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Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz & Pavon (Piperaceae) essential oils from Eastern Ecuador

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ABSTRACT

Essential oils from aerial parts of *Piper aduncum* (Matico) and *Piper obliquum* (Anis del Oriente) of ecuadorian origin were analyzed by GC–FID, GC–MS, ^{13}C NMR and their biological and pharmacological activities were assessed. Chemical composition proved to be unusually different from previous reports for safrole-rich *P. obliquum* (45.8%), while *P. aduncum* main constituent was dillapiol (45.9%). No genotoxic activity was found in the Ames/*Salmonella typhimurium* (TA98 and TA100) assay, either with or without S9 activation. Mutagen-protective properties, evaluated using sodium azide, 2-nitrofluorene and 2-aminoanthracene as mutagens/promutagens, was observed against promutagen 2-aminoanthracene, likely in consequence of microsomial deactivation. Antimicrobial assays have been performed on Gram+/Gram− bacteria, dermatophyte and phytopathogenic fungi and best results were provided by *P. aduncum* against fungal strains with complete inhibition at 500 μg/ml. Preliminary analgesic and antithrombotic activities evidenced the absence of the former in hot plate and edema assays and a limited antiplatelet action against three different agonists (ADP, AA and U46619). Both oils have a very limited antioxidant capacity.

1. Introduction

Piper genus includes about 1000 paleoherb species, with distinctive swollen nodes and drooping spikes, frequently found in the understory and secondary vegetation of tropical forest canopy of the Americas and Asia. Most species are fast-growing aromatic shrubs or vines and many of them have a renowned traditional antiseptic, insecticidal and antimicrobic use (Sengupta and Ray, 1987; Ehringhaus, 1997; Navickiene et al., 2006; de Morais et al., 2007; Fazolin et al., 2007; Duarte et al., 2007). Some Pipers are also employed in folk medicine as analgesics in pain management, toothache and wound treatment (Gatti, 1985). From a phytochemical standpoint, safrole, dillapiol, myristicine and similar methylenedioxyphenyl derivatives have been frequently detected in sensible amounts in the genus (Parmar et al., 1997) and many of these compounds are known to be toxic (Buchanan, 1978) and liable to induce DNA alteration via different mechanisms, often involving hepatic bioactivation (Dietz and Bolton, 2007). Notwithstanding their widespread traditional and commercial use, Piper essential oils have been scarcely evaluated for their mutagenic/genotoxic properties and received little attention on this regard, despite the renowned toxicological profiles of some of the aforementioned substances.

Piper obliquum Ruiz & Pavon is a shrub growing in lowland secondary rainforests of Central and South America. Its leaves are utilized as analgesic or antiarthritic by topic application on the affected body part in Guyana and Ecuador, where the plant is known with the popular name of "Anìs del Oriente", due to its distinct anis-like perfume (Defilippis et al., 2004). Despite the wide number of chemotaxonomic studies performed on the composition of Piper species, a single report on *P. obliquum* from Panama is available (Mundina et al., 1998). The knowledge on possible intraspecific chemodiversity for this specie is thus extremely limited. Putative analgesic, antimicrobial properties and the evaluation of its genotoxic profile and possible intraspecific chemodiversity have not been assessed.

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Piper aduncum L., also known as "matìco", "aperta-ruao", "pimenta longa" or "bamboo piper" is a multistemmed shrub of more than 5 m height, native of the Caribbean but adapted to thrive through the whole tropics, often acting as a weed in disturbed habitats. It presents a wide range of traditional uses and its essential oil is a well-known insecticide, molluschicide and antibacterial (Pohlit et al., 2006). Tea made from leaves and roots is used to treat diarrhea, dysentery, nausea, ulcers, genito-urinary infections and is also traditionally utilized for the control of bleeding as an antihemorrhagic (Bennett et al., 2002; Francis, 2003; Pohlit et al., 2006). It is yet unclear if essential oil constituents may be accounted as responsible or co-responsible for such activities and a certain degree of intraspecific chemodiversity has been pointed out (bin Jantan et al., 1994; Maia et al., 1998; Vila et al., 2005).

In this paper the first report of the chemical composition, genotoxic profile and overview of bioactivity of *P. obliquum* essential oil from Eastern Ecuador is provided, along with the definition of genotoxic profile and bioactive properties of *P. aduncum* essential oil of the same origin.

2. Materials and methods

2.1. Plant material

P. obliquum and *P. aduncum* aerial parts were supplied by Fundacion Chankuap' (Macas, Ecuador) and collected at blooming in January 2006 from wild plants growing in three different locations on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77°15″W/2°35″S) and positively identified by the National Herbary of Pontificia Universidad Catolica del Ecuador (J. Jaramillo). Dried specimens were deposited at the Department of Biology and Evolution, University of Ferrara, Code POB001 and PAD001.

2.2. Essential oils

Essential oils were isolated by a 3 h hydrodistillation of respectively 7 kg of *P. aduncum* and 14 kg of *P. obliquum* aerial parts in a stainless steel distiller equipped with a commercial Clevenger apparatus. Essential oil yield was determined on a volume to dry weight basis. The values for essential oil yield of three distinct distillations corresponding to the three different samplings were averaged. The essential oil samples were stored in glass vials with Teflon-sealed caps at 2 ± 0.5 °C in the absence of light.

2.3. Gas chromatography

Essential oil samples were analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5 ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, Carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the oils was computed by the normalization method from the GC peak areas, without using correction factors.

2.4. GC/mass spectrometry analysis

Essential oil constituents were then analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library. The constituents of the volatile oils were identified by comparing their GC retention times, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in the literature (Adams, 2001). A Varian FactorFour VF-5 ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm) was used. Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, Carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/s; mass range, 29–400 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. The constituents of the volatile oils were identified by comparing their relative ritention time, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns with the above mentioned mass spectra libraries or with those in the literature (Adams, 2001). In order to determine the Kovats index of the components, a mixture of alkenes (C_8–C_{24}) was added to the essential oil before injecting in the GC–MS equipment and analyzed under the same conditions as above.

2.5. NMR spectroscopy

^{13}C NMR spectra were recorded at 100.58 MHz and at temperature of 303 K with a Varian Gemini-400 spectrometer. The essential oils were dissolved in $CDCl_3$ (70 mg/0.8 ml) into a 5 mm NMR and solvent signal was used for spectral calibration (central line of triplet at 77.0 ppm). Chemical shifts (ppm) and peak attribution were made according with those of literature (Kubeczka, 2002), SDBS and Sigma–Aldrich NMR spectra databases and pure standard or mixture of these (safrole, γ-terpinene for *P. obliquum* and dillapiol, γ-terpinene, *cis*-ocimene for *P. aduncum*). For *P. obliquum* were identified via ^{13}C NMR safrole, γ-terpinene, α-terpinene, terpinolene and for *P. aduncum cis*-ocimene, *trans*-ocimene, γ-terpinene, 4-terpineol, piperitone and dillapiol. DEPT, ^1H and bidimensional NMR experiments were used to attribute signals for compounds in low proportions (Rezzi et al., 2002; Agnihotri et al., 2005; Guerrini et al., 2006).

2.6. Biological activities

All the following biological activities of *P. obliquum and P. aduncum* essential oils were compared to those achieved with synthetic positive controls and commercial *Thymus vulgaris* essential oil, in order to provide a direct reference with an essential oil reputed for its antioxidant and antibacterial properties. Data reported for each assay are the average of three determinations of three independent experiments.

2.6.1. Microorganisms

Biological activities (antifungal and antibacterial activity) of *P. obliquum* and *P. aduncum* essential oils were performed by employing the standard disks diffusion technique according to a previously described methodology (Guerrini et al., 2006). The culture media and conditions employed for ATCC strains were in accordance with American Type Culture Collections protocols.

2.6.2. Antibacterial activity

Mother cultures of each microorganism were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures Petri dishes with proper sterile media with the aim of obtaining the microorganisms concentration of 10^5 and 10^6 CFU/ml for yeasts and bacteria, respectively. Aliquot of dimethyl sulphoxide (DMSO) was added to the essential oils and different amounts of the solution deposited on sterile paper disks (6 mm diameter, Difco) in order to obtain a 0.01–0.5 mg/ml concentration range. The lowest concentration of each essential oil showing a clear zone of inhibition was taken as the minimum inhibitory concentration (MIC). Controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution.

2.6.3. Antifungal activity

Cultures of the fungi were grown on potato dextrose agar (PDA, Difco, Detroit, MI, USA) for phytopathogens and on sabouraud dextrose agar (SDA, Difco, MI, USA) for dermatophytes. Antifungal activity was checked by dissolving the extracts in DMSO and aseptically added them to sterile media at 45 °C in order to obtain the two concentrations of 50, 100 and 500 μg/ml. The DMSO concentration in the final solution was adjusted to 0.3%. Controls were set up with equivalent quantities of DMSO. The cultures were obtained by transplanting mycelium disks (diameter 10 mm) from a pure mother culture in stationary phase on thin cellophane sheets at 26 ± 1 °C until the logarithmic growth phase was reached. Subsequently the cultures were transferred to Petri plates with media containing extracts diluted to the above-mentioned final concentrations. The fungal growth was daily evaluated by measuring the diameter of the cultures for five (phytopathogens) or eight (dermatophytes) days from the treatment onset (kept as 0 time). There were three replicates for each treatment.

2.6.4. Antioxidant activity

Radical scavenging and antioxidant properties were performed in different assays, namely DPPH (1,1-diphenyl-2-picrylhydrazyl) bleaching test, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) test and Photochemilumiscence according to previously described methods (Sacchetti et al., 2005; Scartezzini et al., 2005).

2.6.5. Platelet aggregation studies

Blood from male Guinea pig was obtained by cardiac puncture after CO_2 euthanasia, collected in plastic tubes and anticoagulated with sodium citrate 3.8% 1 part citrate: 9 part blood. After centrifugation for 15 min at $180 \times g$ to obtain platelet rich plasma (PRP), the remaining blood was centrifuged again 10 min

2000 g to obtain platelet poor plasma (PPP). PRP from guinea pig was used to perform aggregation in the aggregometer PAP-4D (Biodata, Horsham, PA, USA) following Born's turbidimetric method (Born, 1962). Aggregation was recorded as the percent change in light transmission: the baseline was set using PRP and maximal transmission using PPP. PRP was preincubated at 37 °C for 5 min with solvent (DMSO, final concentration 0.5%) or the compound under study before addition of the platelet aggregatory agent. Maximal aggregation was induced stimulating platelets with 3 μM ADP, 50 μM arachidonic acid (AA) or 1 μM U46619. Tests were performed within 3 h to avoid platelet inactivation. The effects of test compounds and aspirin were expressed as percent inhibition compared with control samples. DMSO at 0.5% did not interfere with platelet aggregation.

2.6.6. Antinociceptive activity

The writhing test was performed according to Koster's method (Koster et al., 1959). Briefly, vehicle or 100 mg/kg essential oils were orally administered to mice 1 h before intraperitoneal injection of 0.2 ml of 0.6% acetic acid. After treatment with the algogen agent, mice were placed in observational chambers and the number of writhes of each mouse was counted over a period of 30 min.

2.6.7. Essential oils mutagenic activity

Essential oil dissolved in DMSO was tested with *Salmonella typhimurium* strains TA98 and TA100 (100 μl per plate of a fresh overnight culture) with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic system (S9 mix), using plate incorporation assay (Maron and Ames, 1983). The concentrations of the test sam-

Table 1
Chemical composition of *Piper aduncum* and *Piper obliquum* essential oils

Compound[a]	Method	RT	RI[b]	*P. aduncum* (%[c])	*P. obliquum* (%[c])
α-Thujene	RI, MS[d]	5.223	930	0.35	0.97
α-Pinene	RI, MS	5.395	939	1.35	1.75
Camphene	RI, MS	5.848	954	0.05	0.52
β-Pinene	RI, MS	6.935	979	1.27	2.01
Myrcene	RI, MS	7.867	991	0.73	1.3
α-Phellandrene	RI, MS	8.295	1003	1.06	0.33
3-Carene	RI, MS	8.578	1005	0.07	0.54
α-Terpinene	RI, MS, NMR[e,f]	8.953	1017	0.82	6.24
p-Cymene	RI, MS	9.367	1025	0.73	2.6
Limonene	RI, MS	9.544	1027	1.6	0.12
1,8-Cineole	RI, MS	9.610	1031	1.3	0.18
Z-Ocimene	RI, MS, NMR[g]	10.534	1037	2.23	1.13
E-Ocimene	RI, MS, NMR[g]	11.197	1050	10.39	1.51
γ-Terpinene	RI, MS, NMR[f]	11.569	1060	2.42	17.12
Sabinene hydrate *cis*	RI, MS	12.011	1070	0.42	–
Isoterpinolene	RI, MS	13.449	1088	0.12	–
Terpinolene	RI, MS, NMR[f]	13.558	1089	0.82	11.46
p-Cymenene	RI, MS	13.997	1093	0.69	–
Linalool	RI, MS	14.830	1097	1.82	0.7
Sabinene hydrate *trans*	RI, MS	15.232	1122	0.24	–
allo-Ocimene	RI, MS	17.222	1132	0.36	0.17
Camphor	RI, MS	17.478	1146	0.05	0.25
Terpinen-4-ol	RI, MS, NMR[g]	20.933	1177	3.14	0.06
α-Terpineol	RI, MS	22.349	1189	0.06	–
cis-Piperitol	RI, MS	22.744	1196	0.05	–
trans-Piperitol	RI, MS	24.169	1208	0.13	–
Piperitone	RI, MS, NMR[g]	28.632	1253	8.47	tr
Safrole	RI, MS, NMR[f]	32.858	1287	–	45.86
δ-Elemene	RI, MS	38.118	1338	0.08	0.24
Methyl decanoate	RI, MS	38.358	1345	–	0.05
α-Ylangene	RI, MS	40.549	1375	0.08	–
α-Copaene	RI, MS	41.901	1377	0.27	0.5
β-Bourbonene	RI, MS	42.675	1388	–	0.05
β-Cubebene	RI, MS	43.816	1388	0.05	–
β-Elemene	RI, MS	44.161	1391	0.45	–
α-Gurjunene	RI, MS	45.463	1410	0.13	–
β-Caryophyllene	RI, MS	46.271	1419	2.57	0.7
β-Copaene	RI, MS	47.582	1432	0.09	0.11
Aromadendrene	RI, MS	48.396	1441	0.05	–
α-Humulene	RI, MS	50.021	1455	0.65	0.09
γ-Muurolene	RI, MS	53.341	1480	1.01	0.24
Bicyclogermacrene	RI, MS	55.093	1498	1.45	0.39
α-Muurolene	RI, MS	56.063	1500	0.07	0.32
δ-Amorphene	RI, MS	56.554	1512	0.14	–
Cubebol	RI, MS	56.975	1515	0.84	–
Nonadecane	RI, MS	57.562	1517	–	0.31
Myristicin	RI, MS	57.834	1519	0.89	0.28
Humulene epoxyde II	RI, MS	61.638	1608	0.18	–
Dillapiol	RI, MS, NMR[f]	62.985	1678	45.92	0.6
Total				0	0
Extraction Yield				0.8 (0.66–0.92)	0.16 (0.1–0.19)

[a] Compounds are listed in order of elution from Varian FactorFour VF-5 ms.
[b] KI, Retention indices calculated on Varian FactorFour VF-5 ms.
[c] RA%, relative area percentage (peak area relative to total peak area %, calculated on Varian FactorFour VF-5 ms).
[d] Mass spectrometry.
[e] Nuclear magnetic resonance.
[f] For *P. obliquum*.
[g] For *P. aduncum*.

ples used were 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2} and 10^{-1} μg plate^{-1}. The plate for negative control contained 100 μl of DMSO, with or without S9 mix. The positive control plates for the first contained 2 μg plate^{-1} of 2-aminoantracene for both TA98 and TA100 strains. The positive control plates without S9 mix contained 2 μg plate^{-1} of 2-nitrofluorene for TA98 strain and 1 μg plate^{-1}of sodium azide for TA100 strain. A sample was considered mutagenic when the observed number of colonies was at least 2-fold over the spontaneous level of revertants (Maron and Ames, 1983). The colonies were counted manually after 48 h of incubation at 37 °C using a Colony Counter 560 Suntex (Antibioticos, Italy). Lyophilized post-mitochondrial supernatant S9 mix (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). Before its use, the S9 mix was filtered through a 0.45 μm Millipore disposable filter.

2.6.8. Antimutagenic activity and toxicity

The inibithory effect of essential oils (10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2} and 10^{-1} μg plate^{-1}) on mutagenic activity of direct acting mutagen 2-nitrofluorene (2 μg plate^{-1}) and sodium azide (1 μg plate^{-1}), was examined in plate incorporation assay, derived from mutagenicity test as described by Maron and Ames (1983) with some minor modifications, using tester strain TA98 and TA100, respectively. The inhibitory effect of essential oils on mutagenic activity of the indirectly acting mutagen 2-aminoanthracene (2 μg plate^{-1}) was examined in plate incorporation assay, using tester strain TA98 and TA100 with S9 mix. The inhibition rate for mutagenic was calculated according to the formula: inhibition rate (%) = $(A - B) \times 100/A$, where A are revertants in positive control, and B are revertants in the extract sample, having subtracted the spontaneous revertants. The significant differences ($P \leq 0.05$) between the means of revertants per plate of the samples in relation to the mutagens were calculated using the post hoc Tuckey honest significant difference test (HSD)(Spjotovoll and Stoline, 1973). This statistical test has been already applied to antimutagenic activity of extracts of natural substances in the *Salmonella*/microsome assay by Horn and Vargas (2003). A critical point, affecting the outcome of the interaction between an antimutagen and a testing bacterial strain is the overlapping of the citotoxic and antimutagenic dose concentration. In other worlds, it is important to confirm that the dose-dependent disappearance of the mutant colonies is not a result of cell-killing. For this purpose a simple survival assay for the treated bacteria must be performed to evaluate a cytotoxic lowest effective dose (LED) or a highest uneffective dose (HUD). In order to verify the toxicity of the analysed samples on bacterial cells and evaluate the HUD, a toxicity test was performed (Maron and Ames, 1983). A fresh 15-h culture was diluted 10^5 times to give a 1–2 $\times$ 10^4 bacteria ml^{-1} solution. The test samples at several concentrations (10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1} μg plate^{-1}) diluted in DMSO, mixed with 2 ml of molten top agar, were plated with 0.1 ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 μmoles of L-histidine and 0.05 μmoles of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of solution. 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. The final criterion used to interpret the results of significant decrease in the number of *Salmonella* revertants was statistical analysis ($P \leq 0.05$) in the Tuckey HSD test combined with HUD comparison. When the modulator dose concentration is statistically effective and it ranges below or coincide with HUD, the samples were considered to present sign of the effect (antimutagenicity). HUD for *P. obliquum* toxicity was easily evaluated by visual estimation. HUD for *P. aduncum* is hardly derivate by visual estimation,

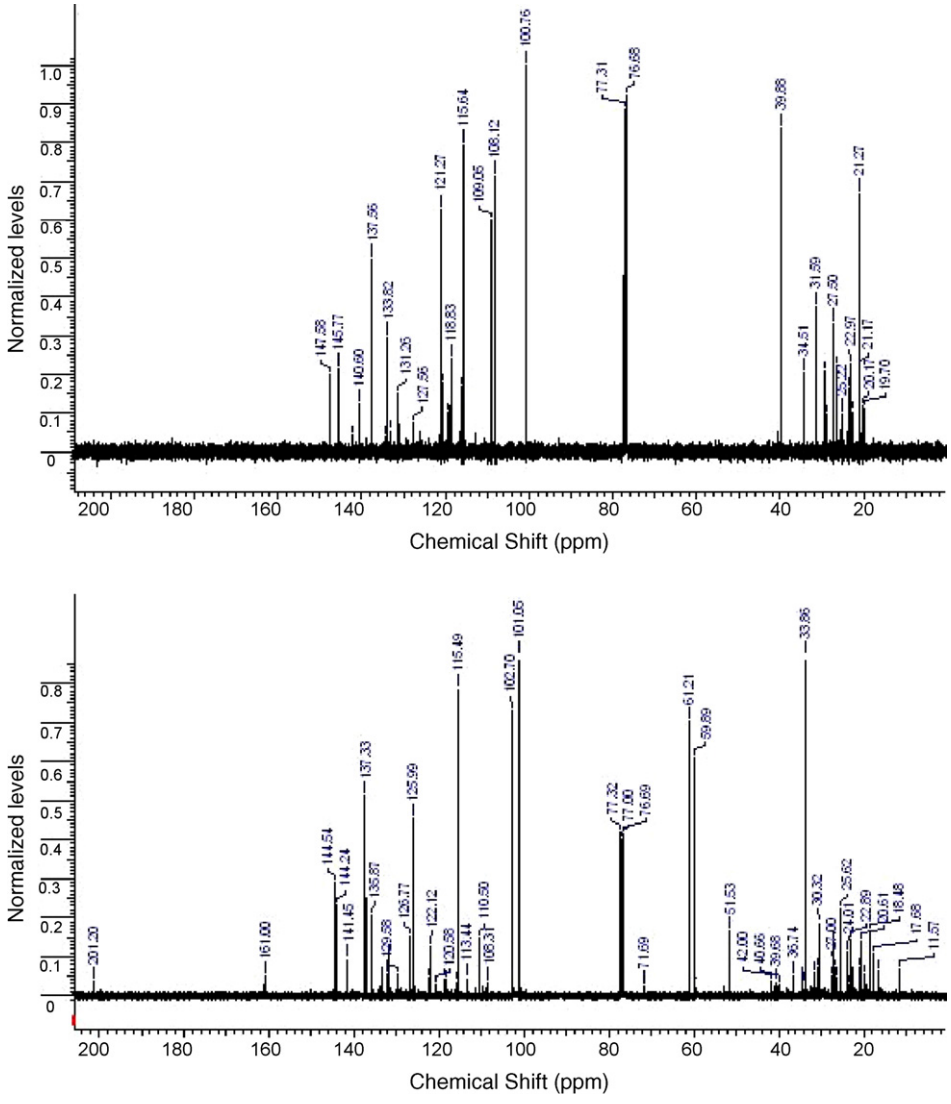

Fig. 1. ^{13}C NMR of *Piper obliquum* and *Piper aduncum* essential oils. For peak assignment see Table 2.

Table 2
Resonance assignments, chemical shifts of constituents identified in essential oil of *P. obliquum* and *P. aduncum* in ^{13}C NMR Spectra

Chemical shift (ppm) oil	Chemical shift (ppm) standard and/or literature	Intensity[a]	Identified peak *P. obliquum*	Intensity[a]	Identified peak *P. aduncum*
11.57	11.64[b]	–	–	6.9	(E)-Ocimene
16.78	16.85,18.89[c]	–	–	5.4	4-Terpineol
17.68	17.69[d], 17.70[b]	–	–	10.8	(Z)/(E)-Ocimene
18.48[e]	18.52[f]	–	–	16.4	Piperitone
19.70	19.71[g]	11.2	Terpinolene	–	–
19.68	19.73[d]	–	–	5.2	(Z)-Ocimene
20.17	20.18[g]	11.8	Terpinolene	–	–
20.61[e]	20.63[f]	–	–	16.1	Piperitone
21.17	21.23[g]	21.8	α-Terpinene	–	–
21.24	21.29[d]	66.9	γ-Terpinene	9.9	γ-Terpinene
22.90	22.77[g]	10.3	α-Terpinene	–	–
22.89	22.96[d]	22.7	γ-Terpinene	19.9	γ-Terpinene
23.25	23.28[c]	–	–	6.1	4-Terpineol
23.40	23.41[g]	16.5	Terpinolene	–	–
24.01[e]	23.93[f]	–	–	11.4	Piperitone
25.22	25.37[g]	9.8	α-Terpinene	–	–
25.62	25.66[d], 25.66[b]	–	–	25.8	(Z)/(E)-Ocimene
25.76[e]	25.89[f]	–	–	10.9	Piperitone
26.41	26.44[d]	–	–	4.7	(Z)-Ocimene
26.58	26.63[g]	22.2	Terpinolene	–	–
27.00	27.15[c]	–	–	7.3	4-Terpineol
27.26	27.46[b]	–	–	14.0	(E)-Ocimene
27.50	27.57[d]	33.1	γ-Terpinene	4.9	γ-Terpinene
28.94	29.14[g]	9.6	α-Terpinene	–	–
29.49	29.53[g]	21.0	Terpinolene	–	–
30.32[e]	30.56[f]	–	–	18.5	Piperitone
30.74	30.93[c]	–	–	7.1	4-Terpineol
31.41	31.45[g]	18.8	Terpinolene	–	–
31.59	31.65[d]	37.5	γ-Terpinene	5.9	γ-Terpinene
33.86	33.90[d]	–	–	100.0	Dillapiol
34.45	34.54[g]	9.6	α-Terpinene	–	–
34.51	34.56[d]	20.2	γ-Terpinene	4.0	γ-Terpinene
34.55	34.60[c]	–	–	5.0	4-Terpineol
36.74	36.84[c]	–	–	5.1	4-Terpineol
39.88	39.89[d]	83.9	Safrole	–	–
51.53[e]	51.56[f]	–	–	16.5	Piperitone
59.89	59.73[d]	–	–	63.7	Dillapiol
61.21	61.24[d]	–	–	61.3	Dillapiol
71.69	71.71[c]	–	–	3.0	4-Terpineol
76.68		92.1	$CDCl_3$	41.5	$CDCl_3$
77.00		86.2	$CDCl_3$	40.2	$CDCl_3$
77.31		88.7	$CDCl_3$	41.9	$CDCl_3$
100.76	100.74[d]	100.0	Safrole	–	–
101.05	101.09[d]	–	–	91.8	Dillapiol
102.70	102.76[d]	–	–	73.0	Dillapiol
108.12	108.12[d]	71.2	Safrole	–	–
109.05	109.06[d]	60.0	Safrole	–	–
110.50	110.48[b]	–	–	14.8	(E)-Ocimene
113.44	113.47[d]	–	–	4.4	(Z)-Ocimene
115.49	115.34[d]	–	–	78.5	Dillapiol
115.64	115.59[d]	79.4	Safrole	–	–
115.93	115.98[d]	16.7	γ-Terpinene	3.5	γ-Terpinene
116.31	116.36[g]	12.2	α-Terpinene	–	–
118.41	118.54[c]	–	–	4.1	4-Terpineol
118.83	118.86[d]	24.0	γ-Terpinene	4.4	γ-Terpinene
119.42	119.64[g]	10.1	α-Terpinene	–	–
120.74	120.75[g]	17.6	Terpinolene	–	–
121.27	121.25[d]	62.7	Safrole	–	–
122.12	122.44[b]	–	–	12.6	(E)-Ocimene
122.46	122.49[d]	–	–	4.5	(Z)-Ocimene
125.99	126.08[d]	–	–	45.2	Dillapiol
126.77[e]	126.78[f]	–	–	15.1	Piperitone
127.56	127.56[g]	7.5	Terpinolene	–	–
129.58	129.60[d]	–	–	5.4	(Z)-Ocimene
131.25	131.22[d]	15.1	γ-Terpinene	2.3	γ-Terpinene
131.70	131.72[d], 131.76[b]	–	–	14.2	(Z)/(E)-Ocimene
132.08	132.00[d]	–	–	5.3	(Z)-Ocimene
133.19	132.93[g]	5.4	α-Terpinene	–	–
133.63	133.57[d], 133.78[b]	–	–	8.8	(Z)/(E)-Ocimene
133.77	133.80[c]	–	–	3.8	4-Terpineol
133.82	133.82[d]	29.6	Safrole	–	–
134.21	134.15[g]	3.9	Terpinolene	–	–
135.87	136.97[d]	–	–	20.4	Dillapiol
137.33	137.39[d]	–	–	51.1	Dillapiol

Table 2 (*Continued*)

Chemical shift (ppm) oil	Chemical shift (ppm) standard and/or literature	Intensity[a]	Identified peak *P. obliquum*	Intensity[a]	Identified peak *P. aduncum*
137.56	137.56[d]	49.8	Safrole	–	–
137.57	137.66[d]	–	–	12.9	Dillapiol
140.60	140.60[d]	12.3	γ-Terpinene	–	–
141.45	141.67[b]	–	–	9.3	(E)-Ocimene
142.49	142.38[g]	4.3	α-Terpinene	–	–
144.24	144.22[d]	–	–	23.2	Dillapiol
144.54	144.56[d]	–	–	28.9	Dillapiol
145.77	145.77[d]	21.4	Safrole	–	–
147.58	147.58[d]	19.8	Safrole	–	–
161.00[e]	160.50[f]	–	–	4.8	Piperitone
201.20[e]	200.11[f]	–	–	3.6	Piperitone

[a] Relative to the highest signal, set to 100.

[b] Peyton, J., 1982. J. Org. Chem. 47, 4165–4167: experimental value confirmed by dept, ^{1}H and bidimensional NMR experiment.

[c] Saito, T., Hayamizu, K., Yanagisawa, M., Yamamoto, O., Spectral Database for Organic Compounds, SDBS (free site organized by National Institute of Advanced Industrial Science and Technology (AIST), Japan http://www.aist.go.jp/RIODB/SDBS/).

[d] Standard experimental value in $CDCl_3$.

[e] Experimental value in $CDCl_3$ deduced by comparison to those obtained in C_6D_6 and literature (Kubeczka, K.H., 2002. Essential oils analysis. Wiley, NY) and confirmed by dept, ^{1}H and bidimensional NMR experiment.

[f] Kubeczka, K.H., 2002. Essential oils analysis. Wiley, NY.

[g] Sigma–Aldrich NMR spectra database.

therefore we fixed it at the lower dose just before LED. LED for *P. aduncum* was statistically evaluated, with Poisson assumptions, by using the stepwise collapsing of the homogeneous control and dose counts (Khromov-Borisov et al., 2000). All data were analyzed for homogeneity of variance using Levene's test (a robust test against normality). All computations were made by employing the statistical software SPSS Ver. 10.0 and personal developed software (for estimation of LED).

3. Results and discussion

Table 1 reports the composition for each component of *P. obliquum* and *P. aduncum* essential oils, whose composition resulted uniform from the three different samplings. For fingerprinting purposes, 1H NMR analyses were performed (Fig. 1); resonances and chemical shifts for identified constituents were assigned as listed in Table 2 . Thirty-three compounds accounting for 98.7% and 46 compounds accounting for 95.7% of the total were respectively identified. For *P. obliquum*, data collected were discordant from those available in the literature and main constituents, in particular, were different. The only report available on *P. obliquum* essential oil is from Panama and describes an oil rich in β-caryophyllene (27.6%) with a pattern dominated by sesquiterpenes (78% approximately) (Mundina et al., 1998). The sample from eastern Ecuador was instead extremely rich in safrole (45.86%), γ-terpinene (17.12%) and terpinolene (11.46%), thus evidencing in a preliminary way a different profile and suggesting the hypothesis of phytochemical intraspecific variability. If these data will be confirmed by more extensive samplings, *P. obliquum* may be considered as a fast-growing potential renewable source of safrole, being most of the natural sources of this compound woody plants and trees found in endangered habitats like *Ocotea pretiosa* and *Sassafras albidum*.

On the contrary, *P. aduncum* essential oil has been thoroughly evaluated and different profiles have been described from different parts of the world. Barring botanical misinterpretations and different collection times, Matico is seemingly presenting some degrees of chemical polymorphism. Different chemical profiles are known: one from Bolivia in which the main constituent is 1,8-cineole (40%), one from Panama rich in sesquiterpenes like β-caryophyllene and aromadendrene and, finally, a chemotype frequently found in the Americas, South East Asia and Oceania, in which dillapiol is predominant (30–90%) (Smith and Kassim, 1979; Gottlieb et al., 1981; bin Jantan et al., 1994; de Morais et al., 1998; Pino et al., 2004; Vila et al., 2005; Rali et al., 2007; Arze et al., 2008). Some Brazilian accessions are rich in linalool or nerolidol and surprisingly devoid of phenylpropenes (Mesquita et al., 2005; Navickiene et al., 2006; de Oliveira et al., 2006). *P. aduncum* essential oil from eastern Ecuador proved to be abundant in dillapiol, as often reported from the Amazon. Piperitone (8.4%) and *trans*-ocimene (19%) were also present.

Given the lack of information regarding biological activities of *P. obliquum*, the unique composition of *P. aduncum* essential oil from amazonian Ecuador and the presence in both of putative noxious substances, evaluation of their mutagenicity and a number of biological activities were performed. Essential oils can be topically applied or orally administered and careful examination of possible mutagenic properties is required to confirm and assure the safety of their use. Moreover, some evidences of possible genotoxic activities exerted by essential oils are emerging (Lazutka et al., 2001). Notwithstanding the traditional use of *P. aduncum* and *P. obliquum* and the growing commercial availability of Matico in the worldwide market of natural remedies (Pohlit et al., 2006), the mutagenic/antimutagenic behaviour of their essential oils have not been investigated before. It should be emphasized that tradition in use is by no means warrants of safety, particularly regard to mutagenicity/carcinogenicity, where a complex set of cause–effect relationships, signs and symptoms is involved and not easily recognized by the population (Souza Brito and Souza Brito, 1993). By using the plate incorporation assay, no mutagenic activity of *P. aduncum* and *P. obliquum* essential oils (Fig. 2), was detected when investigated on *S. typhimurium* tester strains TA98 and TA100, either with or without S9 activation. Range concentration was limited by toxicity up to 10^{-2} μg plate^{-1}. Lack of mutagenic activity may be somehow surprising, given the known hepatocarcinogenic activity of safrole and other allylbenzenes (Miller et al., 1983), yet in literature are reported questionable results about safrole mutagenicity by using Ames *Salmonella* reversion assay (Sekizawa and Takayuki, 1982). Dorange et al. (1977) reported that safrole was mutagenic in strain TA1535 in the presence of liver enzymes. On the other hand, Wislocki et al. (1977) reported that safrole was not mutagenic in strain TA100 or in TA1535. They observed, however, that the safrole metabolite (safrole-2′,3′-epoxide) was mutagenic in both strains TA100 and TA1535 even in the absence of S9. Our negative result was in agreement with Sekizawa and Takayuki (1982), who used strains TA98 and TA100 with and without metabolic activation. Moreover, it must be underscored that these authors tested pure chemicals rather then evaluating a phytocomplex as an essential oil, in which different substances are or may be simultaneously active, and may lead to different result than the administration of a single substance. Essential oils are utilized *in toto* and thus

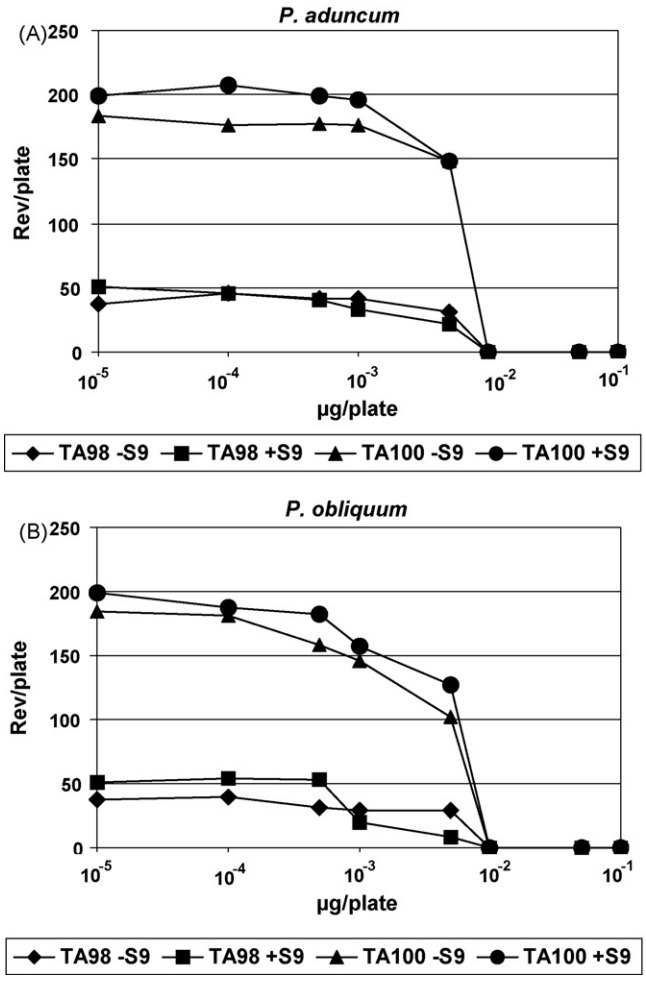

Fig. 2. Comparative mutagenicity in *Salmonella typhimurium* TA98 and TA100 (Ames test) of *P. aduncum* (Matico) (A) and *P. obliquum* (Anis del Oriente) (B) oils assessed with (+S9) and without (−S9) metabolic activation.

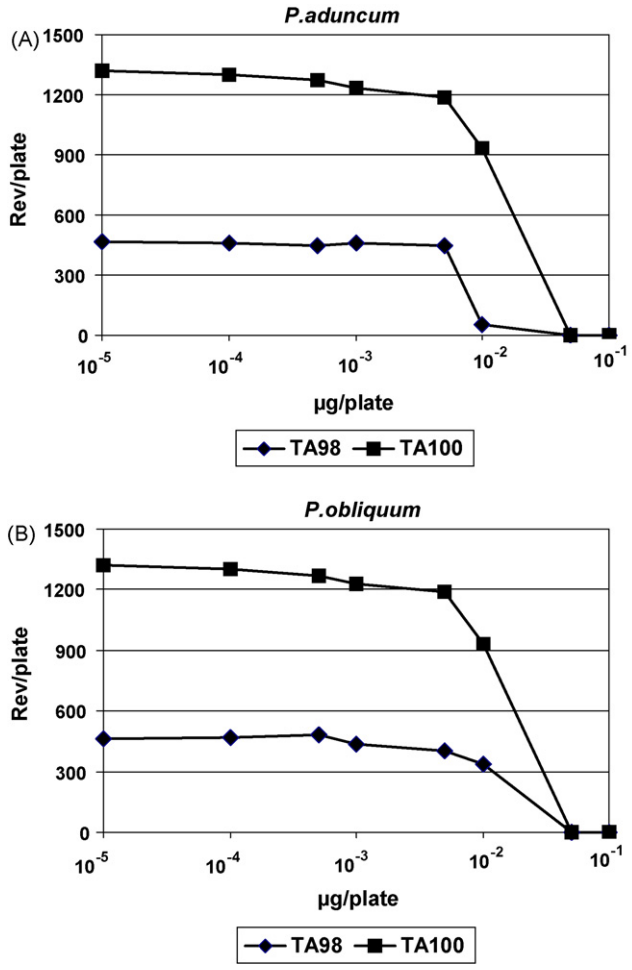

Fig. 3. Comparative antimutagenic effect in *S. typhimurium* TA98 and TA100 of *P. aduncum* (Matico) (A) and *P. obliquum* (Anis del Oriente) (B) oils, assessed without metabolic activation.

their evaluation as a phytocomplex is more representative of their veritable effects. Similar conclusions were obtained in a different genotoxic test performed on safrole and dillapiol containing *P. auritium* and *P. sanctum* (Muraleedharan et al., 1989; Ramos Ruiz et al., 1996; Deciga-Campos et al., 2007). *P. aduncum* and *P. obliquum* essential oils when tested for mutageno-protective efficacy in the Ames *Salmonella*/microsome assay have not shown any significant statistical effects of increasing amounts of both essential oils on the activity of directly acting mutagens 2-nitrofluorene and sodium azide (Fig. 3). In *P. aduncum* HUD for toxic effect has been settled at 10^{-3} μg plate^{-1} for both TA98 and TA100 (Fig. 4). Significant offset differences from revertants of 2-nitrofluorene and sodium azide ($P < 0.05$) was, for both chemicals and according to Tuckey HSD test, settled at 10^{-2} μg plate^{-1} (Fig. 3). In *P. obliquum* toxic HUD has been settled for both strains at 10^{-3} μg plate^{-1} (Fig. 4), and statistical offset differences was settled at 10^{-2} μg plate^{-1} for both chemicals (Fig. 3). Therefore, the decrease of revertants depicted in Fig. 3 has to be assigned to the toxicity exerted by the essential oils. Different results were obtained when strains were exposed to *P. aduncum* and *P. obliquum* oils in presence of indirectly acting mutagen 2-aminoanthracene, which acts as a genotoxic compound through S9 mix. In this case, both oils could induce a slight decrease the HUD values, which results statistically significant, according to Tuckey HSD test. In *P. aduncum* HUD for toxic effect has been settled at 10^{-3} μg plate^{-1} for both TA98 and TA100, and offsets of statistically significant differences for revertants of 2-aminoanthracene ($P < 0.05$) assumed the values of 10^{-3} and 5×10^{-4} μg plate^{-1} for TA98 and TA100, respectively. In this case we can assume a significant inhibition rate of 23 and 28% for TA98 and TA100 respectively (Fig. 5). In *P. obliquum* HUD for toxic effect has been settled at 10^{-3} μg plate^{-1} for both TA98 and TA100, and offsets of statistically significant differences, assumed the value of 5×10^{-4} μg plate^{-1} for both strains (Fig. 5). Therefore, we can assume a significant inhibition rate of 15 and 14% for TA98 and TA100, respectively.

The mechanism by which *P. obliquum* essential oil inhibits the mutagenicity of 2-aminoanthracene is unknown. However, some suggestions can be made on the basis of the present set of data. Since there is an evident difference in the protective activity of the oil against direct and indirect mutagens and since allylbenzenes and safrole are well known inhibitors of microsomial enzymes yet at 50 μM (Ueng et al., 2005; Budzinski et al., 2000), it can be therefore suggested that safrole or other constituents of the essential oil may interact sinergically with some specific enzymes in the S9 liver homogenates, which are necessary for activation of chemical mutagens. Thus, these oils antimutagenic effect could be explained by the interaction of their constituents with cytochrome P-450 activation system, leading to a reduction of the mutation ratio caused by 2-aminoanthracene, as conformed by other evidences regarding essential oils (Evandri et al., 2005). Regarding the antimicrobial properties of the oils, it must be noted that both were scarcely active if compared with *T. vulgaris* essential oil against both Gram positive

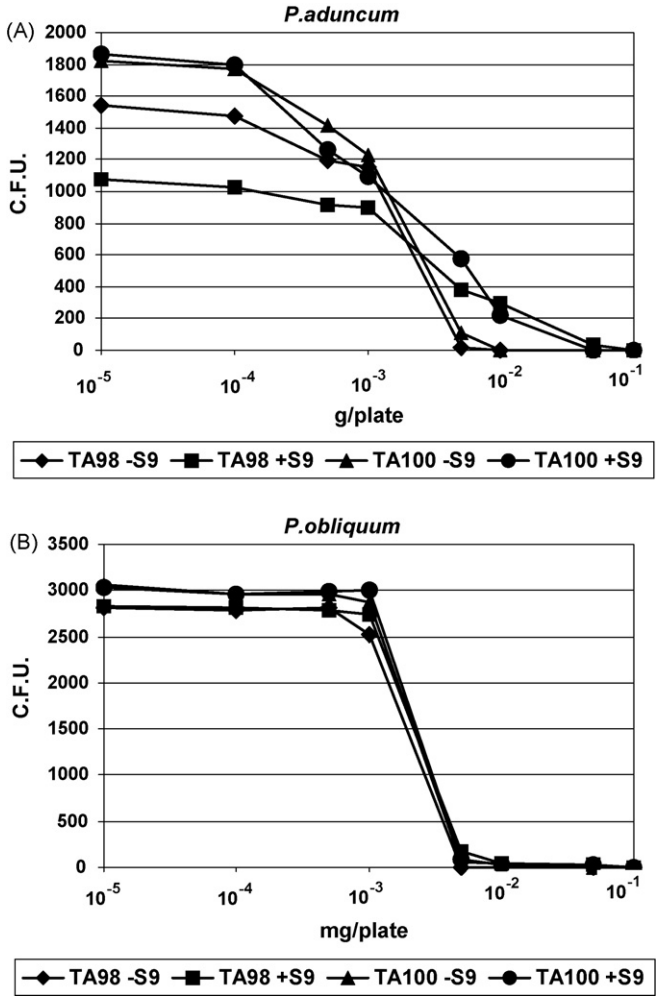

Fig. 4. Comparative toxicity effect in *S. typhimurium* TA98 and TA100 of *P. aduncum* (Matico) (A) and *P. obliquum* (Anis del Oriente) (B) oils, assessed with (+S9) and without (−S9) metabolic activation, in order to evaluate the toxicity highest uneffective dose, beyond which C.F.U. reduction could be assigned as antimutagenic effect.

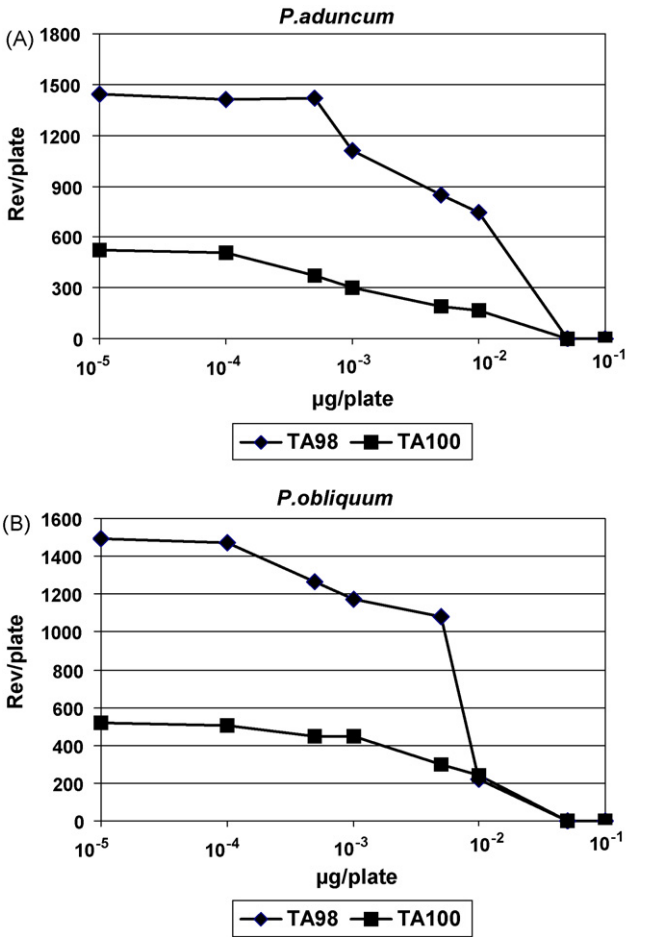

Fig. 5. Comparative antimutagenic effect in *S. typhimurium* TA98 and TA100 of *P. aduncum* (Matico) (A) and *P. obliquum* (Anis del Oriente) (B) oils, assessed with metabolic activation.

(*Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis, Enterococcus foecalis*) and Gram negative (*Klebsiella oxytoca*, *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis)* bacteria (Table 3). If referred to *P. aduncum*, such data are seemingly discordant with those available in the literature suggesting a lower effectiveness of our Ecuadorian sample (Duarte et al., 2007). The efficacy of the oils on fungal strains was instead noticeable (Table 4). In particular, *P. aduncum* essential oil was active in a dose-dependent manner against both dermatophytes and phytopathogens, inducing complete inhibition of *Magnaporthe grisea* and *Trichophyton* species at 500 mg/ml. It also exerted a good efficacy at lower doses (50 μg/ml) against *M. grisea*, evidencing a potency three times higher than Triciclazole. Such activity fits with previous antifungine evaluations of Matico essential oils rich in dillapiol (Pohlit, 2007) and allylbenzenes (Muraleedharan and Basil, 1990). The activity of *P. obliquum* was instead negligible.

Neither oil was active as radical scavenger at 20 μl/ml in DPPH and ABTS assays and only in photochemiluminescence an antioxidant activity comparable to Trolox was obtained (3.7 and 3.9 mmol Trolox/g for *P. aduncum* and *P. obliquum*, respectively). In view of the purported analgesic properties ascribed to *P. aduncum* and *P. obliquum* in folk medicine, preliminary tests were conducted in order to evaluate the possible contribution of essential oils to noci-

Table 3
Antimicrobial properties of *P. aduncum* and *P. obliquum*

	P. aduncum MIC (mg/ml)	*P. obliquum*	*T. vulgaris*	Cloramphenicole MIC (μg/ml)
Gram−				
Escherichia coli ATCC 4350	0.23	4.79	0.06	2.5
Pseudomonas aeruginosa ATCC 17934	5.24	>4.79	0.18	5
Klebsiella oxytoca ATCC 29516	>5.24	>4.79	0.4	1.25
Proteus mirabilis ATCC 29852	5.24	4.79	0.4	2.5
Gram+				
Enterococcus foecalis ATCC 29212	>5.24	>4.79	0.11	5
Micrococcus luteus ATCC 9622	5.24	>4.79	0.11	1.25
Bacillus subtilis ATCC 7003	5.24	>4.79	0.11	2.5
Staphylococcus aureus ATCC 29213	>5.24	>4.79	0.11	2.5

Table 4
Antifungal activity of *P. aduncum* and *P. obliquum* against phytopathogens and dermatophytes

Strain	*Magnaporthe grisea* ATCC[a] 64413, inhibition (%)			*Pythium ultimum*[b], inhibition (%)			*Botrytis cinerea* ATCC 48339, inhibition (%)		
	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)
P. aduncum	61.93	83.03	98.17	4.52	24.62	56.78	15.83	23.75	70.51
P. obliquum	5	8.64	30.46	+	12.17	25.68	2.22	8.41	19.92
	24.1[c]	62[c]	94[c]	100[d]	100[d]	100[d]	89.7[e]	100[e]	100[e]

Strain	*Trichophyton mentagrophytes* CBS[f] 160.66, inhibition (%)			*Trichophyton tonsurans* CBS 493.76, inhibition (%)			*Nanizzia cajetani* IHME[g] 3441, inhibition (%)		
	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)	50 (μg/ml)	100 (μg/ml)	500 (μg/ml)
P. aduncum	+	58.68	100	+	46.02	100	+	25	84
P. obliquum	12.25	17.35	20.41	+	+	+	+	+	4
Ketoconazole	100	100	100	100	100	100	100	100	100

[a] American type culture collection.
[b] Institute of Vegetal Pathology, University of Bologna, Italy.
[c] Triciclazole.
[d] Piraclostrobin.
[e] Boscalid.
[f] Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands.
[g] Institute of Hygiene and Epidemiology—Mycology, Brussels, Belgium.

Table 5
Antinociceptive and antiplatelet properties of *P. aduncum* and *P. obliquum*

	100 mg/kg os		IC50 (μg/ml)		
	Hot plate	Edema	ADP	AA	U46619
P. aduncum	Inactive	Inactive	196 (169–218)	149 (105–210)	168 (155–182)
P. obliquum	Inactive	Inactive	164 (120–225)	106 (61–187)	188 (165–214)

ception. Both tests, however, gave negative results (Table 5). On the contrary, both oil were moderately active in the antiplatelet assays performed on three inducers of aggregation: ADP, arachidonic acid (AA) and U46619, a stable tromboxane A_2 agonist. If compared with essential oils previously screened (Tognolini et al., 2006) the activity of *P. aduncum* and *P. obliquum* were not as high as those provided by oils rich in phenypropenes, but nevertheless not negligible (Table 5).

In conclusion, *P. obliquum* and *P. aduncum* essential oils can be considered genotoxically safe at the tested conditions and *P. aduncum* provided interesting antifungine properties. This activity is valuable towards an extension of the employ of this drug as new phytotherapeutic or preservative ingredient, besides its consolidated ethnomedical use.

Conflict of interest

The authors declare that there are no conflicts of interest.

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