Cat's Claw Oxindole Alkaloid Isomerization Induced by Cell Incubation and Cytotoxic Activity against T24 and RT4 Human Bladder Cancer Cell Lines

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Key words
- *Uncaria tomentosa*
- Rubiaceae
- oxindole alkaloids
- isomerization
- incubation conditions
- cytotoxic activity
- T24 and RT4 human bladder cancer cell lines

received Dec. 7, 2012
revised June 25, 2013
accepted July 15, 2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0033-1350742
Published online August 23, 2013
Planta Med 2013; 79: 1413–1420 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

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Abstract

The antitumor activity of *Uncaria tomentosa*, a native vine from the Amazonian rainforest, has been ascribed to pentacyclic oxindole alkaloids occurring in its bark. Former studies have shown that this activity, as well as its intensity, depends on whether cat's claw alkaloids occur as original compounds or isomerized derivatives. This work addresses this aspect, using T24 and RT4 human bladder cancer cell lines for that purpose. Bark samples were extracted by dynamic maceration, prepurified with cross-linked polyvinylpyrrolidone and properly fractioned by an ion exchange process to obtain an oxindole alkaloid purified fraction. Alkaloid isomerization was induced by heating it under reflux at 85 °C. Samples collected after 5, 15, and 45 min of heating were analyzed by HPLC-PDA, freeze-dried at once, and separately assayed using the non-isomerized purified fraction for comparison purposes. The latter showed significant and dose-dependent cytotoxic activity against both T24 and RT4 cancer cell lines (IC_{50}: 164.13 and 137.23 µg/mL, respectively). However, results for both cell lines were equivalent to those observed for isomerized samples ($p > 0.05$). The alkaloid isomerization induced by the incubation conditions (buffered medium pH 7.4 and temperature 37 °C) helps to explain the similar results obtained from non-isomerized and isomerized samples. Mitraphylline, speciophylline, uncarine F, and, to a lesser degree, pteropodine were more susceptible to isomerization under the incubation conditions. Thus, the alkaloid profile of all fractions and their cytotoxic activities against T24 and RT4 human bladder cancer cell lines are determined to a large extent by the incubation conditions.

Abbreviations

POA:	pentacyclic oxindole alkaloids
TOA:	tetracyclic oxindole alkaloids
PPE:	prepurified extract
OAPF:	oxindole alkaloid purified fraction
OAPF 5′:	oxindole alkaloid purified fraction after 5 min heating under reflux
OAPF 15′:	oxindole alkaloid purified fraction after 15 min heating under reflux
OAPF 45′:	oxindole alkaloid purified fraction after 45 min heating under reflux

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Uncaria tomentosa (Willd.) DC. (Rubiaceae), popularly known as cat's claw, is a woody vine native to the Amazonian rainforest. Aqueous preparations from its bark are extensively used in folk medicine to deal with rheumatism, arthritis, viral infections, and cancer [1, 2]. Moreover, the immunostimulant and antitumor activities are well documented [1, 3].

Three classes of bioactive compounds from its bark are recognized, namely, polyphenols, quinovic acid glycosides, and mainly tetracyclic (TOA) and pentacyclic oxindole alkaloids (POA) [3]. The antitumor activity is ascribed to POA, and many studies about it have been performed using specific cell lines and alkaloids as isolated compounds [4–10]. Nonetheless, it has often been disregarded as to whether these alkaloids represent original forms or isomerized forms arising from them.

At this point it should be noted that cat's claw oxindole alkaloids are rather susceptible to isomerization. This isomerization proceeds via a re-

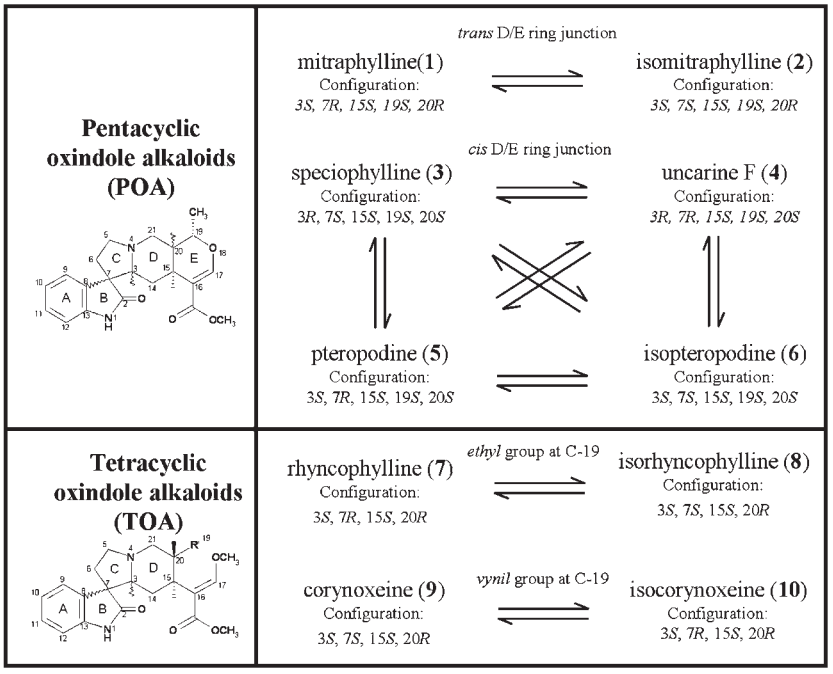

Fig. 1 Isomerization scheme for oxindole alkaloids from cat's claw [11, 12].

versible elimination/addition involving the stereogenic centers C-3 and C-7, and its rate is dependent on pH, temperature, and solvent polarity [11, 12]. For the POA, the equilibrium composition of the various isomers is defined by the nature of the D/E ring junction, which remains intact during isomerization (**Fig. 1**). The interconvertible alkaloids, mitraphylline (**1**) and isomitraphylline (**2**), show a *trans* D/E ring junction, whereas the other four interconvertible isomeric forms [speciophylline (**3**), uncarine F (**4**), pteropodine (**5**) and isopteropodine (**6**)] have a *cis* configuration [11]. With regard to TOA, the various groups attached to the angular carbons C-20 and C-15 show a fixed relative orientation: one pair of interconvertible alkaloids, rhyncophylline (**7**) and isorhyncophylline (**8**), has an ethyl group, and the other pair, corynoxeine (**9**) and isocorynoxeine (**10**), has a vinyl group attached to C-20 [12].

POA can induce dissimilar antitumor activities when evaluated singly. Nonetheless, these activities seem to be related strongly to whether the cat's claw alkaloids were assayed as original or isomerized forms. For instance, pteropodine (**5**) acted only on ovarian carcinoma (SK-OV-3) while the respective isomerized form isopteropodine (**6**) was inactive [4]. In the same work, the author reported that speciophylline (**3**) had a broad set of activities against malignant melanoma (SKMEL), epidermoid carcinoma (KB), ductal carcinoma (BT-549), and ovarian carcinoma (SK-OV-3), while mitraphylline (**1**) and its isomerized form isomitraphylline (**2**) were ineffective [4]. Conversely, uncarine F (**4**), pteropodine (**5**), and isopteropodine (**6**) (all of them interconvertible alkaloids) were able to significantly inhibit the proliferation of human lymphoblastic leukemic T cells (CCRF-CEM-C7H2) and leukemic cell lines (HL60 and U-937) whereas isomitraphylline (**2**) was less active, and mitraphylline (**1**) was entirely ineffective [5, 6].

Further evidence suggests that the cell lines assayed in cytotoxic and antiproliferative studies also play an important role. For instance, both pteropodine (**5**) and its interconvertible form isopteropodine (**6**) showed similar cytotoxic activities against non-small cell lung carcinoma (H460), cervical carcinoma (ME180), prostate carcinoma (DU145), reticular lymphosarcoma (LSR), and stomach carcinoma (C678) [7]. In contrast, isopteropodine (**6**) was more effective against medullary thyroid carcinoma (MTC-SK) than pteropodine (**5**) [8].

The issue remains open to questions regarding several relevant works in which only one isomer was assayed singly considering the high susceptibility of oxindole alkaloids toward isomerization. For instance, mitraphylline (**1**) inhibited growth and showed cytotoxic activity against glioma (GAMG), neuroblastoma [SKNBE (2)], Ewing's sarcoma (MHH-ES-1), and breast cancer (MT-3) human cell lines [9, 10]. However, the interconversion of mitraphylline (**1**) to isomitraphylline (**2**) throughout the treatment period was neglected.

Thus, this work aimed to verify the influence of cat's claw alkaloid isomerization on cytotoxic activity specifically against T24 and RT4 human bladder cancer cell lines. For that purpose, a chemically well-defined oxindole alkaloid purified fraction was obtained and characterized. Additionally, the isomerization of the alkaloid fraction was induced by heating under reflux and was also monitored throughout the cell incubation period by HPLC-PDA.

Materials and Methods

Plant material and extraction procedure

A stem bark sample of *Uncaria tomentosa* (Willd.) DC. (batch 023), collected in Peru, in May 2005, was kindly gifted by Quimer Ervas e Especiarias (São Paulo, Brazil). A voucher specimen (J. Campos, Nº 10500) was deposited at the Herbarium of Universidad Nacional Mayor de San Marcos (Lima, Peru). The sample was comminuted in a cutter mill (SK1 Retsch) provided with a 2-mm steel sieve. Specific extraction conditions were employed to obtain the maximum oxindole alkaloid yield [13]. Briefly, a 25.0 g powdered sample was extracted by 2 h dynamic maceration in a

magnetic stirrer (IKA RH basic 1) using a hydroethanolic solution 63% (v/v) and a plant : solvent ratio of 0.5 : 10 (w/v). Temperature and magnetic stirring speed were kept constant at 23 ± 1 °C and 300 rpm, respectively, throughout the extraction process. The extractive solution was filtered through a paper filter (Whatman, № 2), concentrated under vacuum at 40 °C up to half of its original weight (Büchi R-114), and immediately freeze-dried (Modulyo 4 L; Edwards).

Obtaining the oxindole alkaloid purified fraction (OAPF)

Briefly, a 0.82 g sample of freeze-dried crude extract was dissolved in 200 mL of hydroethanolic solution 40% (v/v), and then mixed with 8.2 g of cross-linked polyvinylpyrrolidone (PVPP) (Divergan RS; BASF) to remove the polyphenols. The mixture was stirred magnetically at 300 rpm for 1 h at room temperature (23 ± 1 °C) and filtrated through a paper filter. The residue was washed with 100 mL of hydroethanolic solution 40% (v/v). Both the washing liquid and filtrate were pooled (300 mL) and acidified to pH 3.0 with a formic acid solution 10% (v/v), and the resultant solution was coded as PPE.

The PPE was fractioned following a four-step ion exchange process using a glass column (2.7 × 50 cm) previously filled with a strong anionic resin (Dowex Marathon; Sigma-Aldrich). The flow rate was kept constant at 5.0 mL/min throughout the processing.

First step (first pass fraction): 300 mL of PPE were poured onto the column and drained off. Non-adsorbed compounds were washed out with 150 mL of hidroethanolic solution 40% (v/v), and all remaining liquid was drained off again. Both eluates were discarded.

Second step (neutralization): Prior to extraction of the alkaloids, the column was conditioned by washing it with 300 mL of ammonium acetate buffer 0.3 M (pH 7.0), with the eluate obtained being discarded.

Third step (extraction): The alkaloid extraction was accomplished by adding 300 mL of hydroethanolic solution 80% (v/v) onto the column. The first 40-mL subfraction eluated was discarded, and the next 260 mL were acidified with formic acid solution 10% (v/v) to pH 5.5, concentrated under vacuum up to 50 mL at 40 °C, and then freeze-dried at once. The product obtained was coded as OAPF.

Fourth step (resin washing): The resin bed was washed with 100 mL of ethanol, and then regenerated with 200 mL of an HCl solution 5% (v/v). Both eluates were discarded.

The pH (Digimed DM-22), conductivity (Quimis), and oxindole alkaloid profile were monitored in all process steps by HPLC-PDA analysis. In addition, process reproducibility was assayed by repeating the same procedure five times, using two glass columns separately with similar dimensions (2.7 × 50 cm), and the individual content of the POA and TOA alkaloids in OAPF were determined by HPLC-PDA analysis. The results are expressed by RSD % (relative standard deviation).

HPLC-PDA analysis of oxindole alkaloids

Both POA and TOA contents were assayed by an HPLC-PDA method [14] using mitraphylline (**1**) (Phytolab, purity > 99%) as the external standard. Briefly, the analyses were performed employing an HPLC-PDA Proeminence (Shimadzu). A Gemini-NX RP-18 column (250 × 4.6 mm i. d., 5 µm) (Phenomenex) protected by an RP-18 guard column was used. The mobile phase consisted of ammonium acetate buffer 10 mM (pH 7.0) (A) and acetonitrile (B) in a linear gradient program. The flow rate was kept constant at 1.0 mL/min and the analyses were conducted at room temperature (23 ± 1 °C). The detection was performed at 245 nm. The total alkaloid content was obtained by the sum of individual alkaloid contents **1**–**6** for POA, and **7** and **8** for TOA. The results are expressed by the mean of three determinations.

Monitoring of alkaloid isomerization in purified process

Separately, 2-mL aliquots of crude extract, as well as PPE and OAPF after the acidifying step, were collected and appropriately diluted in acetonitrile : water (50 : 50, v/v), and the individual contents of POA (**1**–**6**) and TOA (**7** and **8**) were determined by HPLC-PDA. Each individual alkaloid content was expressed as the percentage ratio in relation to the total alkaloid content of either POA or TOA [$(C_{individual}/C_{total}) \times 100$].

Isomerization of oxindole alkaloids in OAPF induced by heating under reflux

Freeze-dried 40-mg OAPF samples were dissolved in 120 mL of hydroethanolic solution 40% (v/v) and submitted to 120 min heating under reflux at 85 °C. To determine the isomerization kinetic, 1-mL aliquots were collected after 5, 10, 15, 20, 30, 45, 60, 90, and 120 min, properly diluted in acetonitrile : water (50 : 50, v/v) and analyzed by HPLC-PDA. In parallel, 40-mL aliquots were collected after 5 min (OAPF 5′), 15 min (OAPF 15′), and 45 min (OAPF 45′) of heating under reflux, concentrated under vacuum at 40 °C up to 20 mL, and immediately freeze-dried.

Cytotoxicity assay

Cytotoxicity was measured by the MTT assay based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), which turns into purple formazan crystals by viable and metabolic active cells [15]. Both T24 and RT4 human bladder cancer cell lines obtained from the American Type Culture Collection (ATCC) were maintained in RPMI and DMEM culture medium, respectively, containing 0.5 U/mL penicillin/streptomycin antibiotics and supplemented with 10% (v/v) fetal bovine serum. Bladder cancer cell lines were plated (96-well plate) at 3×10^3 (T24) and 8×10^3 (RT4) per well and after reaching semiconfluence, the cultures were treated as described below. Separately, 7.0-mg samples of OAPF, OAPF 5′, OAPF 15′, and OAPF 45′ were firstly dissolved in 100 µL of DMSO (Sigma-Aldrich). The aliquots of these solutions were transferred to a 96-well plate to yield concentrations of 10, 25, 50, 100, 150, and 200 µg/mL. The negative controls were performed with the addition of culture medium (cell viability control) or DMSO (vehicle control) in the absence of treatment. Cisplatin (Sigma-Aldrich, purity > 99%) was used as a positive control. The cell cultures were maintained at 37 °C in a humid atmosphere saturated with 5% CO_2 (Thermo Scientific). After 48 h of treatment, the culture medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). After removing the PBS, 90 µL of culture medium and 10 µL of MTT reagent (Sigma-Aldrich) (5 mg/mL) were added to each well. The cells were incubated for 3 h and the solution was then separated from the precipitate. Separately, 100-µL aliquots of DMSO were added to the wells and the optical absorbance was measured at 570 and 630 nm using an ELISA plate reader (Spectramax M5; Molecular Devices). The results were expressed as the percentage of cell viability in relation to the DMSO group. The inhibitory concentration (IC_{50}) was determined by three independent experiments, and each experiment was performed in triplicate.

Alkaloid	Recovery (%) $\bar{X} \pm SD^2$	Alkaloid content (%, w/w)[1]	
		OAPF $\bar{X} \pm SD^2$	Crude extract $\bar{X} \pm SD^2$
Mitraphylline (**1**)	81.70 ± 2.96	17.36 ± 2.35	1.05 ± 0.01
Isomitraphylline (**2**)	87.10 ± 4.49	7.88 ± 0.50	0.43 ± 0.02
Speciophylline (**3**)	85.03 ± 4.46	8.57 ± 1.55	0.59 ± 0.01
Uncarine F (**4**)	94.87 ± 3.13	2.17 ± 0.19	0.10 ± 0.01
Pteropodine (**5**)	90.91 ± 3.50	18.72 ± 1.32	1.00 ± 0.01
Isopteropodine (**6**)	84.46 ± 8.55	6.89 ± 0.70	0.31 ± 0.01
Total POA	86.22 ± 2.16	62.04 ± 6.22[3]	3.48 ± 0.06[3]
Rhyncophylline (**7**)	53.75 ± 12.15	3.31 ± 0.70	0.24 ± 0.01
Isorhyncophylline (**8**)	90.04 ± 3.74	3.81 ± 0.40	0.20 ± 0.01
Total TOA	70.19 ± 7.78	7.16 ± 1.11[4]	0.44 ± 0.01[4]

[1] Alkaloid content expressed as % (w/w, freeze-dried sample); [2] values represent the mean ± standard deviation; [3] sum of alkaloid contents in **1**–**6**; [4] sum of alkaloid contents in **7** and **8**. Note: Pentacyclic oxindole alkaloids (POA) and tetracyclic oxindole alkaloids (TOA)

Table 1 Oxindole alkaloid recoveries in the ion exchange process and alkaloid content in the oxindole alkaloid purified fraction (OAPF) and crude extract (n = 3).

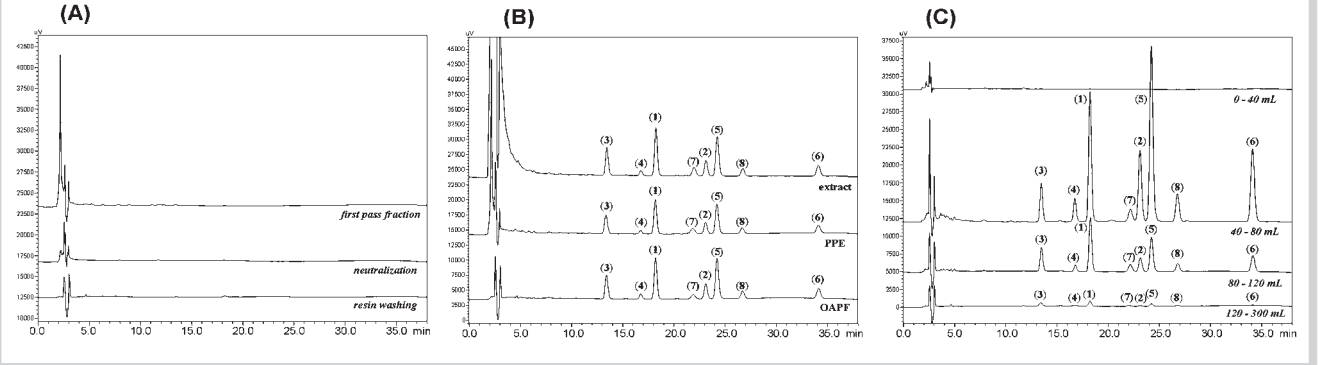

Fig. 2 HPLC-PDA profiles of oxindole alkaloids in different steps of the ion exchange process at 245 nm showing the absence of alkaloids in eluates from the *first pass fraction, neutralization*, and *resin washing* steps (**A**); presence and maintenance of alkaloid profile in oxindole alkaloid purified fraction (OAPF) from both the crude extract and prepurified extract (PPE) (**B**); the distribution of alkaloids in the subfractions from the *third step* eluate (**C**). Note: mitraphylline (**1**), isomitraphylline (**2**), speciophylline (**3**), uncarine F (**4**), pteropodine (**5**), isopteropodine (**6**), rhyncophylline (**7**), and isorhyncophylline (**8**).

Monitoring of alkaloid isomerization in cell treatment

The alkaloid isomerization was monitored and expressed as described above (see item Monitoring of alkaloid isomerization in purified process). Separately, 3 mg samples of OAPF, OAPF 5′, OAPF 15′, and OAPF 45′ were first dissolved in 100 µL of DMSO in the same way as for the cytotoxity assay. Separately, a 10-µL aliquot of each solution was transferred to wells (6-well plate) and properly diluted with phosphate buffered saline (PBS, pH 7.4) to yield concentrations of 100 µg/mL. Aliquots of 500 µL were collected at 0, 6, 12, and 48 h incubation times at 37 °C in a humid atmosphere saturated with 5% CO_2, appropriately diluted in acetonitrile : water (50 : 50, v/v) and analyzed by HPLC-PDA.

Statistical analysis

The results were statistically evaluated by one-way ANOVA followed by Tukey's test (Minitab 14® software) and $p < 0.05$ values were considered significant.

Supporting information

Oxindole alkaloid isomerization in heating under reflux, cytotoxic activity of oxindole alkaloid purified fractions against T24 and RT4 human bladder cancer cell lines, reproducibility of the ion exchange process, and monitoring of pH and conductivity from ion exchange process eluates are available as Supporting Information.

Results and Discussion

For obtaining the OAPF with satisfactory recovery, the POA : ion exchange resin ratio of 1.0 : 1000 (w/w) (equivalent to 0.82 g of freeze-dried crude extract per 30.0 g of resin) was established previously. It allowed a total alkaloid recovery of 86.22% with respect to the OAPF (**Table 1**). Both POA and TOA eluted almost entirely in the *third step* were practically undetectable in the other steps of the process (**Fig. 2**). Other experimental conditions tested were unsuccessful. Using a 1.5 : 1000 ratio (w/w), for instance, the alkaloid recovery decayed to 75.77%, but it dropped to near zero with a POA : ion exchange resin ratio of 3.0 : 1000 (w/w).

When considered separately, POA and TOA recoveries were 86.22% and 70.19%, respectively (**Table 1**). In comparison to the crude extract, it corresponds to an 18- and 16-fold concentration increase, in that order (**Table 1**). The reproducibility of the fractioning process was 7.53–10.12% and 5.00–13.58% for POA and TOA, respectively (**Table 1S**, Supporting Information), which was considered satisfactory bearing in mind the matrix and process complexity.

The high ionic charge of a purified fraction can lead to inconsistent results in the cytotoxicity assay. Maximum conductivity levels caused by high ammonium acetate concentration were recorded in the first 40-mL subfraction of the *third step* eluate (**Table 2S**, Supporting Information), besides traces of oxindole alka-

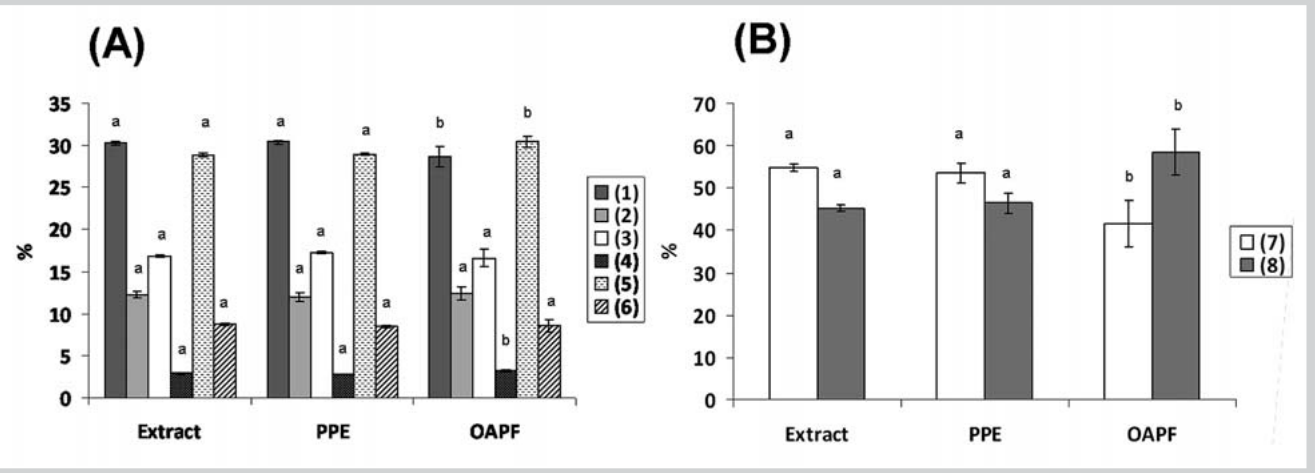

Fig. 3 Comparative alkaloid profile of pentacyclic oxindole alkaloids (POA) (**A**) and tetracyclic oxindole alkaloids TOA (**B**) in the crude extract, prepurified extract (PPE), and oxindole alkaloid purified fraction (OAPF) (n = 3), Results are expressed as ($C_{individual}/C_{total}$) × 100. For POA, C_{total} represents the sum of contents for **1**–**6**, while for TOA, C_{total} represents the sum of contents for **7** and **8**; [a, b] The same letters indicate an individual alkaloid relative content statistically equivalent using Tukey's test ($p > 0.05$). Note: mitraphylline (**1**), isomitraphylline (**2**), speciophylline (**3**), uncarine F (**4**), pteropodine (**5**), isopteropodine (**6**), rhyncophylline (**7**), and isorhyncophylline (**8**).

loids (**Fig. 2C**). Thus, the first subfraction was discarded, and the following ones were pooled yielding a 260-mL fraction. As the isomerization rate is slower in acid medium than in the basic one [11, 12], the pooled subfractions were acidified to pH 5.5, but not to pH 4.0, to keep the ionic charge at a low level.

Isomerization while obtaining OAPF was monitored through the ion exchange process (**Fig. 3**). As can be seen by comparing content data between PPE and the crude extract, the percent of contents of isomitraphylline (**2**), speciophylline (**3**), and isopteropodine (**6**) in OAPF were statistically equivalent ($p > 0.05$) after the ion exchange process (**Fig. 3A**). Conversely, the percent of contents of mitraphylline (**1**), uncarine F (**4**), and pteropodine (**5**) differed significantly from each other ($p < 0.05$), but most likely it was due to the difference among the recovery data of these alkaloids (**Table 1**) rather than a resulting isomerization induced by the process. Likewise, the lower recovery of rhyncophylline (**7**) (53.75%) compared to isorhyncophylline (**8**) (90.04%) (**Table 1**) was responsible for a differential TOA profile in OAPF (**Fig. 3B**).

Alkaloid isomerization of OAPF was induced by 120 min of heating under reflux in hydroethanolic solution 40% (v/v) (**Fig. 1S**, Supporting Information). The mitraphylline (**1**) was converted to isomitraphylline (**2**) (*trans* D/E ring junction), and speciophylline (**3**), uncarine F (**4**), and pteropodine (**5**) were converted to isopteropodine (**6**) (*cis* D/E ring junction) similar to what has been previously observed from bark cat's claw crude extract [14]. Likewise, isorhyncophylline (**8**) was isomerized to rhyncophylline (**7**).

Aiming to evaluate the influence of alkaloid isomerization on cytotoxic activity, samples were collected after 5, 15, and 45 min of heating under reflux and nominated OAPF 5′, OAPF 15′, and OAPF 45′, respectively. The OAPF, as well as the OAPF 5′, OAPF 15′, and OAPF 45′, showed significant cytotoxic activity against T24 and RT4 human bladder cancer cell lines after 48 h treatment, with the response being dose-dependent (**Fig. 2S**, Supporting Information).

As recognized at an early point, the cytotoxic activity of oxindole alkaloids occurs by a selective induction of apoptosis via activation of caspase [5, 8, 16–18], without altering the cellular viability of nonmalignant cells, as shown in fibroblasts from the African green monkey kidney (VERO), normal human bone marrow, human skin fibroblast (HF-SAR), and human embryonic kidney epithelial (293 T) [4, 6, 8, 19]. In relation to human bladder cancer cells lines, specifically T24 and RT4, the oxindole alkaloids can act inhibiting the Wnt-signaling pathway downstream of beta-Catenin [19] in the same way that occurs in human colorectal cancer cell lines, since patients with bladder cancer exhibit a hypermethylation in the APC tumor suppressor gene, a regulator of the Wnt-signaling pathway [20]. It is expected, therefore, that OAPF may be acting in a similar way, but further studies are needed to confirm this hypothesis.

Although the oxindole alkaloid profiles were different after heating under reflux (**Table 2**), the IC_{50} values from all samples collected at different times of heating were statistically equivalent ($p > 0.05$) for both cell lines (**Table 3**).

At first sight, it seems contradictory considering various literature data, according to which singly evaluated oxindole alkaloids showed dissimilar antitumor activities regarding type and intensity [4–10]. A further alternative explored in this work to explain this apparent paradox implied a possible alkaloid isomerization throughout the incubation period. Taking into account that oxindole alkaloids can be easily isomerized, the alkaloid profile was monitored in buffered medium (PBS, pH 7.4; 37 °C in a humid atmosphere saturated with 5% CO_2) simulating the conditions employed in the cytotoxicity assay. Under these conditions, the oxindole alkaloids were converted to isomerized forms throughout the incubation time. Thus, after 6 h incubation, the initial alkaloid profile in OAPF became comparable to those observed in OAPF 5′ and OAPF 15′ (**Table 4**), and basically no substantial difference could be assessed when compared to that from the most isomerized sample collected after heating under reflux (OAPF 45′). Afterwards, the alkaloid profile remained virtually unchanged after 12 h and 24 h incubation. Like the alkaloid interconversion observed when the OAPF was treated by heating under reflux, mitraphylline (**1**) was converted to isomitraphylline (**2**) (*trans* D/E ring junction), and speciophylline (**3**), uncarine F (**4**), and pteropodine (**5**) were converted to isopteropodine (**6**) (*cis* D/E ring junction). In relation to the TOA, isorhyncophylline (**8**) was isomerized to rhyncophylline (**7**). Although the temperature and pH can decidedly elicit the oxindole alkaloid isomerization [11,

Table 2 Influence of heating under reflux time on the alkaloid content of oxindole alkaloid purified fraction (n = 3).

Alkaloid	OAPF		OAPF 5′		OAPF 15′		OAPF 45′	
	$\bar{X} \pm SD$[3]	% ± SD[4]	$\bar{X} \pm SD$[3]	% ± SD[4]	$\bar{X} \pm SD$[3]	% ± SD[4]	$\bar{X} \pm SD$[3]	% ± SD[4]
Mitraphylline (**1**)	18.90 ± 0.39	27.72 ± 0.24	13.04 ± 0.82	18.94 ± 0.39	10.50 ± 0.21	14.25 ± 0.43	8.83 ± 0.03	12.00 ± 0.04
Isomitraphylline (**2**)	9.01 ± 0.22	13.22 ± 0.49	14.85 ± 0.32	21.59 ± 0.48	19.86 ± 0.22	26.95 ± 0.03	21.87 ± 0.02	29.73 ± 0.03
Speciophylline (**3**)	8.23 ± 0.87	12.05 ± 1.12	1.58 ± 0.21	2.28 ± 0.20	2.00 ± 0.10	1.57 ± 0.04	1.12 ± 0.02	1.53 ± 0.03
Uncarine F (**4**)	2.54 ± 0.11	3.73 ± 0.10	2.44 ± 0.18	3.54 ± 0.11	1.16 ± 0.02	2.72 ± 0.16	1.61 ± 0.01	2.19 ± 0.01
Pteropodine (**5**)	21.32 ± 0.06	31.28 ± 0.41	20.53 ± 0.91	29.83 ± 0.09	19.24 ± 0.57	26.11 ± 1.03	15.35 ± 0.03	20.86 ± 0.04
Isopteropodine (**6**)	8.17 ± 0.35	12.00 ± 0.62	16.38 ± 0.62	23.81 ± 0.37	20.94 ± 1.42	28.40 ± 1.65	24.79 ± 0.06	33.69 ± 0.08
Total POA [1]	68.17 ± 0.95		68.82 ± 2.99		73.71 ± 0.77		73.58 ± 0.03	
Rhyncophylline (**7**)	3.18 ± 0.35	43.35 ± 2.98	3.40 ± 0.10	48.77 ± 2.23	3.82 ± 0.34	51.18 ± 3.57	4.05 ± 0.11	50.98 ± 0.59
Isorhyncophylline (**8**)	4.12 ± 0.07	56.65 ± 2.98	3.58 ± 0.36	51.23 ± 2.23	3.63 ± 0.20	48.82 ± 3.57	3.90 ± 0.10	49.02 ± 0.59
Total TOA [2]	7.30 ± 0.33		6.98 ± 0.42		7.45 ± 0.17		7.94 ± 0.12	

[1] Sum of contents for **1**–**6**; [2] sum of contents for **7** and **8**; [3] alkaloid content expressed as % (w/w, freeze-dried sample) by mean ± standard deviation; [4] alkaloid profile expressed as ($C_{individual}/C_{total}$) × 100 by mean ± standard deviation. For POA, C_{total} represents the sum of contents for **1**–**6**, while for TOA, C_{total} represents the sum of contents for **7** and **8**. Note: Oxindole alkaloid purified fraction (OAPF) after 5 min (OAPF 5′), 15 min (OAPF 15′), and 45 min (OAPF 45′) of heating under reflux

Table 3 IC_{50} values of oxindole alkaloid purified fractions evaluated in T24 and RT4 human bladder cancer cell lines (n = 3).

	IC_{50} (μg/mL) $\bar{X} \pm SD$[1]	
Sample	T24	RT4
OAPF	164.13 ± 10.12[a]	137.23 ± 11.77[b]
OAPF 5′	175.21 ± 35.04[a]	124.22 ± 17.84[b]
OAPF 15′	154.86 ± 16.61[a]	132.25 ± 25.33[b]
OAPF 45′	182.83 ± 19.35[a]	153.00 ± 21.12[b]
Cisplatin [2]	1.98 ± 0.86	5.15 ± 1.85

[1] Values represent the mean ± standard deviation; [2] standard cytotoxic compound; [a] results statistically equivalent ($p > 0.05$) for the T24 cell line; [b] results statistically equivalent ($p > 0.05$) for the RT4 cell line. Note: Oxindole alkaloid purified fraction (OAPF) after 5 min (OAPF 5′), 15 min (OAPF 15′), and 45 min (OAPF 45′) of heating under reflux

12], this as a broad and almost complete interconversion was unexpected under the mild incubation conditions employed.

This also implies that the antitumor activity of cat's claw oxindole alkaloids can be more intricate to evaluate than one can presume, with still more single isomers. This seems critical mainly for alkaloids more susceptible to isomerization, mitraphylline (**1**), speciophylline (**3**), uncarine F (**4**), and pteropodine (**5**) to a lesser extent. As reported earlier, the speciophylline (**3**) drops to 5% of the initial concentration after 2 h at 37 °C and the isomerized forms (**4**-**6**) rise instantly [1]. Besides, after 6 h incubation, significant reductions were also recorded for mitraphylline (**1**) (55%), speciophylline (**3**) (85%), and pteropodine (**5**) (20%) in relation to their initial concentrations in OAPF (**Table 4**).

Since former *in vitro* studies evaluating the antitumor activity of cat's claw alkaloids usually mention an incubation time exceeding 12 h in buffered medium pH 7.4 at 37 °C [4–10], the activity found presumably can be associated to alkaloids and their isomerized forms instead of a particular single alkaloid initially assayed. It especially concerns mitraphylline (**1**), speciophylline (**3**), uncarine F (**4**), and pteropodine (**5**), but not isomitraphylline (**2**) and isopteropodine (**6**), which seemed to be less susceptible to isomerization [11].

In that sense, the cytotoxic activity against nine cancer cell lines was reported for cat's claw preparations with different oxindole alkaloid profiles. Nevertheless, no specific relationship was established between activity and alkaloid profiles [21,22]. The incubation conditions employed probably also led to isomerization of the oxindole alkaloids throughout the period of treatment, which might have disguised this correlation.

In conclusion, the non-isomerized fraction (OAPF) and heat-induced isomerized fractions (OAPF 5′, OAPF 15′, and OAPF 45′) showed similar cytotoxic activities against human bladder cancer cell lines (T24 and RT4). The alkaloid isomerization occurred early in the cytotoxicity assay under rather mild incubation conditions (pH 7.4 at 37 °C), resulting in very similar alkaloid profiles for all fractions. Therefore, the cytotoxic activities against T24 and RT4 human bladder cancer cell lines can be associated, to a large extent, with the isomerization reactions observed under the incubation conditions.

Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

Conflict of Interest

The authors declare that there is no conflict of interest.

Table 4 Alkaloid profiles of purified fractions after different incubation times under the same conditions of cell treatment (n = 3).

	Incubation time (h)															
	0 (% ± SD[1])				6 (% ± SD[1])				12 (% ± SD[1])				48 (% ± SD[1])			
Alkaloid	**OAPF**	**OAPF 5′**	**OAPF 15′**	**OAPF 45′**	**OAPF**	**OAPF 5′**	**OAPF 15′**	**OAPF 45′**	**OAPF**	**OAPF 5′**	**OAPF 15′**	**OAPF 45′**	**OAPF**	**OAPF 5′**	**OAPF 15′**	**OAPF 45′**
Mitraphylline (**1**)	29.03 ± 0.23	19.15 ± 0.47	14.94 ± 0.18	12.04 ± 0.15	13.28 ± 0.09	11.96 ± 0.10	11.79 ± 0.02	11.32 ± 0.15	11.83 ± 0.38	11.23 ± 0.07	11.52 ± 0.03	11.30 ± 0.06	11.71 ± 0.28	11.21 ± 0.08	11.64 ± 0.03	11.45 ± 0.11
Isomitraphylline (**2**)	12.10 ± 0.14	20.80 ± 0.87	26.70 ± 0.13	29.24 ± 0.06	27.05 ± 0.04	28.28 ± 0.38	29.96 ± 0.05	30.06 ± 0.06	29.03 ± 0.57	29.10 ± 0.41	30.33 ± 0.04	30.12 ± 0.14	29.25 ± 0.43	29.15 ± 0.29	30.07 ± 0.02	29.80 ± 0.10
Speciophylline (**3**)	14.42 ± 0.19	2.23 ± 0.50	1.52 ± 0.17	1.47 ± 0.07	1.92 ± 0.06	1.85 ± 0.02	1.79 ± 0.04	1.70 ± 0.04	2.27 ± 0.23	2.04 ± 0.08	1.86 ± 0.04	1.79 ± 0.09	1.89 ± 0.11	1.67 ± 0.08	1.44 ± 0.03	1.28 ± 0.06
Uncarine F (**4**)	3.83 ± 0.01	3.78 ± 0.11	3.07 ± 0.06	2.44 ± 0.02	4.47 ± 0.04	4.18 ± 0.04	3.78 ± 0.07	3.23 ± 0.05	4.10 ± 0.10	3.86 ± 0.09	3.59 ± 0.05	3.19 ± 0.07	3.44 ± 0.21	3.20 ± 0.03	3.00 ± 0.04	2.91 ± 0.03
Pteropodine (**5**)	30.29 ± 0.16	29.72 ± 0.43	27.20 ± 0.04	21.42 ± 0.01	24.80 ± 0.09	24.26 ± 0.45	22.80 ± 0.12	19.80 ± 0.18	22.02 ± 0.13	21.72 ± 0.34	20.96 ± 0.11	19.45 ± 0.11	18.97 ± 0.01	19.14 ± 0.34	19.15 ± 0.42	19.45 ± 0.24
Isopteropodine (**6**)	10.34 ± 0.12	24.30 ± 0.19	26.56 ± 0.24	33.40 ± 0.16	28.49 ± 0.16	29.47 ± 0.87	29.88 ± 0.17	33.90 ± 0.48	30.75 ± 1.40	32.05 ± 0.65	31.74 ± 0.27	34.15 ± 0.15	34.74 ± 1.03	35.63 ± 0.61	34.71 ± 0.33	35.10 ± 0.05
Rhynchophylline (**7**)	59.37 ± 0.45	56.68 ± 3.40	55.23 ± 1.61	53.61 ± 0.29	56.64 ± 0.02	58.28 ± 0.67	59.81 ± 0.14	61.71 ± 1.51	55.36 ± 0.58	56.92 ± 0.38	58.36 ± 0.68	60.37 ± 0.83	55.54 ± 1.28	57.50 ± 0.64	59.84 ± 0.52	60.89 ± 0.64
Isorhyncophylline (**8**)	40.63 ± 0.45	43.32 ± 3.40	44.77 ± 1.61	46.39 ± 0.29	43.36 ± 0.02	41.72 ± 0.67	40.19 ± 0.14	38.29 ± 1.51	44.64 ± 0.58	43.08 ± 0.38	41.64 ± 0.68	39.63 ± 0.83	44.46 ± 1.28	42.50 ± 0.64	40.16 ± 0.52	39.11 ± 0.64

[1] Results are expressed as ($C_{individual}/C_{total}$) × 100 by mean ± standard deviation. For POA, C_{total} represents the sum of contents for **1**–**6**, while for TOA, C_{total} represents the sum of contents for **7** and **8**. Note: Oxindole alkaloid purified fraction (OAPF) after 5 min (OAPF 5′), 15 min (OAPF 15′), and 45 min (OAPF 45′) of heating under reflux

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