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Antigenotoxic, Antioxidant and Lymphocyte Induction Effects Produced by Pteropodine

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Abstract: Pteropodine is a heterohimbine-type oxindole alkaloid specifically isolated from 'Cat's claw' (*Uncaria tomentosa*), a plant that has shown cytostatic, anti-inflammatory and antimutagenic properties and is used in traditional medicine to cure a number of diseases. In this report, we studied the ability of pteropodine to decrease the rate of sister-chromatid exchanges and micronucleated polychromatic erythrocytes in mice administered doxorubicin. We also determined its capacity to induce lymphocyte production in mice as well as its free radical scavenging potential by applying the DPPH assay. We found pteropodine (100–600 mg/kg) to significantly decrease the frequency of sister-chromatid exchanges and micronucleated polychromatic erythrocytes in mice administered with 10 mg/kg of doxorubicin. Furthermore, we determined that pteropodine partially corrected bone marrow cytotoxicity induced by doxorubicin, as it showed an improvement in the rate of polychromatic erythrocytes. Besides, 600 mg/kg of pteropodine increased 25.8% of the production of lymphocytes over the control value along a 96-hr assay, and it exhibited a strong capacity to trap the DPPH-free radical (98.26% with 250 μg/ml). Our results establish that pteropodine is an effective antimutagen in the model used, and suggest that pteropodine deserves further research in the area of cell protective potential and its mechanism of action.

Uncaria tomentosa, commonly known as 'Cat's claw', is a Rubiaceae plant native to Peru that is used in traditional medicine to treat several health disorders, such as arthritis, bursitis, rheumatism, herpes, allergies, ulcers, candidiasis, menstrual disorders, bowel and intestinal disorders, HIV infection, different types of cancer. It is also used as adjuvant in chemotherapy or radiotherapy. For these purposes the bark or root is usually prepared as infusion, or both are also used in extracts or tinctures [1,2]. Research on the biomedical properties of the plant has confirmed its stimulating action on the immune function as well as its cytostatic, anti-inflammatory and antimutagenic effects [3–6]. A further investigation has revealed its antiproliferative activity on the growth of the human breast cancer cell line MCF7 [7].

A number of constituents of the plant have been identified and chemically characterized. These include quinovic acid glycosides, oxindol alkaloids, proanthocyanidins, polyphenols, triterpenes, and sterols such as β-sitosterol, stigmasterol and campesterol [8–11]. Six oxindol alkaloids have been isolated from the plant, including pteropodine also called uncarine C (fig. 1), which is a heterohimbine-type oxindol that has been reported to show an apoptotic effect in leukaemic lymphoblasts and to participate in the improvement of memory impairment induced by the dysfunction of cholinergic systems in the brain of mice [12–15]. These data suggest that pteropodine could act synergistically with other constituents of *U. tomentos*a in one or more of the reported actions of the plant.

Based on the abovementioned information, our laboratory has undertaken the evaluation of the genotoxic and antigenotoxic potential of β-sitosterol and pteropodine. In an earlier study, we found that neither of the two compounds produced genotoxicity in mouse [16]. We have also determined that β-sitosterol protects mouse cells from the DNA damage induced by doxorubicin, and that it is a lymphocyte inducer and a free radical scavenging agent [17]. With respect to pteropodine, we have found no information

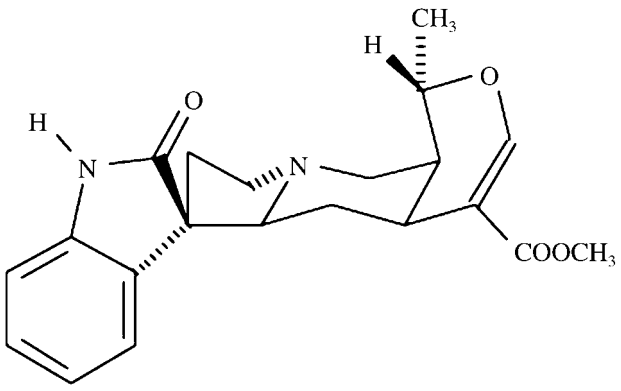

Fig. 1. Chemical structure of pteropodine.

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regarding its effect as a genoprotector agent. In the present study, we have demonstrated that pteropodine was a strong antigenotoxic agent against the SCE and micronuclei induced by doxorubicin in mice, that it stimulated the lymphocyte production in the same animal, and it had a significant free radical scavenging potential.

Materials and Methods

Chemicals and animals. Pteropodine (99% pure) was obtained from Profiqua (Mexico City), whereas 5-bromodeoxyuridine (BrdU), doxorubicin, colchicine and 1,1-diphenyl-2-pycril-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). The Giemsa and Wright stains were obtained from Merck (Mexico City). Sodium citrate, sodium chloride, potassium phosphate and sodium phosphate were purchased from Baker S.A. (Mexico City).

The animals, male mice (NIH) weighing 25 ± 2 g, were obtained from the Mexican National Institute of Rehabilitation. They were maintained in metallic cages at 23 ± 1° and a 12-hr dark–light cycle (8 a.m. to 8 p.m.), consuming food (Rodent Laboratory chow 5001, Purina) and water *ad libitum*. The experimental protocol was approved by the Committee of Ethics and Biosecurity of the Mexican National School of Biological Sciences.

Lethal dose 50. We determined the LD_{50} using a method consisting of two steps and only 13 animals that were intraperitoneally administered with pteropodine [18]. Mortality among mice was observed up to 7 days; the LD_{50} was obtained as the geometric mean between the minimal lethal dose and the maximal sublethal dose. With this procedure we confirmed the previously reported LD_{50} of 771 mg/kg for pteropodine [16].

Sister chromatid exchange, cellular proliferation kinetics and mitotic index. For this assay, six experimental groups were intraperitoneally injected with the compounds. These groups comprising five mice each were as follows: a negative control group administered with 0.4 ml of distilled water, a positive control group treated with 10 mg/kg of doxorubicin and four groups that were first injected with 100, 200, 400 and 600 mg/kg of pteropodine, and 30 min. later with 10 mg/kg of doxorubicin. The selected dose of the mutagen was based on previous studies that have shown significant genotoxic effects without systemic toxicity [16,17]. Pteropodine was dissolved in distilled water (in a water bath at 37° for 7 min.); also, the doses used in this study had shown no genotoxic effect in an earlier report [16].

A 50 mg tablet of BrdU partially coated with paraffin was subcutaneously implanted in each animal, and 1 hr later the animals were injected with the chemicals. Twenty-one hours after the tablet implantation, the mice were intraperitoneally injected with 5 mg/kg of colchicine and left for 3 hr; then, the animals were cervically dislocated, their femurs dissected and the obtained bone marrow was placed in a solution of KCl (0.075 M) at 37° and incubated for 30 min. The cell suspension was centrifuged for 10 min. at 625 ×g, the supernatant was discarded and the cells were fixed in a solution of methanol–acetic acid (3 : 1). The fixation process was repeated at least twice. For the staining process, two drops of each cell suspension were deposited onto ethanol cleaned slides and treated so as to differentiate the sister chromatids [16,19].

The cytogenetic analysis per mouse was made as follows: (i) the rate of SCE was determined in 60 second-division metaphases; (ii) cellular proliferation kinetics (CPK) was determined in 100 metaphases, identifying the cells in first (M1), second (M2) and third (M3) cellular division. With these data, we determined the average generation time (AGT) using the formula AGT = [21/(1M1 + 2M2 + 3M3)] × (100); and (iii) the mitotic index (MI) was determined in 1000 cells. A statistical test of the obtained data was initially made with a one-way ANOVA, followed by a two-tailed Student's t-test using the program Instat 2.

Micronucleated polychromatic erythrocytes (MNPE). Six groups, with five individuals each, were organized for this assay. The compounds were inoculated intraperitoneally. A negative control group was treated with distilled water, another group was administered 10 mg/kg of doxorubicin, and four more groups were first treated with 100, 200, 400 and 600 mg/kg of pteropodine, and after 30 min. with 10 mg/kg of doxorubicin.

Initially, we obtained two drops of blood from the tail of each mouse and smeared them onto ethanol-cleaned slides; then, the cells were fixed in methanol for 3 min. and stained 15 min. with a 4% Giemsa solution made in PBS (pH = 6.8) [20]. Subsequently, the tested chemicals were administered to the mice and the blood cells were obtained and stained as indicated above at 24, 48, 72 and 96 hr. To determine the antigenotoxic potential of pteropodine, we scored the rate of MNPE in 1000 polychromatic erythrocytes per mouse, and for the cytotoxic evaluation the rate of polychromatic erythrocytes/normochromatic erythrocytes in 1000 cells per mouse were determined [20]. A statistical test of the obtained data was initially made with a one-way ANOVA, followed by a two-tailed Student's t-test using the program Instat 2.

Lymphocyte counting. Six groups of mice with five individuals each were intraperitoneally injected with the tested compounds. Four of these groups were administered with pteropodine (100, 200, 400 and 600 mg/kg), and another group with 0.4 ml of distilled water. The last group was injected with the immunostimulant agent α-interferon (0.01 µl/kg), which is a dose that falls within the therapeutic range reported for rat and human beings, and one that has been applied to increase the lymphocyte production in mouse [17,21]. Twenty-four hours after the chemical administration we obtained two drops of blood from the tail of each mouse and smeared them on ethanol-cleaned slides; the cells were fixed in methanol and stained with Wright solution for 5 min., the colour was accentuated with distilled water for 6 min., and the slides were rinsed with tap water. We then made a differential count of white blood cells including neutrophiles, eosinophiles, basophiles, lymphocytes and monocytes. A statistical test of the obtained data was initially made with a one-way ANOVA, followed by a two-tailed Student's t-test using the program Instat 2.

Free radical scavenging potential. This study is based on the measurement of substances that scavenge the stable radical DPPH. Following the method described by Russo *et al.* [22], we prepared an 86 µM solution of DPPH in ethanol, and afterward we added 4, 8, 15, 30, 60, 125 and 250 µg/ml of pteropodine. The absorbance of the mixture was measured at 517 nm, 10 min., after having added the tested chemical. The experiment was made in triplicate, and percentage of the scavenging activity was calculated using the formula [(control absorbance − pteropodine absorbance)/control absorbance)] × (100). Results were evaluated with a linear regression analysis.

Results

The results obtained for sister-chromatid exchanges (SCE), AGT and mitotic index (MI) are indicated in table 1. With respect to the rate of SCE, mice treated with doxorubicin revealed an increase of about six times the level observed in the negative control group. However, the SCE values in animals treated with the four doses of pteropodine, and then with doxorubicin, showed a significant decrease, almost reaching the control level. The same table shows a homogeneous AGT value in the four tested doses of pteropodine

Table 1.

Sister chromatid exchanges (SCE), average generation time (AGT), and mitotic index (MI) in mice treated with pteropodine (PT) and doxorubicin (DX).

Agent	Dose (mg/kg)	SCE $\bar{X}$ ± S.D.	M1 (%)	M2 (%)	M3 (%)	AGT (hr)	MI (%) $\bar{X}$ ± S.D.
Control	–	*2.51 ± 0.12	34	51	15	12.45	5.94 ± 0.21
DX	10	14.06 ± 0.12	39	50	11	12.47	6.16 ± 0.19
PT + DX	100 + 10	*3.56 ± 0.18	33	53	14	13.16	6.21 ± 0.17
PT + DX	200 + 10	*4.32 ± 0.24	38	52	10	13.29	6.00 ± 0.19
PT + DX	300 + 10	*4.53 ± 0.42	38	50	12	12.32	6.42 ± 0.18
PT + DX	600 + 10	*3.10 ± 0.36	36	53	11	12.37	5.96 ± 0.20

Distilled water (0.4 ml) was used as control agent.
The results were obtained in the bone marrow cells. $\bar{X}$ ± S.D. represents the mean ± S.D. of five mice per group. SCE data were determined in 60 second-division cells per mouse. M1, M2, and M3 correspond to the rate of cells in first, second, and third cellular division, respectively. AGT was determined in 100 cells per mouse, and MI in 1000 cells per mouse.
*Statistically significant difference with respect to the value obtained with DX. Data were evaluated with a one-way ANOVA, followed by a two-tailed Student's t-test, $P \leq 0.05$.

and doxorubicin, with a variability of not more than 30 min. among them, and without statistical differences as to the value of the control group. The results for MI likewise presented no modifications among the experimental groups.

The results concerning the determination of MNPE are shown in table 2. In this assay, doxorubicin induced micronuclei since the first observation (at 24 hr) and had a mean value of 21.9 along the 4-day treatment, whereas animals previously treated with 100 mg/kg of pteropodine had a mean of 6.4 MNPE. In fact, the antigenotoxic effect of pteropodine was detected with all tested doses. The highest reduction of MNPE was achieved with 600 mg/kg of the oxindol at 24 hr (89%). The results regarding the frequency of polychromatic erythrocytes are shown in table 3. With the inoculation of doxorubicin, we observed about a threefold inhibition of the index along the evaluated schedule indicating a strong cytotoxic effect of the chemical; however, adding pteropodine before the mutagen gave rise to a significant correction of the damage. With the high dose of pteropodine, the mean value of the index was only 15.2% lower than that obtained in mice treated with the vehicle alone.

As regards the induction of lymphocytes (table 4), we found a significant effect of pteropodine with the four tested doses at all evaluated times. The highest induction was determined with the administration of 600 mg/kg of pteropodine. The other types of blood cells in pteropodine-treated animals were found in a range similar to those determined for the control animals.

Finally, with the DPPH assay, we demonstrated a strong capacity of pteropodine to trap free radicals (fig. 2). The mean and S.D. of the optical density (OD) obtained for the tested concentrations (μg/ml) of pteropodine (PT) were as follow: $OD_{PT4} = 0.227 \pm 0.003$; $OD_{PT8} = 0.215 \pm 0.001$; $OD_{PT15} = 0.200 \pm 0.002$; $OD_{PT30} = 0.177 \pm 0.002$; $OD_{PT60} = 0.149 \pm 0.001$; $OD_{PT125} = 0.067 \pm 0.001$; $OD_{PT250} = 0.005 \pm 0.001$; $OD_{control} = 0.321 \pm 0.003$. The effect was expressed in a concentration-dependent manner reaching a DPPH radical inhibition as high as 98% with 250 μg/ml of pteropodine. The data corresponded to a linear tendency ($y = 0.32x + 27$) with a correlation coefficient $r = 0.91$ ($P = 0.0017$).

Discussion

Pteropodine is one of the most important pentacyclic oxindoles that has been identified as a constituent of Cat's claw, a plant which is known for its curative properties for various

Table 2.

Micronucleated polychromatic erythrocytes (MNPE) in mice administered with pteropodine (PT) and doxorubicin (DX).

Agent	Dose (mg/kg)	MNPE ($\bar{X}$ ± S.D.) 24 hr	48 hr	72 hr	96 hr
Control	–	*1.4 ± 0.16	*1.3 ± 0.20	*1.3 ± 0.09	*1.45 ± 0.30
DX	10	19.1 ± 0.26	21.7 ± 0.29	24.3 ± 0.31	22.82 ± 0.22
PT + DX	100 + 10	*7.6 ± 0.17	*7 ± 0.21	*5 ± 0.07	*6 ± 0.16
PT + DX	200 + 10	*5.5 ± 0.25	*6.3 ± 0.08	*6.1 ± 0.22	*6 ± 0.23
PT + DX	300 + 10	*5.1 ± 0.12	*4.5 ± 0.11	*4.3 ± 0.28	*4.4 ± 0.22
PT + DX	600 + 10	*2.1 ± 0.23	*2.7 ± 0.20	*3.7 ± 0.10	*3 ± 0.12

Distilled water (0.4 ml) was used as control agent.
The results were obtained in 1000 polychromatic erythrocytes per mouse. $\bar{X}$ ± S.D. represents the mean ± S.D. of five mice per group.
*Statistically significant difference with respect to the value obtained with DX. Data were evaluated with a one-way ANOVA, followed by a two-tailed Student's t-test, $P \leq 0.05$.

Table 3.

Polychromatic erythrocytes (PE, %) with respect to the number of normochromatic erythrocytes in mice treated with pteropodine (PT) and doxorubicin (DX).

Agent	Dose (mg/kg)	PE (%) $\bar{X}$ ± S.D. 24 hr	48 hr	72 hr	96 hr
Control	–	*1.9 ± 0.11	*2.0 ± 0.11	*2.0 ± 0.04	*2.1 ± 0.32
DX	10	0.5 ± 0.26	0.6 ± 0.12	0.7 ± 0.22	0.6 ± 0.11
PT + DX	100 + 10	*0.8 ± 0.22	*0.9 ± 0.08	*1.0 ± 0.29	*0.8 ± 0.20
PT + DX	200 + 10	*0.9 ± 0.20	*1.1 ± 0.24	*1.0 ± 0.24	*1.0 ± 0.26
PT + DX	300 + 10	*1.2 ± 0.06	*1.5 ± 0.21	*1.6 ± 0.22	*1.6 ± 0.05
PT + DX	600 + 10	*1.8 ± 0.15	*1.7 ± 0.16	*1.6 ± 0.24	*1.6 ± 0.18

Distilled water (0.04 ml) was used is control agent.
The results were obtained in 1000 erythrocytes per mouse. $\bar{X}$ ± S.D. represents the mean ± S.D. of five mice per group.
*Statistically significant difference with respect to the value obtained with DX. Data were evaluated with a one-way ANOVA, followed by a two-tailed Student's t-test, $P \leq 0.05$.

diseases. However, the biomedical effects of pteropodine itself are poorly studied. The chemical has been shown to enhance the responses evoked by both acetylcholine and 5-hydroxytryptamine in Xenopus oocytes in which rat cortex total RNA was translated [13]; in addition, its antiproliferative and apoptotic effects have been demonstrated in a specific type of cancerous cells [7]. With respect to pentacyclic alkaloids from *U. tomentosa*, their administration has been reported to produce a moderately beneficial effect in patients with rheumatoid arthritis [23] and to significantly enhance the proliferation of normal human resting or weakly activated B and T lymphocytes [2,24]. Our present finding with respect to the induction of lymphocyte proliferation by pteropodine is congruent with the latter activity as well as with the known immunostimulant effect determined with the administration of *U. tomentosa* [6,25]. Moreover, our positive results with all doses tested encourage future studies to evaluate the application of pteropodine in human beings.

There are no previous studies regarding antigenotoxicity and the cancer chemoprevention potential of pteropodine. Interestingly, however, various oxindoles are known to possess antiangiogenic activity [26,27] and some have also been reported as protein kinase inhibitors [28,29]. This last activity is relevant in light of the key role that protein kinase complexes play in the cell cycle regulation which can be altered in cancer development; it is significant, moreover, because protein kinase inhibitors have been successfully studied as chemopreventive agents [30–32]. Besides melatonin, a chemical structurally related to pteropodine because of its indole structure, is known as an antioxidant, an antigenotoxicant and an enhancer of the lifespan in mice and rats [33,34]. Also, ascorbigen (an indole-containing derivative of L-ascorbic acid) as well as its analogues have been shown to inhibit tumour growth and to protect against bacterial and viral infection, besides exhibiting immunomodulatory activity [35].

Our present data clearly indicate that pteropodine reduced the SCE and MNPE produced by doxorubicin in mouse, showing a protective effect on the *in vivo* DNA damage. Furthermore, the results of our study suggest that the pteropodine capacity to inhibit DNA damage may be related with its efficient free radical trapping ability, as shown in the DPPH assay. This activity also agrees with its capacity to

Table 4.

Induction of lymphocytes in mice treated with pteropodine (PT).

Agent	Dose	Lymphocyte (%) $\bar{X}$ ± S.D. 24 hr	48 hr	72 hr	96 hr
Control	–	44.5 ± 0.10	45.3 ± 0.21	46.1 ± 0.14	47.4 ± 0.02
α-Interferon	0.01 µl/kg	50.3 ± 0.26	*56.1 ± 0.22	*60.6 ± 0.22	*56.0 ± 0.11
PT	100 mg/kg	*58.5 ± 0.22	*61.1 ± 0.08	*66.5 ± 0.09	*68.8 ± 0.12
PT	200 mg/kg	*59.8 ± 0.20	*61.3 ± 0.24	*65.8 ± 0.24	*74.4 ± 0.26
PT	300 mg/kg	*62.1 ± 0.06	*65.6 ± 0.21	*74.3 ± 0.22	*76.7 ± 0.03
PT	600 mg/kg	*68.4 ± 0.15	*67.1 ± 0.16	*72.5 ± 0.24	*78.8 ± 0.08

Distilled water 0.4 ml of distilled water.
$\bar{X}$ ± S.D. represents the mean ± S.D. of five mice per group.
*Statistically significant difference with respect to the control value. Data were evaluated with a one-way ANOVA, followed by a two-tailed Student's t-test, $P \leq 0.05$.

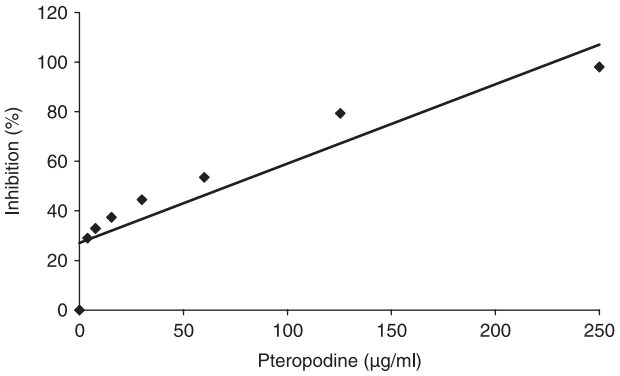

Fig. 2. DPPH radical scavenging activity of pteropodine (PT). Absorbance at 517 nm was scored after 10 min. at room temperature, and the percentage DPPH radical scavenging activity was calculated according to the following equation: %DPPH radical scavenging = [(Control absorbance – PT absorbance)/control absorbance)] (100). $y = 0.32x + 27$. $r = 0.91$.

reduce chromosome alterations induced by doxorubicin, an antineoplastic agent that damages DNA (among other mechanisms) by reducing a quinone molecule to a semi-quinone radical [36]. Besides, the present results agree with those reported for β-sitosterol, a compound also found in *U. tomentosa*, and they show significant antigenotoxicity against doxorubicin and radical scavenging potential in the DPPH assay [17]. In summary, the cell protective action of pteropodine found in the present investigation, together with similar effects determined for *U. tomentosa* and some of its constituents, and compounds chemically related to pteropodine, strongly suggests the importance of confirming our results in other experimental models so as to consider pteropodine a candidate for cancer chemoprevention.

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