it, giving important evidence, using the micronucleus test, even if no specific evidence was identified about the putative chemicals responsible (Dos Santos et al., 2008). *C. regelianus* was instead studied for the possible antitumor role of its essential oil from leaves using different tumor cell lines, identifying the role of ascaridole and edoperoxides in exerting antiproliferative capacity against *in vitro* cancer cells and suggesting an action mechanism similar to that of artemisinin related compounds (Bezerra et al., 2009). Finally, *C. flavens* essential oil from leaves was tested for anticancer (i.e., antiproliferative) activity against human lung carcinoma and human colon adenocarcinoma cell lines, suggesting the sesquiterpenes α-cadinol, β-elemene and α-humulene as the compounds most responsible for the cytotoxic properties of the phytocomplex (Sylvestre, Pichette, Longtin, Nagau, & Legault, 2006). As a preliminary step of a research pathway that laid the foundation of the in-depth investigation reported here, we recently demonstrated the non-mutagenic activity of *C. lechleri* bark essential oil (Ames test and *S. cerevisiae* D7 assay). The anti-mutagenic properties of the terpene phytocomplex were also tested against ethyl methane sulphonate (EMS) employing *S. cerevisiae* D7 strain, and against 2-nitrofluorene, sodium azide and 2-aminoanthracene for TA98 and TA100 Ames strains (Rossi et al., 2011).

Croton lechleri Müll. Arg. (sin. *Croton draconoides* Müll. Arg.), a small-sized Amazonian tree belonging to the *Euphorbiaceae* family, is mainly known for traditional uses of its sap which was investigated in depth from a chemical and bioactivity perspective. In our previous related research, *C. lechleri* stem bark essential oil was studied for its chemical composition and bioactivities, suggesting its employment as a functional food constituent (Rossi et al., 2011, and references therein). In continuing research on *C. lechleri* essential oil's applicative functional profile, the anti-mutagenic potential was here reported by performing the Ames test against heterocyclic amines, known to be indirect mutation inducers produced during cooking of protein-rich foods (Robbana-Barnat, Rabache, Rialland, & Fradin, 1996). In fact, the Ames assay is well established and it is an effective strategy to check phytocomplexes or pure compounds for their potential chemopreventive role. It is also an ubiquitously accepted mutagenicity and antimutagenicity test and a good predictive tool for carcinogens (83% of mutagens found in the Ames test are also carcinogens) (Ames, Durston, Yamasaki, & Lee, 1973; Edenharder, Worf-Wandelburg, Decker, & Platt, 1999; Rossi et al., 2011). Cytotoxic and antiproliferative activity was then assayed against human colon carcinoma (LoVo) and human hepatocellular carcinoma (HepG2) cells, to evaluate possible further anticancer evidence of this Amazonian plant derivative.

2. Materials and methods

2.1. Plant material and isolation of essential oil

Three different stocks of *C. lechleri* Müll. Arg. stem barks collected in September 2006 from wild adult trees growing in the outskirts of the Juyukamentsa village (Morona-Santiago province, Ecuador) were subjected to steam distillation and the essential oil obtained was then treated to prevent degradations, as previously reported (Rossi et al., 2011).

2.2. Chemical characterization of the essential oil

The essential oil was chemically characterised through gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and the chemical structure of the main compounds was confirmed by GC–MS and proton Nuclear Magnetic Resonance (^{1}H NMR) (Rossi et al., 2011).

2.3. Chemicals

Chromatographic grade solvents and pure compounds used for bioassays were purchased from Sigma–Aldrich Italy (Milano, Italy) except for the heterocyclic amines (HCAs), namely the quinolines 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) supplied by Toronto Research Chemicals Inc. (Toronto, Canada). Dimethyl sulfoxide (DMSO, Sigma-Aldrich Italy) was used as solvent for HCAs and *C. lechleri* essential oil dilutions. All the microbial culture media were from Oxoid Italia (Garbagnate, Italy). Lyophilized post-mitochondrial supernatant S9 fraction (Aroclor 1254-induced,

Sprague–Dawley male rat liver in 0.154 M KCl solution), commonly used for the activation of promutagens to mutagenic metabolites, was purchased from Molecular Toxicology, Inc. (Boone, NC, USA) and stored at −80 °C. The components of S9 mix were: 8 mM $MgCl_2$, 32.5 mM KCl, 5 mM G6P, 4 mM NADP, 0.1 m sodium phosphate buffer pH 7.4 and S9 at the concentration of 0.68 mg/mL of mix.

2.4. Ames test: Mutagen-protective activity of C. lechleri essential oil against HACs

2.4.1. Tester bacterial strain

To evaluate the mutagen-protection capacity of *C. lechleri* stem barks essential oil in presence of the HACs previously cited, the tester strains *S. typhimurium* TA98 and TA100 kindly supplied by Prof. R. Barale (Pisa University, Italy) were used. For all assays, an inoculum of thawed permanent culture was added to 20 ml of Nutrient Broth and incubated a 37 °C in an orbital shaker (120 rpm) until reaching a microbial concentration of approximately 2×10^8 bacteria/ml.

2.4.2. C. lechleri essential oil cytotoxicity: Highest Uneffective Dose (HUD)

To correctly set up the assay to test the possible mutagen-protection capacity of the essential oil, the Highest Uneffective Dose (HUD) of the *C. lechleri* essential oil was determined in order to define the range of essential oil concentration avoiding cytotoxic interferences. Following the indications previously reported (Rossi et al., 2011), the *C. lechleri* HUD was determined for the wide concentration range from 0.01 to 10.00 mg/plate, with and without metabolic activation (S9 mix) with the object to avoid overlapping of the cytotoxic and antimutagenic results which would be therefore indistinguishable. In other words, HUD is of crucial importance to set up mutagen-protection experiments to confirm that the dose-dependent disappearance of the mutant colonies is not a result of cell-killing (Maron & Ames, 1983; Rossi et al., 2011). The essential oils were diluted in DMSO, mixed with 2 ml of molten top agar and plated with 0.1 ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 μmoles of L-histidine and 0.05 μmol of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of solution. Negative controls were set up with 100 μl/plate of DMSO with or without S9 mix. The colony-forming units (CFU) were assessed after the plates were incubated at 37 °C for 48 h and compared with that of control where no test samples were added. HUD for *C. lechleri* essential oil was evaluated by visual estimation (colonies counting) of three independent experiments and integrated by statistical analyses.

2.4.3. Mutagenic activity of heterocyclic amines (HCAs)

The mutagenic activity of the HCAs was determined on the basis of the counted CFU in plates treated with IQ, MeIQ, MeIQx, Glu-P-1 and Glu-P-2 at concentrations comprising between 10^{-10} and 10^{-5} mol/plate (Edenharder et al., 1999). The results were then compared to those recorded in negative control plates to check significant evidence of direct and indirect induced mutagenicity by HCAs. Therefore, to reach the target, mutagenic assays were performed in triplicate with and without metabolic activation (S9 mix).

2.4.4. Mutagen protection of C. lechleri essential in presence of HCAs

The potential mutagen protection of *C. lechleri* essential oil with TA98 tester strain system was determined for a concentration range from 0.01 to 0.10 mg/plate in presence of HCAs. Negative controls were set up with 100 μl/plate of DMSO with or without S9 mix. HCAs were all used at 10^{-9} mol/plate to check the protective capacity of the essential oil in presence of a clear indirect mutagenicity mediated by the presence of S9 mix. Given the different concentration at which the different HCAs induced direct mutagenicity (without S9 mix), the protective activity of the essential oil was checked in presence of IQ and MeIQ at 10^{-7} mol/plate, while for MeIQx, Glu-P-1 and Glu-P-2 the concentration tested was 10^{-5} mol/plate. The inhibition rate for mutagenic induction was computed considering the data obtained from three independent experiments, with and without metabolic activation, according to the formula: inhibition rate $(\%) = (A - B) \times 100/A \pm$ standard deviation, where A are revertants in positive control, and B are revertants in the essential oil samples, having subtracted the spontaneous revertants. Data were expressed also as CFU/plate ± standard deviation.

2.5. DNA methyltransferase inhibition

C. lechleri essential oil (10^{-4} to 10^{-1} mg/ml concentration range) was analysed for the DNA methyltransferase 1 activity by the EpiQuik™ DNA methyltransferase assay kit and instructions provided by the manufacturer (EpiQuik DNA Methyltransferase 1 Activity/Inhibitor Screening Assay Kit, Epigentek Group Inc., New York, NY, USA). This kit yields accurate measurements of methylcytosine content as a percentage of total cytosine content. Briefly, in an assay with this kit, the unique cytosine-rich DNA substrate is stably coated on the strip wells. These wells are specifically treated to have a high DNA absorption ability. The Dnmt1 enzyme transfers a methyl group to cytosine from *S*-adenosyl-L-methionine to methylate DNA substrate. The methylated DNA can be recognised with anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be colorimetrically quantified through an enzyme-linked immunosorbent assay-like reaction using 5-methylcytosine antibody. The amount of methylated DNA is proportional to the OD intensity and the degree of DNA methylation can be calculated using the following formula:

$$\%\text{Methylation} = [\text{OD}(\text{sample} - \text{blank})/2\text{OD}(\text{positive control} - \text{blank})] \times 100$$

where OD represents the optical density, blank is buffer without DNA, positive control is methylated control DNA.

2.6. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay

Two cancer cell lines, human colon carcinoma LoVo (ECACC No.: 87060101) and human hepatocellular carcinoma HepG2 (ATCC No.: CRL-11997) were used. The LoVo and HepG2 cells were cultured in RPMI 1640 and D-MEM medium, respectively, both supplemented with 10% foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin.

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay (MTT, Sigma, Italy) was used to determine the ability of metabolically active cells to reduce the yellow tetrazolium salt (MTT) forming insoluble purple formazan crystals. The assay for each sample concentration was performed in triplicates and the culture plates were kept at 37 °C with 5% (v/v) CO_2 for one day. After incubation, 100 μl of medium was removed from each well. Subsequently, 100 μl of 0.5% w/v MTT, dissolved in phosphate buffered saline, was added to each well and allowed to incubate for a further 4 h. After 4 h of incubation, 100 μl of DMSO was added to each well to dissolve the formazan crystals. Absorbance values at 570 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy) and expressed as IC_{50}, which is the concentration to reduce the absorbance of treated cells by 50% considered as cytotoxicity with reference to the control (untreated cells).

Cells counts and viability were performed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 2×10^4 cells/ml. 100 μl of the above concentration were cultured in 96-well plates for one day to become nearly confluent. Concentrations ranging from 0.05 to 1 mg/ml of the samples were prepared from the stock solutions by serial dilution in cellular specific medium to give a volume of 100 μl in each well of a microliter plate (96-well). Then cells were cultured with vehicle, essential oil for 24 h.

2.7. Statistical analysis

Relative standard deviations and statistical significance (Student's t test; $P \leqslant 0.05$) were given where appropriate for all data collected. One-way ANOVA and LSD post hoc Fisher's honest significant difference test were used for comparing the bioactive effects of different *C. lecleri* essential oil samples. Student's t test ($P \leqslant 0.05$) combined with the Highest Uneffective Dose (HUD) comparison was used to interpret the results of a significant decrease in the number of *Salmonella* revertants. When the modulator dose concentration is statistically effective and it ranges below or coincides with the HUD, the samples were considered to present sign of the effect (antimutagenicity). All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

3. Results and discussion

C. lechleri essential oil was preliminarily investigated for its chemical composition and bioactivities, for its possible employ as functional food ingredient (Rossi et al., 2011). In particular, the essential oil was characterised as sesquiterpene chemotype, sesquicineole (17.18%), α-calacorene (11.22%), 1,10-di-epi-cubenol (4.72%), β-calacorene (4.31%) and epicedrol (4.07%) being the most abundant compounds accounting for 76.93% of the total (95.84%). The 18.89% of the remaining characterised part is composed of monoterpenes, in particular limonene (4.17%), borneol (2.66%), p-cymene (2.59%) and α-pinene (2.00%) (Fig. 1). Starting from the bioactivity evidences reported for the non-mutagenicity and for the mutagen-protective capacity (Rossi et al., 2011), *C. lechleri* essential oil was subjected to the Highest Uneffective Dose (HUD) evaluation in order to determine the maximum concentration of the terpenic phytocomplex which does not induce cytotoxicity (Table 1).

Therefore, the HUD for toxic effect, with and without metabolic activation through S9 mix, for TA98 and TA100 strains was settled at 0.1 and 0.01 mg/plate respectively (significance $P < 0.05$ according to t test; Fig. 1). This kind of result points out the higher sensitivity of TA100 than TA98 in respect to the toxicity of essential oils. This evidence could be due to the higher permeability of TA100 bacterial cell wall than TA98 towards mono- and sesquiterpenes (Ipek et al., 2005; Vuković-Gačić et al., 2006).

Given the fact that *C. lechleri* essential oil did not exhibit mutagen protective activity for TA100 against the direct mutagen sodium azide and indirect mutagen 2-aminoanthracene at lower concentration than HUD (0.01 mg/plate) (Rossi et al., 2011), and in light of a similar approach reported by related papers (Arimoto-Kobayashi & Hayatsu, 2003), the *S. typhimurium* TA98 strain with and without metabolic activation (S9 mix) was adopted for the following assays with heterocyclic amines (HACs) (Tables 2 and 3). Moreover, the specific mutagenic activity of HACs is much higher in TA98 strain than in TA100, indicating that heterocyclic amines induce frame shift mutations (Frederiksen, 2005).

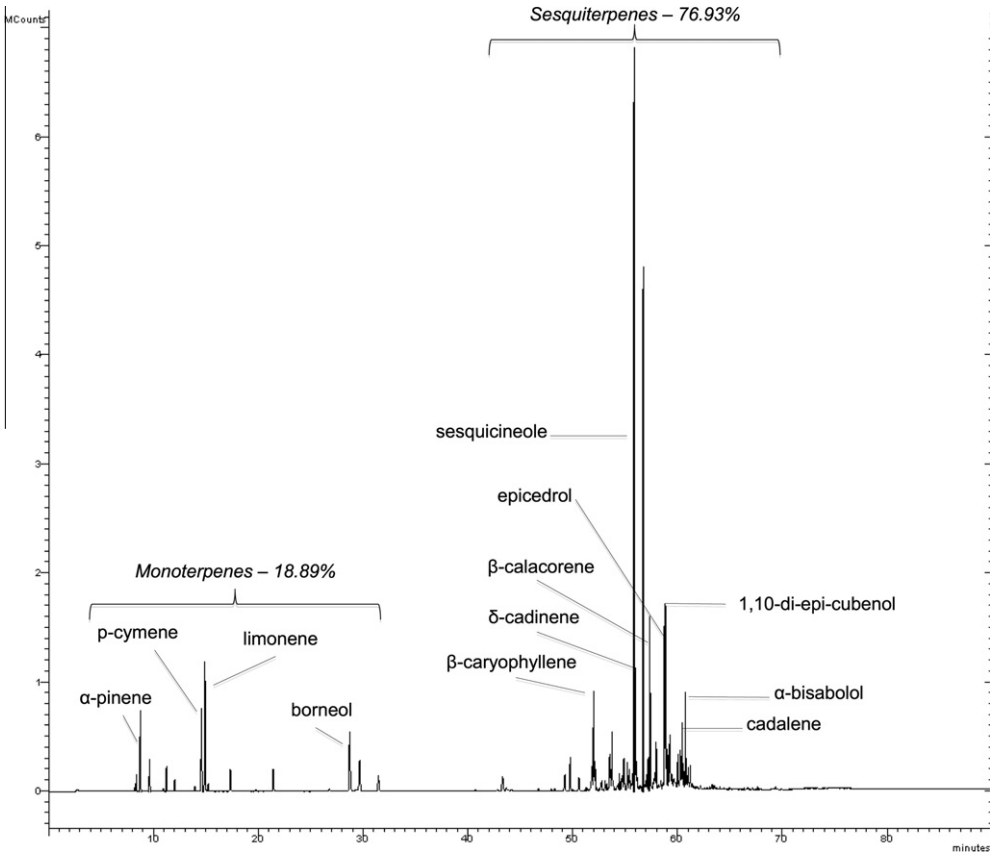

Fig. 1. Gas chromatographic spectrum of *Croton lechleri* stem bark essential oil. Sesquiterpenes were the most abundant compounds being the 76.93% of the total, while monoterpenes accounted for 18.89%.

Table 1
Highest Uneffective Dose (HUD) tested with and without metabolic activation (S9 mix). HUD represents the maximum concentration of *Croton lechleri* essential oil (*C. l.* e.o.) DMSO diluted which does not induce cytotoxic evidences in *S. typhimurium* TA98 and TA100 strains cultures. The HUD data are essential to interpret the results of significant decrease in the number of *Salmonella* revertants. Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as survival percentage ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	CFU/plate	Survival%	CFU/plate	Survival%	CFU/plate	Survival%	CFU/plate	Survival%
	TA98 without S9 mix		TA98 with S9 mix		TA100 without S9 mix		TA100 with S9 mix	
0.000	1682 ± 67	100.00 ± 3.98	3303 ± 61	100.00 ± 1.85	3433 ± 44	100.00 ± 1.28	3587 ± 46	100.00 ± 1.85
0.010	1708 ± 51	101.54 ± 3.03	3337 ± 39	101.03 ± 1.18	3421 ± 52	99.65 ± 1.52	3617 ± 42	101.03 ± 1.18
0.025	1695 ± 47	100.77 ± 2.77	3361 ± 52	101.76 ± 1.55	2452 ± 46*	71.42 ± 1.87*	1814 ± 33*	50.57 ± 1.82*
0.050	1676 ± 41	99.64 ± 2.44	3367 ± 57	101.94 ± 1.73	1516 ± 22*	44.16 ± 1.45*	902 ± 26*	25.15 ± 1.88*
0.075	1634 ± 38	97.15 ± 2.33	3315 ± 48	100.36 ± 1.45	1034 ± 23*	30.12 ± 2.12*	698 ± 23*	19.46 ± 1.29*
0.100	1568 ± 33	93.22 ± 1.96	3289 ± 54	99.57 ± 1.96	128 ± 19*	3.73 ± 1.48*	457 ± 18*	12.74 ± 0.93*
0.250	834 ± 15*	49.58 ± 1.79*	1256 ± 23*	38.03 ± 1.83*	94 ± 12*	2.74 ± 1.27*	127 ± 12*	3.54 ± 0.33*
0.500	31 ± 12*	1.84 ± 0.71*	634 ± 24*	19.19 ± 0.73*	7 ± 2*	0.21 ± 0.05*	16 ± 3*	0.45 ± 0.08*
1.000	–*	0.00 ± 0.00*	94 ± 7*	4.12 ± 0.23*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*
5.000–10.000[a]	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*

(–) no Colony Forming Units (CFU) has been detected because of the cytotoxicity expressed by the *C. lechleri* essential oil.
[a] *C. lechleri* essential oil has been tested until the concentration of 10.00 mg/plate giving always cytotoxicity evidences.
* Significant evidences (cytotoxicity) in light of Student's *t*-test results.

Table 2
Ames test (*Salmonella typhimurium*, strain TA98) to assay mutagen induction (revertants his+/plate) with metabolic activation (S9 mix) of the Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) tested at 10^{-10} to 10^{-5} mol/plate concentration range. HCAs have been diluted in DMSO; negative controls (HCAs = 0 mol/plate) have been set up with 100 μl/plate of DMSO. The results are expressed as Colony Forming Units (CFU)/plate ± standard deviation (s.d.). All the values resulted significant according to Ames computation (Maron & Ames, 1983), except for those achieved for negative controls.

HCAs (mol/plate)	TA98 with S9 mix				
	IQ CFU/plate ± s.d.	MeIQ CFU/plate ± s.d.	MeIQx CFU/plate ± s.d.	Glu-P-1 CFU/plate ± s.d.	Glu-P-2 CFU/plate ± s.d.
0	59 ± 4	59 ± 4	59 ± 4	59 ± 4	59 ± 4
10^{-10}	1060 ± 28	2162 ± 39	561 ± 21	490 ± 35	268 ± 18
10^{-09}	2526 ± 153	2812 ± 64	1434 ± 77	1254 ± 34	1322 ± 139
10^{-08}	4155 ± 169	2864 ± 37	2479 ± 24	4029 ± 154	3320 ± 193
10^{-07}	3824 ± 117	3166 ± 67	3450 ± 31	3463 ± 151	3026 ± 161
10^{-06}	2990 ± 105	2762 ± 73	3069 ± 53	2956 ± 158	2395 ± 116
10^{-05}	2687 ± 107	2228 ± 84	2562 ± 47	2587 ± 108	1838 ± 104

Table 3
Ames test (*Salmonella typhimurium*, strain TA98) to assay mutagen induction (revertants his+/plate) without metabolic activation (S9 mix) of the Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) tested at 10^{-10} to 10^{-5} mol/plate concentration range. HCAs have been diluted in DMSO; negative controls (HCAs = 0 mol/plate) have been set up with 100 μl/plate of DMSO. The results are expressed as Colony Forming Units (CFU)/plate ± standard deviation.

HCAs (mol/plate)	TA98 without S9 mix				
	IQ CFU/plate ± s.d.	MeIQ CFU/plate ± s.d.	MeIQx CFU/plate ± s.d.	Glu-P-1 CFU/plate ± s.d.	Glu-P-2 CFU/plate ± s.d.
0	42 ± 4	42 ± 4	25 ± 2	25 ± 2	25 ± 2
10^{-10}	48 ± 8	45 ± 6	26 ± 2	31 ± 3	26 ± 3
10^{-09}	56 ± 6	51 ± 8	31 ± 3	29 ± 6	27 ± 5
10^{-08}	230 ± 14*	413 ± 39*	29 ± 5	28 ± 3	29 ± 7
10^{-07}	1907 ± 45*	1257 ± 38*	26 ± 6	24 ± 4	23 ± 4
10^{-06}	1404 ± 52*	341 ± 27*	35 ± 5	40 ± 2	38 ± 6
10^{-05}	1334 ± 25*	278 ± 26*	115 ± 9*	94 ± 9*	42 ± 5

* Significant values according to Ames computation (Maron & Ames, 1983).

The mutagen protective capacity of *C. lechleri* essential oil against direct and indirect mutagens (nitrofluorene and 2-aminoanthracene) on TA98 (Rossi et al., 2011) led us to investigate the same capacity employing heterocyclic amines (HCAs). Heterocyclic amines, produced during the cooking process of proteins rich foods, i.e., meat (beef, pork, lamb, and chicken), residues after cooking meat, beef flavors, fish, are potent carcinogenic mutagens when metabolically activated (Arimoto-Kobayashi & Hayatsu, 2003; Aydin et al., 2005; Robbana-Barnat et al., 1996). Although the quantification of HACs in a typical Western diet proves to be difficult, the exposure level to these chemical compounds can be considered similar, to a good approximation, to that of nitrosamines and benzo[a]pyrene (Robbana-Barnat et al., 1996). However, since the real risk to humans is still a matter of debate, WHO and National Health Organizations suggest minimizing the exposure. The HACs formation during cooking at temperatures above 200 °C is mainly due both to pyrolysis of amino acids and to different reaction sequences having amino acids, carbohydrates and creatinine as substrates. The multi-target carcinogen activity of HACs was demonstrated in different laboratory animal models, and the

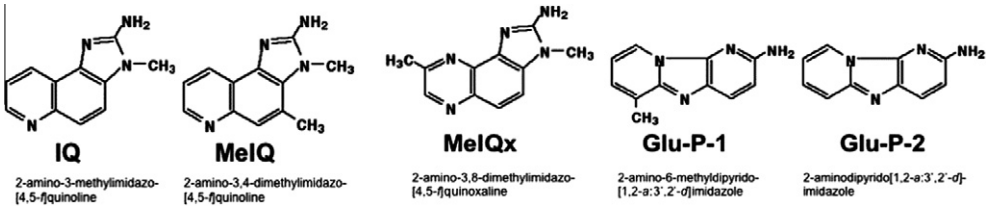

Fig. 2. Chemical structure of the tested Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2).

HACs synergic capacity was related to inducing amino-α-carboniles adducts formation in DNA of cells belonging to stomach, liver, colon, kidneys, prostate and skin tissues (Aydin et al., 2005, and references therein). The HACs considered were the quinolines 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) (Fig. 2). Even if heterocyclic amines are generally reputed to be indirect mutagens, they were used to induce mutagenicity on TA98 strain with and without S9 mix (Tables 2 and 3). All HCAs in presence of S9 mix induced mutagenicity already at 10^{-10} mol/plate, with significant revertants values according to Ames computation (Maron & Ames, 1983), confirming related literature data (Table 2; Edenharder et al., 1999). Interesting evidence emerged by the same assay without metabolic activation, with a direct significant mutagenicity of IQ and MeIQ at 10^{-8} mol/plate (Table 3). In the same conditions, MeIQx and Glu-P-1 gave revertants at 10^{-5} mol/plate while Glu-P-2 resulted inactive as direct mutagen (Table 3). Given the fact that the concentration of 10^{-8} mol/plate is consistent with the amount of HACs in cooked food (Frederiksen, 2005), the results have important implications on the debate about the relationship between heterocyclic amines mutagenesis/cancerogenesis risk and the diet. Results on HUD and to HACs mutagenicity, with and without metabolic activation, drove the mutagen-protective activity of *C. lechleri* essential oil (0.01–0.1 mg/plate concentration range) against all the HACs metabolically activated with S9 mix tested at 10^{-9} mol/plate (Table 4). *C. lechleri* essential oil induced a significant decrease in revertants colonies at 0.05 mg/plate with all HACs. In fact, the reduction was almost of 22% for IQ, 29% for MeIQ, 31% for MeIQx, 20% for Glu-P-1 and of 23% for Glu-P-2. At the highest concentration tested of essential oil (0.1 mg/plate), the highest mutagenic protection was expressed with a revertants reduction of about 69% for Glu-P-2, followed by MeIQx (59%), IQ (42.19%), Glu-P-1 (33%) and MeIQ (30%).

In light of their direct mutagenic activity (Table 3), IQ and MeIQ was tested at 10^{-7} mol/plate to check the possible genotoxic protective activity of *C. lechleri* essential oil (Table 5). As for the metabolic activated conditions, a significant protective activity of essential oil was expressed at 0.05 mg/plate without S9, with a reduction of revertants colonies percentage of almost 39% and 40% for IQ and MeIQ respectively. At 0.1 mg/plate, *C. lecheri* essential oil induced a revertants reduction of 46% for IQ and of 41% for MeIQ in treated *S. typhimurium* TA98 strains. On the contrary, TA98 strains treated with MeIQx and Glu-P-1 at 10^{-5} mol/plate did not exhibit a reduction of revertants colonies in the presence of *C. lechleri* essential oil. Glu-P-2 was not assayed because it did not induce mutagenicity without metabolic activation (Table 6).

The fact that IQ and MeIQ displayed a moderate direct-acting mutagenic activity still inhibited by *C. lecheri* essential oil, leads us to suggest that the essential oil may be involved both early and late in mutagenesis, i.e., by inhibiting metabolic activation via P450, and by either reacting with an ultimate mutagen or blocking its access to DNA. As a matter of fact, the antimutagenic action without S9 mix cannot be regarded as an effective proof to discharge the hypothesis of a promutagen metabolic activation inhibition. Indeed, the antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of IQ were proved only when cytosol activation is involved without hexogen S9 fraction addition (Hatch, Lightstone, & Colvin, 2000). This could be the result of inhibition of either N-OH-IQ formation by a non-450 route or a different pathway of IQ activation. MeIQx, as a case in point, is activated through metabolic pathway under the action of *N*-acetyltransferase forming N2-(guanine-8-yl-MeIQx) adduct with C8 position of guanine (Ochiai et al., 1993). To confirm the hypothesis of a HCAs endogen activation by *S. typhimurium* TA98, an acetyltransferase enzyme was partially purified from this strain and characterised as *O*-acetyltransferase (Saito, Shinohara, Kamataki, & Kato, 1985).

The chemical composition of *C. lechleri* stem bark essential oil (Fig. 1; Rossi et al., 2011) can help to suggest which compounds are responsible for the mutagenic protection. However, the bioactivity of an essential oil, as well as of other kind of phytocomplexes, is hardly due to a single active compound, but it is rather ascribed to a synergic activity of different kinds of chemicals that may not be necessarily the most abundant (Bakkali et al., 2008; Edris, 2007; Voon, Bhat, & Rusul, 2012). Terpenes and their derivatives were found to be potentially useful in the prevention and therapy of several diseases, including cancer. For example, d-limonene (4.20% in *C. lechleri* essential oil) and linalool (0.82% in *C. lechleri* essential oil) are claimed to inhibit, in a dose-dependent manner, the development of mammary, liver, skin, lung, colon, fore-stomach, prostate, cervical and pancreatic carcinomas (Cherng, Shieh, Chiang, Chang, & Chiang, 2007). β-Caryophyllene, a sesquiterpene with interesting protective capacity against oxidative stress (Rossi et al., 2011; Zheng, Kenney, & Lam, 1992) was suggested as a promising potential anticarcinogenic agent. The antimutagenic effects of linalool and β-caryophyllene were also evaluated by the bacterial reverse mutation assay on *S. typhimurium* TA98 and TA100 (Di Sotto, Evandri, & Mazzanti, 2008). Linalool is devoid of antimutagenic activity against 2-nitrofluorene, sodium azide, methyl methane sulfonate and 2-aminoanthracene. In contrast, β-caryophyllene showed a strong antimutagenic activity against 2-nitrofluorene. To our knowledge, related literature does not report any data about inhibition of IQ, MeIQ, MeIQx, Glu-P-1 and Glu-P-2 mutagenicity in the bacterial reverse mutation assay on *S. typhimurium* by terpenes. Even so, it is possible to make suggestions about the correlations between the chemical composition of *C. lechleri* bark essential oil and other structurally related compounds already studied for antimutagenic effect against HCAs. Interesting suggestions came from a quantitative structure–activity relationship (QSAR) database, developed by the combination of inhibitory process results of flavonoids against HCAs mutagenesis with structural evidences, *ab initio* quantum chemical, hydropathic, and antioxidant factors (Hatch et al., 2000). Limited quantitative outcomes emerged from this research strategy supporting the evidence that the inhibition

Table 4
Ames test (*Salmonella typhimurium*, strain TA98) with metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–0.10 mg/plate concentration range) in presence of 1×10^{-9} mol/plate of the heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 with S9 mix									
	HCAs 10^{-9} mol/plate									
	IQ*		MeIQ		MeIQx		Glu-P-1		Glu-P-2	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 5.62	2307 ± 70	100.00 ± 5.58	3672 ± 97	100.00 ± 5.42	1106 ± 51	100.00 ± 5.56	1170 ± 52	100.00 ± 5.63	1267 ± 41
0.010	102.56 ± 5.11	2366 ± 51	94.21 ± 5.52	3459 ± 95	95.42 ± 6.51	1056 ± 51	92.59 ± 5.43	1083 ± 34	96.03 ± 5.30	1217 ± 30
0.025	98.09 ± 4.69	2263 ± 32	93.32 ± 5.07	3427 ± 95	92.94 ± 5.03	1028 ± 51	92.13 ± 4.98	1078 ± 44	94.32 ± 5.15	1195 ± 24
0.050	78.41 ± 2.72*	1809 ± 27*	72.13 ± 4.13*	2649 ± 93*	68.64 ± 4.18*	759 ± 51*	79.20 ± 4.58*	926 ± 58*	77.38 ± 4.22*	981 ± 20*
0.075	68.66 ± 2.04*	1584 ± 23*	70.86 ± 3.98*	2602 ± 93*	54.70 ± 3.80*	605 ± 35*	72.95 ± 4.07*	853 ± 51*	54.77 ± 3.82*	694 ± 18*
0.100	57.81 ± 1.42*	1333 ± 32*	69.85 ± 3.72*	2565 ± 87*	41.25 ± 2.11*	456 ± 11*	66.77 ± 3.81*	781 ± 41*	31.46 ± 2.59*	399 ± 29*

* Significant values (Student *t* test).

Table 5
Ames test (*Salmonella typhimurium*, strain TA98) without metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–1.00 mg/plate concentration range) in presence of 1×10^{-7} moli/plate of the heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 without S9 mix			
	HCAs 10^{-7} mol/plate			
	IQ		MeIQ	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 5.60	1772 ± 46	100.00 ± 5.48	1566 ± 55
0.010	99.29 ± 5.11	1759 ± 40	99.04 ± 5.02	1551 ± 40
0.025	95.48 ± 4.84	1692 ± 38	95.92 ± 4.88	1502 ± 39
0.050	61.16 ± 2.92*	1084 ± 24*	59.64 ± 3.53*	934 ± 44*
0.075	58.19 ± 2.78*	1002 ± 22*	59.06 ± 2.82**	925 ± 32*
0.100	54.25 ± 2.42*	961 ± 31*	58.80 ± 3.78*	921 ± 27*

* Significant values (Student *t* test).

Table 6
Ames test (*Salmonella typhimurium*, strain TA98) without metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–1.00 mg/plate concentration range) in presence of 1×10^{-5} moli/plate of the heterocyclic amines (HCAs) 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 without S9 mix			
	HCAs 10^{-5} mol/plate			
	MeIQx		Glu-P-1	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 3.90	90 ± 3	100.00 ± 4.25	93 ± 4
0.010	100.74 ± 5.21	91 ± 8	96.76 ± 5.03	90 ± 8
0.025	103.33 ± 5.48	93 ± 8	98.92 ± 5.19	92 ± 5
0.050	105.19 ± 5.58	95 ± 8	101.08 ± 5.58	94 ± 5
0.075	98.89 ± 5.18	89 ± 7	102.15 ± 5.64	95 ± 5
0.100	97.42 ± 5.12	88 ± 7	106.83 ± 5.81	99 ± 15

of heterocyclic amine mutagenesis by flavonoids involves the interference with the cytochrome P450 pathway, caused by non-covalent, or eventually covalent, bindings. The widely discussed antioxidant and radical scavenging properties of flavonoids appear to be unrelated to the inhibition of mutagenesis. In fact, the major variables controlling the inhibitory efficacy of the flavonoids in the *S. typhimurium* assay are near-planarity, hydrophobicity and dipole moment.

Relevance of planarity was confirmed in chalcones and structurally related compounds acting as heterocyclic amine mutagenesis inhibitors (Edenharder et al., 1999). If planarity is considered as a structural feature useful as anti mutagenic activity identifier, sesquicineole, the most abundant chemical in *C. lechleri* bark essential oil (17.29%), would be characterised as an effective HCAs mutagenicity inhibitor. On the contrary, α-calacorene (11.29% in *C. lechleri* essential oil) could be structurally related with the inhibitory potency of the flavonoids and chalcones.

Hypothesising that *C. lechleri* essential oil protective activity would be involved in DNA methylation processes, methyltransferase 1 inhibition of essential oil (10^{-4} to 10^{-1} mg/ml concentration range) was tested three times by the EpiQuik™ DNA methyltransferase assay kit. No significant differences between control and treated samples

Table 7
Cytotoxicity expressed as growth inhibition percentage (Inh.%) and IC_{50} (μg/ml) of *Croton lechleri* essential oil (*C. l.* e.o.) against human colon carcinoma LoVo (ECACC No.: 87060101) and human hepatocellular carcinoma HepG2 (ATCC No.: CRL-11997) cell lines. Exposure time 24 h ± s.d. ($n = 3$). Vinblastine (2 g/ml) has been used as positive control.

C. l. e.o. (μg/ml)	LoVo (Inh.%)	HepG2 (Inh.%)
1000	99.12 ± 0.06	88.46 ± 0.04
500	98.97 ± 0.05	87.56 ± 0.04
250	98.92 ± 0.05	85.86 ± 0.03
100	72.66 ± 0.04	63.39 ± 0.02
50	23.53 ± 0.02	20.76 ± 0.01
IC_{50} (μg/ml)	74.95 ± 0.05	82.28 ± 0.03

were found (data not shown), pointing out that the essential oil is not involved in any significant inhibitory effect of DNA methylation.

Given the fact that the Ames assay is an ubiquitously accepted mutagenicity and anti-mutagenicity test and a good predictive tool for carcinogens (83% of mutagens found in the Ames test are also carcinogens) (Ames et al., 1973; Edenharder et al., 1999; Rossi et al., 2011), the stem bark *C. lechleri* essential oil was evaluated for its *in vitro* cytotoxic properties on two human cancer cell lines: colon carcinoma LoVo and hepatocellular carcinoma HepG2. Both human cell lines were capable of forming a homogeneous monolayer on plastic substratum of the culture wells, set for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxic effect of essential oil on the growth of human tumor cell lines, expressed as IC_{50} (Table 7), point out the human colon carcinoma LoVo as the most responsive cell line (IC_{50} = 74.95 μg/ml) with interesting values consistent with those highlighted in other related studies about the antitumor activity of *Croton* spp. essential oils (Bezerra et al., 2009; Sylvestre et al., 2006). This essential oils cytotoxicity can be related to the bioactivity of different terpene-rich extracts from other *Croton* species (Dao et al., 2010; Phan, Phan, Hamada, & Otsuka, 2005). In light of this evidence it could be speculated that the cytotoxic activity of the *C. lechleri* stem bark essential oil would be mainly determined by the most abundant sesquiterpene fractions. In particular, considering the antioxidant capacity reported in a previous related paper (Rossi et al., 2011) as discriminant tool in suggesting the most responsible compounds, the sesquiterpene α- and β-calacorene, δ-cadinene, cadalene, caryophyllene oxide, 1,10-di-epi-cubenol and epicedrol would probably be the most involved compounds in exerting cytotoxicity of the *C. lechleri* stem bark essential oil.

4. Conclusion

C. lechleri stem bark essential showed interesting anti-mutagenic properties at concentrations consistent with its safe use as additive functional phytocomplex in foods (for e.g., as relish for high protein cooked foods). Based on this evidence, a new threshold of HCAs as indirect mutagens was evaluated, together with the determination of direct mutagen capacity of some HCAs at concentrations consistent with their presence in cooked foods. Data reported in this paper also demonstrate that *C. lechleri* stem bark essential oil offers an effective protection against the mutagenic potential of HCAs with and without metabolic activation. In light of the results achieved with the Ames test, and the cytotoxic and antiproliferative assays against colon and hepatocellular carcinomas, it is hard to suggest the potential risk caused by this essential oil in humans, since complex metabolic activation reactions are not adequately represented in *in vitro* assays with exogenous homogenate enzymes. Nevertheless, the data reported in this study provide the framework for the development of new anticancer drugs.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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