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Semi-synthesis of indirubin-3′-oxime from *Strobilanthes cusia* leaves, its acute and sub-chronic toxicity, *in vitro* and *in vivo* antitumor activity in Lewis lung carcinoma bearing mice

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Abstract
Indirubin-3′-oxime (IOX) is known to possess anticancer properties by inhibiting cell proliferation of various human cancer cell lines. In the present study, IOX was prepared directly from an indirubin-rich powder of *Strobilanthes cusia* leaves through an eco-friendly procedure. IOX thus obtained was evaluated for the anticancer activity both *in vitro* and *in vivo*. IOX showed anti-cancer activity against various human cell lines (KB, HepG2, MCF-7, LU-1 and LLC); in particular IOX significantly reduced the survival rate of human oral epidermoid (KB) and human lung cancer (LU-1) cells with IC_{50} values of 2.51 and 3.52 µg/ml, respectively. In the chronic *in vivo* toxicity assay, IOX given *per os* was essentially non-toxic to mice (LD_{50} > 12.0 g/kg). IOX antitumor activity *in vivo* was assessed by use of the Lewis lung carcinoma (LLC) cells transplanted subcutaneously in the mice flank. Mice receiving for 40 days IOX at a daily oral dose of 200 mg/kg b.w. had their life span significantly prolonged by 50% while the tumor mass was significantly (P<0.05) decreased by about 30% as compared to control animals. Since IOX was proved to suppress tumor growth *in vivo* by inhibiting tumor cell proliferation, it is a potential candidate for developing anticancer therapeutic agents.

Keywords: Indirubin-3'-oxime, Lewis lung carcinoma (LLC), cytotoxicity, *Strobilanthes cusia*

1. Introduction
Indirubin-3′-oxime (IOX), a 3, 2-bisindole derivative, is known to be a potential anticancer agent [1]. IOX promotes *in vitro* autophagic and apoptotic death in human acute lymphoblastic leukemia (ALL) JM1, human chronic myelogenous leukemia (CML) cells K562 [2], human lymphoid malignant B-cell (IM9, Reh6) and T- cells (Jurkat, CEM-T). It inhibits *in vitro* proliferation of human leukemia cells LH-60 (with a GI_{50} value of 36.6 µM) [3], as well as MV4-11 and SR4-11 leukemic cell lines [4]. IOX, inhibits β-catein pathway in cancer cells [5]. It was found to inhibit angiogenesis [6], promote *in vitro* apoptosis of human laryngeal adenocarcinoma Hep-2 cells [7], induce apoptosis and tumor cell death in 3 human (A498, CAKI-1, CAKI-2) and 1 murine renal cell cancer (RENCA) cell line [8]. IOX is a potent inhibitor of glycogen synthase kinase-3 and cyclin-dependent kinases (CDKs), which belong to a group of serine/threonine kinases involved in the regulation of cell cycle progression, neuronal function, differentiation and apoptosis [9]. IOX also inhibits protein kinase R, a serine/threonine protein kinase activated by various stress signals and may have a preventive role against the deleterious effects of high glucose in the heart [10]. In addition, IOX has several biological activities of interest for its use in treatment of various deadly diseases. IOX rescues spatial memory deficits and attenuates β-amyloid-associated neuropathology, thus it can be used in the treatment of Alzheimer's disease [11, 12]. It activates Wnt-□-catenin signalling and inhibits adipocyte diffentiation and obersity [13], inhibits P-TEFb function and HIV-1 replication [14], prevents myocardial infarction, reduces blood concentration of lipids [15], prevents platelet aggregation through suppression of ERK and PLCγ2 phosphorylation [16]. It displays anti-inflammatory effects by suppression of influenza a virus-induced cytokines through MAPKs and STAT3 signaling pathways [17]. It enhances the proliferation and differentiation of osteoblastic MC3T3-E1 cells [18], so that it might prove useful in osteoporosis treatment [19, 20].

In the present work, we described the process of direct synthesis of IOX using an indirubin- Rich powder produced from the leaves of *Strobilanthes cusia* (Nees.) O. Kuntze (Acanthaceae) medicinal plant, which is known under the local name of *Cham meo* and is widely distributed in the north Vietnam. IOX was purified by a simple, effective procedure based on a phase-transition process to remove unwanted impurities without use of chromatographic methods or chemical agents. The obtained IOX was evaluated its acute/sub-chronic toxicity and its anticancer activity *in vitro* and *in vivo*. The acute and sub-chronic toxicity of IOX were evaluated in mice and rabbit models, respectively. The *in vitro* cytotoxicity of IOX was assayed on various human tumor cell lines (KB, HepG2, LLC, LU-1 and MCF-7). IOX antitumor activity was investigated in mice bearing tumor induced by LLC cells subcutaneous inoculation. Our results demonstrate that IOX is a novel anti-cancer agent, potentially useful for cancer treatment.

2. Materials and Methods

2.1 General experimental procedures

NMR spectra were determined on a Bruker Avance 500 spectrometer with TMS as internal standard in DMSO-d_6. HR-MS was measured on a Varian FT-MS mass spectrometer. IOX purity was evaluated by a HPLC method, performed on a LC Agilent 1200 Series instrument (Thermostatted automated injector, DAD Detector monitored at an absorption wavelength of 233 nm, column temperature 30 ºC) using a Zorbax Eclipse XDB-C18 column (4,6x150mmx5µm) and a pre-column C_{18} (Agilent Technologies, USA), with a mobile phase of acetonitril – water (95:5 v/v). The flow rate was 0.3 ml/min.

2.2 Plant material and preparation of indirubin-rich powder

The fresh leaves of *Strobilanthes cusia* were collected in the north mountainous provinces of Vietnam. Voucher specimen (C-404) identified by the ethnobotanist Ngo Van Trai (National Institute of Medicinal Materials, Hanoi) was deposited at the Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology.

In brief, the fresh leaves of *Strobilanthes cusia* (1 kg) was soaked in sufficient volume of water for 48-72 hours at room temperature. The whole solution was filtered through a mesh and a cloth bag to remove all pulps and decayed materials and to obtain a leaf filtrate. Isatin agent (6-8 g) dissolved in ethanol was then added to the leaf filtrate. The solution was alkalinized to pH 8.5 – 9.5, stirred well and filtered, to yield a crude indirubin-rich powder. The powder was refluxed in acid solution (pH 3.5 – 5.5) in a water bath and continously stirred for ca. 1 hour. The solids were filtered, washed several times with water until a neutral pH was obtained and dried naturally in the atmosphere or under vacuum to afford a red-violet, odorless highly indirubin-rich powder (>80%).

2.3 Synthesis and purification of indirubin-3'-oxime

IOX was synthesized directly from the indirubin-rich powder prepared from *Strobilanthes cusia* leaves. The powder was added slowly under continuous stirring into a NH_2OH (45-60 g) solution in ethanol for 90 - 120 min. The suspension thus obtained was filtered through a Buchner funnel (3 times) and then filtered again through a porous funnel, stuffed with a 3-5 cm thick layer of silica gel, under a pressure of 100-200 mmHg obtained with a tap-water pump. All filtrates were collected and and acidified to pH 5.0-6.0 and stirred well. Solvent was removed under reduced pressure to yield a concentrated solution, which was diluted with water and allowed to settle down for 1-2 hours at RT. The precipitate thus obtained was filtered, washed with water or ethanol and dried naturally in the atmosphere or under vacuum to afford corresponding crude IOX. Then, the crude IOX was dissolved in alkalined water and extracted with ethyl acetate (3 x 1.0-1.2 L). All extracts were combined and washed with water until neutral, dried over Na_2SO_4 overnight and concentrated under reduced pressure at 40-45 ºC to get the IOX residue. This was dissolved in ethanol, stirred at a temperature of 40-50^{0}C for 30-45 min and allowed to cool down to RT. The precipitate obtained was thus filtered and washed with distilled water. Drying overnight at 50 °C to constant mass gave the corresponding, high-purity (> 98%) in the form of red powder IOX. By TLC on silica gel 60 F_{254} (Merck) with eluent solvent CH_2Cl_2 100%, IOX appeared as a visible red spot at $R_f = 0.35$.

Indirubin-3′-oxime: (3-[1,3-Dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro -2H-indol-2-one). $C_{16}H_{11}N_3O_2$ (M = 277 g/mol) with > 98% purity, m.p. 145-146^{0}C; ^{1}H-NMR (DMSO-d_6, 500 MHz), δ (ppm): 13.46 (1H, s, N-OH); 11.73 (1H, s, H-1'); 10.70 (1H, s, H-1); 8.66 (1H, d, *J*=7.75 Hz, H-4); 8.24 (1H, d, *J*=7.6 Hz, H-4'); 7.40 (1H, m, H-6'); 7.40 (1H, m, H-7'); 7.13 (1H, dt, *J*=1.0, 7.5 Hz, H-6); 7.03 (m, H-5'); 6.97 (1H, dt, *J*=1.0, 7.5 Hz, H-5); 6.90 (1H, d, *J*=7.55 Hz, H-7); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.9 (s, C-2); 151.3 (s, C-3'); 145.2 (s, C-2'); 144.8 (s, C-7'a); 138.3 (s, C-7a); 131.9 (d, C-6'); 127.9 (d, C-4'); 125.9 (d, C-6); 122.9 (d, C-4); 122.6 (s, C-3a); 121.4 (d, C-5'); 120.3 (d, C-5); 116.5 (C-3'a); 111.5 (d, C-7'); 108.8 (d, C-7); 98.9 (s, C-3). ESI-MS *m/z*: 278 $[M+H]^+$, 276 $[M-H]^-$.

2.4 Animals and chemicals

Young adult BALB/c male mice (weighing 24-28 g, 8-10 weeks old) were obtained from the Bioassay Laboratory, Institute of Biotechnology (IBT), VAST. Swiss mice (*Mus musculus)* (18-20 g) and rabbits were obtained from the National Institute of Hygiene and Epidemiology (NIHE), Hanoi. They were maintained in standard RT (22-25 ºC), humidity (50-60%), natural light of day-night cycle (12/12 h) and noise (below 6 dB). The animals were housed in cages and supplied with food and water *ad libitum*. The study was performed in accordance to the guidelines established by the Organization for Economic Cooperation and Development (OECD) [21] for the testing of chemicals, and in compliance with the guide for the care and use of laboratory animals published by the Animal Ethics Committee of Institute of Biotechnology.

Doxorubicine (DOX), dimethyl sulfoxide (DMSO) and ellipticine (ELIP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM medium, fetal bovine serum (FBS), trichloroacetic acid (TCA), acetic acid and phosphate buffer saline (PBS) were obtained from GIBCO-Invitrogen (Carlsbad, CA, USA).

2.5 In *vitro* assessment of IOX cytotoxicity against various cancer cell lines

The *in vitro* assessment of IOX cytotoxicity was performed According to the screening method established by the National Cancer Institute (NCI) based on the rate of cell survival.

2.5.1 Tumor cell lines and cell culture

Human oral epidermoid carcinoma (KB), hepatocarcinoma (HepG2), breast adenocarcinoma (MCF-7), human lung carcinoma (LU-1) and Lewis lung carcinoma (LLC) cell lines were maintained in a DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1% antibiotic PSF (100 units/mL penicilline, 100 µg/mL streptomycin and fungizone), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate at 37^0C and in a humidified 5% CO_2 atmosphere. The MCF-7 cell line was maintained in the DMEM medium supplemented with 5 mL of a 40 µg/L insulin solution.

2.5.2 Cytotoxicity assay

Tumor cell lines were cultivated in a humidified atmosphere of 5% CO_2 / 95% O_2 at 37°C for 48 h. Cell viability was examined by SRB method for cell density determination, based on the measurement of protein content [22]. Viable cells were seeded in the growth medium (180 µL) into 96-microwell plates (4 x 10^4 cells per well) and allowed to attach overnight. The incubation medium was supplemented carefully with IOX (10 µl) in DMSO 10% (stock concentration 4 mg/ml) and the cultivation was continued under the same conditions for additional 48 h. Thereafter, the medium was removed and the cell monolayer attached to the wall was fixed with cold 20% (wt/vol) TCA for 1 h at 4 ºC and stained with 1X SRB staining solution at RT for 30 min, afterwards the unbound dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 515 nm on an ELISA Plate Reader (Bio-Rad). DMSO 10% was used as a blank sample while ellipticine was used as a positive sample. Cytotoxicity, indicated as half inhibition concentration (IC_{50}) and calculated by the program Table Curve Version 4.0, was performed in triplicate at the concentrations of 100 µg/ml; 20 µg/ml; 4 µg/ml and 0.8 µg/ml; (excepted for LU-1 20 µg/ml; 4 µg/ml; 0.8 µg/ml; 0.16 µg/ml). The survival rate (SR) of cells was calculated by the following equation $SR = [(OD_t - OD_0) / (OD_c - OD_0)] \times 100$, where: OD_t: average OD value at day 3; OD_0: average OD value at time-zero; OD_c: average OD value of the blank DMSO control sample.

2.6 Acute toxicity study toward mice

60 healthy albino Swiss mice (*Mus musculus*), both sexes, body weight 18-20 g, were randomly divided into 6 groups (10 mice in each group) and fasted for 15h before the experiment. IOX was prepared in a 2% starch solution at a concentration of 0.2 g/mL. The control group was treated once with 0.4 ml starch solution and the experimental groups (5 groups) were treated with IOX at dosages of 4.0; 6.0; 8.0; 10.0 and 12.0 g/kg body weight. The animals had access to food and water *ad libitum*. Toxic symptoms and mortality were then recorded within 96 h. The body weight was measured at the beginning and once a week for the entire observation period.

2.7 Sub- acute toxicity toward rabbits

Soft capsules VINDOXIM (260 mg) was prepared from Indirubin-3'-oxime (97%, 40 mg) mixed with ingredients including avicel PH101 (140 mg); lactose monohydrate (60 mg); natri starch glyconate (10 mg); natri lauryl sulfate (5 mg); aerosil (2.5 mg); and magnesium stearate (2.5 mg). Ingredient powder from 15 capsules contained indirubin-3'-oxime (97%, 40 mg/each) were dissolved in 14 ml water to prepare Solution A (0.108 capsule /ml). 35 ml of solution A was dissolved further in 70 ml water to prepare solution B (0.036 capsule / ml). Both solution A and B were used for *in vivo* sub-acute toxicity towards rabbits.

21 healthy rabbits, both sexes, body weight 2.0 -2.5 kg, were randomly divided into 3 groups (7 rabbits in each group) and treated with IOX capsule once per day, in the morning, continuously for 28 days. The control group was treated once with 5.0 ml water / kg body. The experimental groups (2 groups) were treated with IOX at dosages of 7.2 mg IOX / kg body weight (5 ml of solution B) and 21.6 mg IOX / kg body weight (5 ml of solution A). The animals had access to food and water *ad libitum*. Toxic symptoms and mortality were then recorded. The body weight was measured at the beginning and once a week for the entire observation period (28 days). Animal body weight, food consumption and activity were checked daily. Toxic symptoms and mortality were monitored daily. Blood samples were collected to evaluate hematological and biochemical parameters. At the end of the study (28 days), all rabbits were sacrificed to isolate organs (liver and kidney) for histological examination.

2.8 Evaluation of IOX antitumor activity *in vivo* in LLC induced tumor bearing mice

In vivo antitumor activity of IOX was determined using tumor-bearing animal models. IOX dosages selected for this experiment were based on existing data on the effective dose and the acute toxicity of IOX in mice.

2.8.1 Tumor transplantation in mice

Exponentially growing 2×10^6 LLC cells, which is the amount needed to induce tumor in 100% of animals, were inoculated subcutaneously into the right flank of the albino mice on Day 0. The mice were kept in a pathogen-free environment, weighted and monitored daily to determine the time of tumor turning-up, as previously described [23].

2.8.2 IOX antitumor activity *in vivo*: study design and treatment

LLC tumor-bearing male mice (n=42) were randomly divided into 7 groups (6 mice / group) including a control group, five IOX-treated groups and a Doxorubicine-treated group (5 mg/kg/day). The control group received only distilled water 0.3 ml/mouse/day for the same schedule as in the treated groups. In IOX-treated groups, mice received IOX daily by oral gavage (50 and 100, low-dose; 200, medium; 400, 500 mg/kg/day, high-dose). Water was used as a vehicle for IOX and Doxorubicine administration. All mice were treated for 45 days.

2.8.3 IOX inhibition of tumor growth in LLC tumor-bearing mice

Tumor volumes were determined with calipers 5 days after cells inoculation [24]. Tumor growth volume was calculated using the formula of Iigo (1991) $V = a \times b^2/2$, where V- tumor volume (mm^3); *a* is the longest diameter and b is the shortest diameter of the tumor. % tumor volume reduction (R(%)) was calculated as $R(\%) = (V_c-V_t)/V_c * 100$, where V_c was the tumor volume in control group and V_t in IOX-treated groups. Tumor inhibition was expressed as inhibition rate (IR in %), which was calculated as $IR = (W_c-W_t)/W_c * 100$, W_c was the tumor weight in control group and W_t in IOX-treated groups. IR % values were used for activity rating and efficacy evaluation. Treatment was considered inactive, moderately and highly active when IR < 25%, IR 25-50% and IR > 50%, respectively. Animal body weight, food consumption and activity were checked daily. Toxic symptoms and mortality were monitored daily. Blood samples were collected to evaluate hematological parameters and organ functions. At the end of the study, all mice were sacrificed to isolate organs (kidney, liver, lung, etc.) for histological examination. The LLC-induced tumors were dissected and weighed.

2.9 Laboratory clinical parameters and histopathological examination for evaluating IOX effects in mice

After 45 days of treatment, blood samples were collected to evaluate hematological parameters including erythrocyte (RBC), leukocyte (WBC), platelets count (PLT), hemoglobin (HGB), and hematocrit (HCT); blood biochemical parameters including creatinine (CREA), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) to evaluate the function of liver and kidney.

At the end of the study, all mice were sacrificed for morphological and metastatic examination of liver and kidney. The dissected organs were stained with hematoxylin and eosin (H&E) solution after fixation with 10% buffered formalin solution. The samples were then embedded in paraffin and cut at a nominal thickness of 4 µm. Microscopic examination was performed for specimens to evaluate the histopathology and antimetastatic effect of IOX.

2.10 Statistical analysis

The result of each experiment was expressed by means ± SD. Statistical analyses and significance, as measured by one-way analysis of variance (ANOVA), were performed using GraphPad PRISM software version 4.0 (GraphPad Software, USA). In all comparisons, $P<0.05$ was considered statistically significant.

3. Results

Reactions for synthesis of Indirubin -3'-oxime from indirubin in indirubin-rich powder prepared starting *Strobilanthes cusia* leaves.

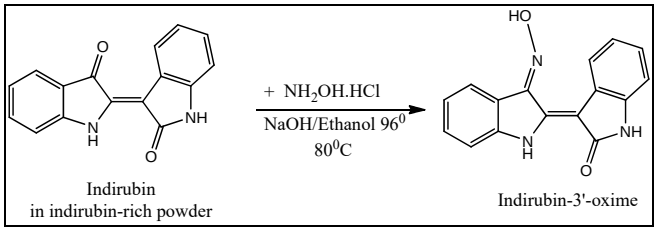

Fig 1: Reaction of indirubin with NH_2OH to produce IOX

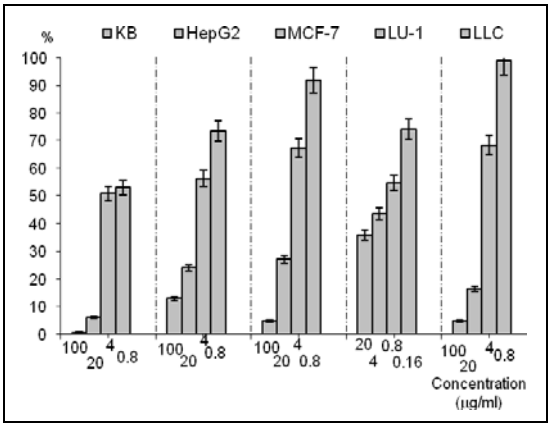

Fig 2: IOX cytotoxicity towards five human cancer cell lines. IOX was dissolved in DMSO at a stock concentration of 4 mg/ml and diluted with DMEM medium a the indicated final concentrations in a 200 µL volume containing tumor cell lines pre-cultured for 24 h and post IOX addition cultivated for another 48 h in a humidified atmosphere of 5% CO_2/ 95% O_2 at 37 °C. Cytotoxicity was indicated by Survival Rate (SR%, columns) (n=3) ($r^2 \geq 0.99$, ± SE, vertical bars; *, $p<0.01$**).**

Table 1: IOX inhibitory effect on survival of various cancer cell lines (Ellipticine – ELIP was used as positive control)

Cell lines	n	IOX IC_{50} (µg/ml)	ELIP IC_{50} (µg/ml)
KB	3	2.51	1.19
HepG2	3	5.79	1.17
MCF-7	3	8.07	1.23
LU-1	3	3.52	1.28
LLC	3	6.64	1.18

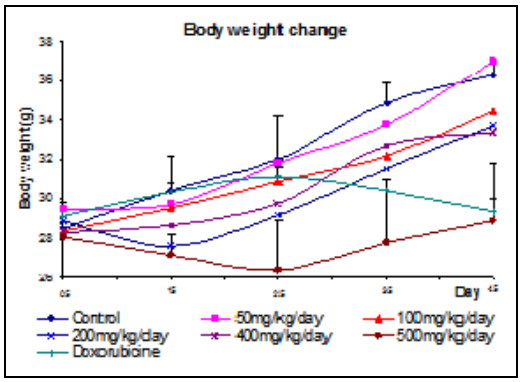

Fig 3: IOX effects on body weight of LLC tumor-bearing mice

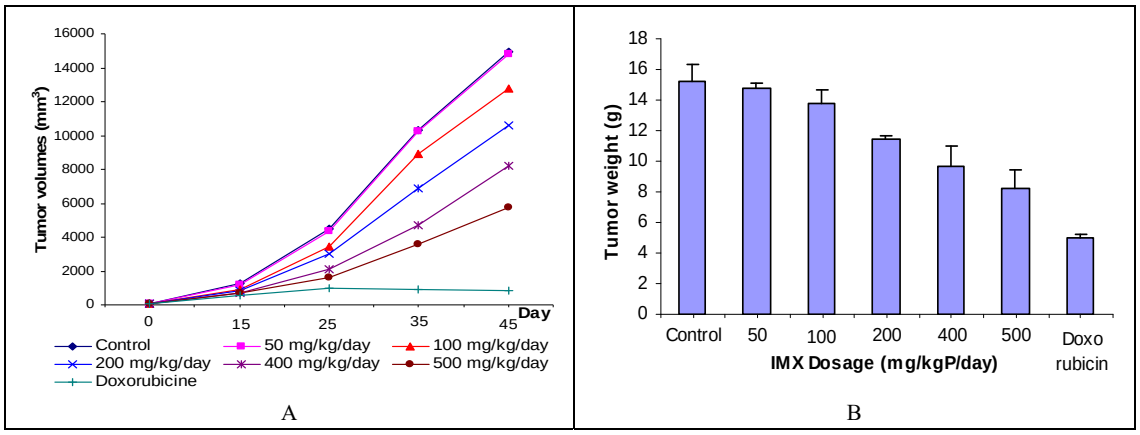

Fig 4: IOX promoted reduction of LLC transplant in (a) tumor volume (mm^3) and (b) tumor weight (g) in mice. LLC cells were implanted subcutaneously into the right flank of animals.

Table 2: Mortality rate and Tumor growth inhibition by IOX in LLC tumor-bearing mice

Treatment group (mg/kg/day)	Mortality rate	% death	Tumor volume (mm^3)	% reduction	Tumor weight (g)	Inhibition rate IR (%)
Control	3/6	50.0	14939.7 ±150.0		15.2 ± 1.1	
IOX 50	2/6	33.3	14774.0 ± 374.2	1.1	14.8 ± 0.4	2.6
IOX 100	1/6	16.7	12774.3 ± 331.1	14.5	13.8 ± 1.0	9.2
IOX 200	0/6	0	10610.7 ± 188.7	29.0	11.4 ± 0.3	24.2
IOX 400	0/6	0	8241.7 ± 134.0	44.8	9.7 ± 1.3	36.3
IOX 500	0/6	0	5757.7± 24.3	42.5	8.2 ± 1.2	45.7
Dox. 5	0/6	0	862.1 ±22.1	94.2	5.0 ± 0.2	66.9

Table 3: Haematological and blood serum biochemical parameters[§] in LLC tumor-bearing mice under IOX treatment

Treatment groups	RBC (10^{12}/L)	WBC (10^9/L)	PLT (10^9/L)	HGB (g/L)	CREA (mmol/L)	SGOT (U/L)	SGPT (U/L)
Day 0 of trial	9.4 ± 0.5	9.7 ± 1.1	523.4 ± 9.8	15.1 ± 0.7	20.1 ± 2.4	84.6 ± 14.9	33.3 ± 12.4
Control	3.9 ± 0.1	37.2 ± 0.2	512.0 ± 5.7	6.4 ± 0.6	22.5 ± 0.7	564.5 ± 9.1	43.5 ± 9.2
IOX 50	4.1 ± 2.9	36.8 ± 0.6	519.0 ± 6.9	6.6 ± 1.0	21.0 ± 0.2	781.0 ± 10.8	42.8 ± 6.6
IOX 100	4.4 ± 3.0	36.0 ± 1.3	520.0 ± 5.66	7.3 ± 0.73	21.1 ± 0.68	967.0 ± 11.85	42.0 ± 4.5
IOX 200	6.1 ± 1.9	34.7 ± 3.2	526.0 ± 7.8	8.8 ± 2.4	20.5 ± 0.7	1030.5 ± 2.8	40.5 ± 3.5
IOX 400	6.9 ± 2.1	28.6 ± 4.1	518.6 ± 8.6	10.8 ± 1.8	20.6 ± 3.7	1047.2 ± 12.6	41.8 ± 4.6
IOX 500	7.4 ± 1.1	25.6 ± 4.1	526.0 ± 6.7	11.3 ± 1.3	20.5 ± 0.7	1059.5 ± 14.8	43.0 ± 9.8

[§] These parameters were evaluated at the end of the trial (45 days of treatment). * $p<0.05$ vs control; & $p<0.05$ vs. day 0.

4. Discussion

4.1 Preparation of indirubin-rich powder and synthesis and purification of indirubin-3′-oxime (IOX)

In almost previous publications so far, indirubin and its derivatives are totally synthesized by condensation reaction under reflux between isatins and indoxyl acetate derivatives in alkalined methanol [25]. Besides using classical synthetic chemistry, indirubin can be produced from various indigo-bearing plants, such as woad leaves (*Isatis tinctoria*), Japanese indigo leaves *Polygonum tinctorium*, French indigo *Indigofera tinctoria,* Chinese indigo *Baphicacanthus cusia* syn. *Strobilanthes cusia*. *Strobilanthes cusia* (Nees) O. Ktze. is a shrub or subshrub growing widely in Vietnam and Asian countries like China, Malaysia, Thailand, and Laos. In Vietnam, *Strobilanthes cusia*, local name Cham meo, grows very popular in the northern mountainous provinces. It has been used not only for dyeing clothing and textiles, but also as a medicinal plant using for the prevention and treatment of numerous ailments since many years in Vietnam traditional medicine [26]. Indirubin contained in the plant is an active ingredient of a traditional Chinese medicine preparation Danggui Longui Wan, which has potent anti-leukemia activity against myelocytic leukemia [27]. In traditional method, indirubin was formed as a by-product with very low yield (< 5%) in the process producing *Indigo Naturalis* from the fresh leaves of *Strobilanthes cusia* [18]. In our method, indirubin is produced as the major compound in the form of indirubin-rich powder by adding isatins into fermented aqueous solution of the fresh *Strobilanthes cusia* leaves. The indirubin-rich powder obtained by this method appeared as red-violet and odorless solid with high yield (>85%) is the starting material for the synthesis of Indirubin-3′- oxime (Fig.1).

Indirubin-3′- oxime, an active indirubin derivative, has a better water solublity and thus has many valuable biological activities than indirubin. As described elsewhere, IOX could be synthesized by the reaction of hydroxylamin with indirubin, which was purified from indigo through a number of complex processes. Such process had some limitations, for examples the reaction was being carried out in complex facilities, at high temperature (at least 115 ºC), using toxic and costly pyridine as a solvent [18]. The use of strong oxidizing agents (H_2O_2,

KMnO4, etc.) to remove impurities raised the production cost and reduced the performance of synthesis of IOX. These disadvantages and the high-cost reagents made the process difficult to implement on a large scale. Moreover, indirubin partly decomposes when exposed to light [28], so that the purification removing indirubin and unwanted by-products from the mixture of indigo raw materials and from the main product, indirubin-3'-oxime, is a difficult and not efficient task.

The semi-synthetic process for IOX production described in this study directly from indirubin-rich powder is noteworth for its simplicity. It is carried out in normal solvents like water, ethanol and the synthesis of IOX is directly from indirubin-containing plant powder without the need of indirubin purification.

Indirubin-rich powder is added to NH2OH ethanol solution (pH = 10-12) at 80 ºC to produce IOX (Fig. 1). After reaction, the suspension is filtered through silica gel layer. The filtrate is acidified and dilluted with water. The red product is filtered and washed to obtain the corresponding crude indirubin-3′-oxime.

The reaction is carried out in ethanol, a less toxic and common solvent and at a relatively lower temperature, this representing a more eco-friendly procedure. IOX is purified by a simple, effective method based on phase transition processes whereby IOX is precipitated and subsequently refined without the use of chromatographic methods or chemical agents for removal of unwanted impurities. The purity of IOX which was assessed by HPLC with a C-18 column, reached up to 98% (Supplement Information).

IOX has been proven to induce apoptosis and exhibit good antitumor activity in previous studies [29]. Therefore, to investigate the efficacy of IOX in cancer prevention and therapy, we aimed to study the acute/sub-chronic toxicity and the antitumor activity of IOX *in vitro* and *in vivo*. This was completed through a cytotoxicity assay and an *in vivo* assay on tumor-bearing mice model using highly metastatic LLC lung cancer cells.

4.3 *In vitro* cytotoxicity assay

IOX cytotoxicity, was evaluated according to the SRB method, in terms of survival rate of several human carcinoma cell lines (Fig. 3). As shown in Tab.1, IC50 values of IOX were in range between 2.51 and 8.07 µg/ml. IOX reduced most significantly the survival rate of KB and LU-1 human lung cancer cell lines, with IC50 values of 2.51 and 3.52 µg/ml, respectively. In all cell lines tested, ELIP displayed a slightly greater activity as compared to IOX.

4.4 IOX acute toxicity in mice

After oral administration of IOX, no significantly abnormal animal behavior was evident during the whole observation period except for a reduced motor activity within the first 2 h after delivery of the highest dose (500 mg/kg/day). Food consumption by all animals of the 5 experimental groups showed not significant differences as compared to the control group. Only 2 out of 10 mice receiving IOX at the dose of 12.0 g/kg body weight died within 96 h after oral administration, so that peroral LD50 could not be determined. According to the recommendations of WHO and OECD for oral acute toxicity assessment, a chemical entity showing an LD50 above 5000 mg/kg is considered nontoxic. Since IOX LD50 was presumably > 10,000 mg/kg (10 g/kg), it was considered safe for oral administration while its maximum tolerated dose (MTD) by the same administration route was presumed to be greater than 100 mg/kg (Supplement information).

4.5 IOX sub-acute toxicity in rabbits

In the sub-acute toxicity study, experimental rabbit groups treated with IOX did not show any sign of toxicity and mortality in compare to the control group. After 28 days of oral administration, no significant changes in the body weight among control and tested rabbit groups were observed. Haematological parameters, including red blood cells, white blood cells, plateletts, hematocrit and hemoglobin; biochemical parameters including SGOT, SGPT, creatinin, total bilirubin, and total protein at the beginning (day 1st), in the medium (day 14th) and at the end of experiment (day 28th) showed no significant changes among control and tested rabbit groups. The findings of histopathological evaluation on all organs (heart, liver, kidney, lung, stomach and spleen) did not relieve any abnormalities or of toxicological significance after 28 days across both control and IOX-capsule treated rabbit groups. Liver and kidney inflammation were observed in two samples of tested groups (Supplement information).

4.6 IOX antitumor activity in LLC tumor- bearing mice

Based on the previous toxicological findings, IOX dosages in mice were selected as follows: 400 and 500 mg/kg as high-dose level, 200 mg/kg as medium-dose level and 100 and 50 mg/kg as low-dose level. IOX antitumor activity *in vivo* was investigated in 42 albino-mice inoculated with LLC cells into the flank to induce subcutaneously a tumor. Thereafter, tumor-bearing mice (n= 6 per group) were treated with water only as negative control (group 1); IOX at different dosages (50, 100, 200, 400, 500 mg/kg/day) to evaluate its tumor growth inhibiting effect (group 2 – 6); and doxorubicine (5 mg/kg/day) as positive control (group 7). The same study design and selected dosages was published elsewhere [30].

4.6.1 Mortality and clinical trend

The animals were observed daily prior to and following drug administration for signs of toxicity and mortality throughout the trial period (45 days). There were no deaths or overt signs of toxicity after daily oral administration of IOX, even at the highest dose tested. At the trial end, 3 mice died (50%) in the control and in the 50 mg/kg/day IOX dose group, while 2 mice died (33.3%) of the IOX 100 mg/kg/day dose group. No sign of toxicity and mortality were found in the positive control group (doxorubicine) and in the groups treated with IOX doses of 200, 400 and 500 mg/kg/day (Tab.2). This indicates that IOX from 200 mg/kg/day dosage upwards protects mice from cancer-induced death and prolongs their life span.

4.6.2 Body weight changes

Throughout IOX trial, mice body weight was recorded as a general health index (Fig. 4). Control mice gained weight during the observation period, as well as the IOX-treated mice at low dose-level (50 and 100 mg/kg/day); on the contrary, the

body weight of IOX treated mice at 500 mg/kg/day dose was significantly lower than that of the controls from day 25 to day 45. Water and food consumption by the animals of all groups were not much different each other.

4.6.3 IOX inhibition of tumor growth

In control mice, LLC tumor transplant volume reached about 0.0625 cm3 value on day 5 after cell inoculation and increased rapidly to ca. 14.9 cm3 on day 45. IOX treatment at high-dose (400 and 500 mg/kg/day), however, significantly inhibited tumor growth, the corresponding volumes reaching ca. 10.6 and 8.2 cm3 values with a reduction (R(%)) of 44.8% and 61.5%, respectively (P<0.05) (Tab.2, Fig. 5a). Moreover, rapidly growing tumors translated into a rapid increase in tumor weight. In IOX-treated groups, tumor weights were much lower as compared to those of the control group. At the trial end, tumors in the latter group weighted 15.17 g, while in IOX-treated groups (400 and 500 mg/kg/day) weights were only 9.67 and 8.23 g (36.3 and 45.7% reduction vs. control), respectively (Fig.5b and Tab.2).

At the trial end, IOX treatment with medium and high-doses (200 - 500 mg/kg/day) inhibited LLC tumor growth by a rate of 26.2-45.7%, so that its antitumor efficacy can be rated as moderate; IOX treatment with oral doses up to 200 mg/kg/day can reduce somewhat tumor growth.

4.6.4 Hemocytometrical and blood biochemical parameters

LLC tumor transplant growth in mice caused marked effects on blood cytometric and biochemical parameters. Erythrocyte count and hemoglobin content were reduced by about 60% in tunor-bearing mice at the end of the trial as compared to the animals at the 0 day. This tumor-induced anemia was counteracted in a dose-dependent manner by IOX. In fact, after the highest dose treatment the above-mentioned parameters were reduced by only 22-25%. Whether this effect simply comes from the antineoplastic activity of IOX or from its stimulation of hematopoiesis is an interesting hypothesis worth to be investigated. Tumor growth was also accompanied by about a four-fold increase in white blood cell count. This phenomenon that underscores the normal immune response of the body against tumor or to specific antibodies [31] was partly reversed by IOX treatment, as reported in Table 3. PLT counts and some biochemical parameters, i.e. serum creatinine and SGPT (Serum Glutamic Pyruvic Transaminase) were unmodified either by tumor growth or by IOX treatment. This finding indicates that IOX organ (kidney, in particular) toxicity is relatively small. At variance, the enzyme SGOT (Serum Glutamic Oxaloacetic Transaminase), increased by about seven-fold during tumor growth, thus indicating toxic effects of the tumor toward liver. This effect was slightly further increased by IOX. In the absence of proper controls, i.e. healthy mice treated for the same period of time with IOX at the highest dose, we can suspect that IOX per se diplays some toxic effects toward liver.

4.6.5 Histopathological evaluation and microscopic findings

All mice from the control and IOX-treated groups were examined histologically. Either in livers or kidneys was examined and no gross abnormalities or lesions at autopsy in died and 40-day surviving mice were noted. Liver necrosis was observed in one sample of control group. The findings of histopathological evaluation on all organs (data not shown) did not relieve any abnormalities after IOX oral administration or of toxicological significance across the different dose-groups.

Although it was reported that subcutaneous implantation of LLC cells would metastatize inner organs such as liver, kidney, spleen, lung and stomach, there were no signs of metastasis in those organs, in both control and IOX-treated animals. In liver and lung of mice in control group, it appeared some white abnormal lumps, which were then detected as necrosis, but not malignant cells. Thus, further experiments should be carried out to clarify whether IOX could prevent metastasis *in vivo*.

5. Conclusion

In our report, IOX is synthesized and purified in high yield directly through an eco-friendly procedure from an indirubin – rich powder, which is produced from fresh Strobilanthes cusia leaves. From the present biological findings, IOX may be, considered safe or fairly non-toxic to mice when administered per os and it is well tolerated up to the 200 mg/kg/day dose at which it displayed clear antitumor activity in a tumor-bearing mice model where it exerted growth inhibition. Both volume and weight of LLC-induced tumor, in fact, were significantly reduced at medium daily doses. Although additional clinical toxicological investigations need to be performed for safety assessment in human beings, IOX may be considered a powerful candidate for the development of therapeutic, anti-cancer agents.

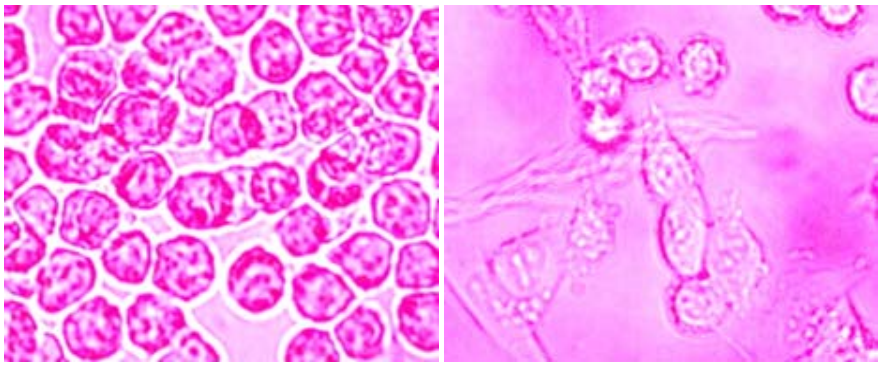

Fig 5: LLC cells were cultured *in vitro*

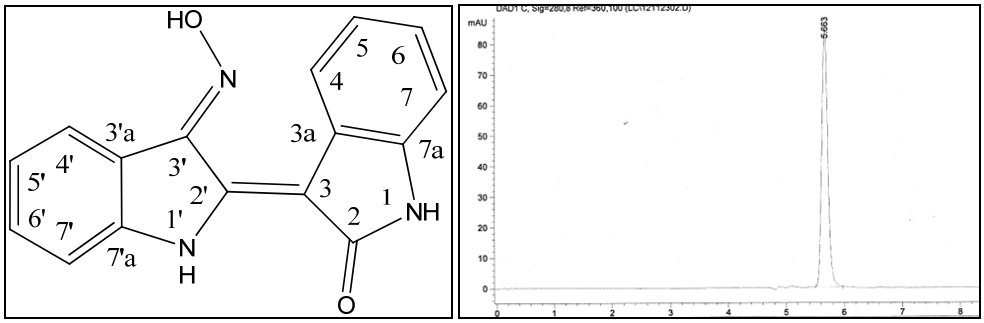

Fig 6: Molecular structure and HPLC chromatogram (C-18 Column) of IOX

Table 4: Evaluation of acute toxicity of IOX in mice

Group	Oral dosage (g IOX / kg body weight)	Number of tested mice in group	Number of dead mice	Ratio (%)
1	4.0 g/kg	10	0	0
2	6.0 g/kg	10	0	0
3	8.0 g/kg	10	0	0
4	10.0 g/kg	10	0	0
5	12.0 g/kg	10	2	20.0

Table 5: Effect of the subchronic oral administration of VINDOXIM to rabbit body weight (n=21)

Experimental time	*Control*		*Tested group 1*		*Tested group 2*		*p*
	Initial weight (kg)	**Percent weight gain (%)**	**Initial weight (kg)**	**Percent weight gain (%)**	**Initial weight (kg)**	**Percent weight gain (%)**	
Before Experiment (m_0)	2.07±0.15		2.05±0.16		2.08±0.15		
After 1 week (m_1)	2.14±0.16	↑3.38	2.09±0.16	↑1.95	2.14±0.16	↑2.88	$P_{T1\text{-}C} > 0.05$
After 2 week (m_2)	2.18±0.16	↑5.31	2.14±0.15	↑4.39	2.16±0.15	↑3.85	$P_{T2\text{-}C} > 0.05$
After 3 week (m_3)	2.23±0.15	↑7.73	2.17±0.14	↑5.85	2.22±0.15	↑6.73	
After 4 week (m_4)	2.26±0.16	↑9.18	2.24±0.13	↑9.27	2.26±0.15	↑8.65	
$P_{pre\text{-}cons}$	<0.05		<0.05		<0.05		

Table 6: Effect of the subchronic oral administration of Vindoxim to haematological parameters of experimental rabbits (n=21)

Parameters	**n**		**Control**	**Group T1**	$P_{to, t1,t2}$	**Group T2**	$P_{to, t1,t2}$
Red blood cells (x 10^{12}/ L)	7	t_0	5.5 ± 0.8	5.0 ± 1.6		5.8± 0.4	
		t_1	5.0 ± 0.6	4.8± 1.7		5.3± 0.8	
		t_2	5.4 ± 0.4	5.8 ± 0.3		5.7 ± 0.5	
White blood cells (x 10^9/ lit)	7	t_0	9.4 ± 2.3	7.8 ± 2.6		7.5 ± 1.2	
		t_1	7.7 ± 1.6	6.7 ± 2.3		7.1 ± 1.1	
		t_2	9.1 ± 1.9	8.7 ± 2.3		7.7 ± 1.8	
Plateletts (x 10^9/ lit)	7	t_0	484.9 ± 185.6	427.4 ± 145.4		417.6 ± 61.5	
		t_1	457.7 ± 107.0	431.0 ± 143.2	*> 0.05*	438.5 ± 104.6	*>0.05*
		t_2	588.7 ± 167.4	528.3 ± 114.5		460.6 ± 91.0	
Hematocrit (%)	7	t_0	33.9 ± 3.4	30.2 ± 9.5		34.5 ± 1.5	
		t_1	33.3 ± 1.9	29.4 ± 9.7		33.4 ± 2.1	
		t_2	32.9 ± 2.0	34.4 ± 1.4		33.3 ± 2.4	
Hemoglobin (g/dL)	7	t_0	11.1 ± 1.0	9.8 ± 3.1		11.3 ± 0.5	
		t_1	10.5 ± 0.6	9.4 ± 3.1		9.8 ± 3.4	
		t_2	10.6 ± 0.5	11.0 ± 0.2		11.0 ± 0.5	
				$P_{C\text{-}T1} > 0.05$		$P_{C\text{-}T2} > 0.05$	

t_0: Before experiment. t_1: after 14 days. t_2: after 28 days of oral administration of VINDOXIM

Table 7: Effect of the subchronic oral administration of Vindoxim to biochemical parameters of experimental rabbits (n=21)

Parameters	**n**		**Control**	**Group T1**	$P_{to, t1,t2}$	**Group T2**	$P_{to, t1,t2}$
SGOT (IU/ L)	7	t_0	34.1 ± 15.7	28.9 ± 14.3	*> 0.05*	25.5 ± 19.9	*> 0.05*
		t_1	33.4 ± 7.2	30.6 ± 12.9		32.5 ± 10.4	
		t_2	24.0 ± 6.1	32.4 ± 18.6		19.8 ± 4.1	
SGPT (IU/ L)	7	t_0	50.9 ± 13.1	49.2 ± 17.8		48.3 ± 22.4	

		t_1	54.3 ± 15.3	50.4 ± 19.5		51.1± 19.2	
		t_2	69.4 ± 12.7	63.7 ± 13.4		55.8 ± 13.3	
Creatinin (µmol/L)	7	t_0	93.3 ± 15.2	86.3 ± 27.3		100.9 ± 12.6	
		t_1	87.7 ± 17.3	81.5 ± 28.3		90.0 ± 18.3	
		t_2	97.0 ± 13.0	111.4 ± 10.0		107.6 ± 10.6	
Urea (mmol/L)	7	t_0	5.7 ± 1.3	5.2 ± 2.0		5.9 ± 0.8	
		t_1	4.8 ± 1.3	4.5 ± 1.7		5.2± 1.0	
		t_2	7.2 ± 1.4	7.7 ± 1.0		7.1 ± 0.4	
Total Bilirubin (µmol/L)	7	t_0	2.6 ± 0.3	2.2 ± 0.7		2.3 ± 0.2	
		t_1	2.5 ± 0.5	2.3 ± 0.8		2.3 ± 0.5	
		t_2	2.6 ± 0.3	2.7 ± 0.2		2.7 ± 0.3	
Total Protein (g/L)	7	t_0	57.7 ± 3.0	51.4 ± 17.0		56.5 ± 2.4	
		t_1	54.9 ± 2.3	49.3 ± 16.4		57.3 ± 3.1	
		t_2	59.4 ± 2.3	60.9 ± 4.6		59.1 ± 2.8	
				$P_{C-T1} > 0.05$		$P_{C-T2} > 0.05$	

Table 8: Histopathological evaluation on all organs by microscopic observation

Organ	Code	Control	Code	Group 1	Code	Group 2
Liver	M18	Normal liver tissue	M1	Focal hepatic lesions	M9	Normal liver tissue
Kidney		Glomerular hyperemia. Localized interstitial nephritis		Normal kidney tissue		Normal kidney tissue
Liver	M20	Normal liver tissue	M7	Normal liver tissue	M13	Normal liver tissue
Kidney		Normal kidney tissue		localized interstitial nephritis		Normal kidney tissue
Liver	M21	Hepatitis portal of entry	M8	Hepatitis portal of entry	M14	Necrotic hepatitis
Kidney		Normal kidney tissue		Localized interstitial nephritis		Normal kidney tissue

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