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In Vitro Testing for Genotoxicity of Indigo Naturalis Assessed by Micronucleus Test

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In the field of cosmetic dyes, used for coloring the hair and skin, there is a clear tendency to replace the widely used synthetic dyes by natural colorants, such as henna and mixtures of henna with indigo. The aim of this study was to estimate the genotoxicity of water and DMSO solutions of indigo naturalis (prepared from *Indigofera tinctoria* leaves) using the cytokinesis-blocked micronucleus (CBMN) assay in the human metabolically active HepG2 cell line. The cytotoxic effects of indigo solutions were first assessed by propidium iodide and fluorescein-diacetate simultaneous staining. For both solutions, cytotoxicity was always under 10%. Data obtained in the CBMN assay (for all concentrations tested) indicated that the frequency of MN (micronuclei) in exposed cells was no higher than the control. Both the water and DMSO solutions showed the same behavior. These results indicate that indigo naturalis exhibits neither cytotoxicity, nor genotoxicity for all concentrations tested, which may justify excluding indigofera and its components from the list of carcinogenic agents.

Keywords: *Indigofera tinctoria*, Indigo naturalis, HepG2 cells, Genotoxicity, Micronucleus test.

In the field of cosmetic dyes, used for coloring the hair and skin, there is a clear tendency to replace the widely used synthetic dyes by natural colorants, such as henna and mixtures of henna with indigo. Henna gives a red to orange color but if it is combined with indigo (Figure 1), it yields a brown color, so-called henna black. Indigo-related pigments are widely used, not only in cosmetics, but also in textile, pottery and mural paintings [1]. Blue dye indigo is extracted principally from the leaves of *Indigofera tinctoria* Linn., a perennial shrub, belonging to the family *Fabaceae* (alt. *Leguminosae*), cultivated in India, China and other countries.

Indigo is also widely used in traditional Chinese and Indian medicine to treat various inflammatory diseases and dermatosis [2], epilepsy, nervous disorders, bronchitis and liver ailments [3,4]. Indigo is said to be good for mouth ulcers and, externally, in ointment form, is used for infected ulcers, psoriasis, hemorrhoids and sore nipples [5-8].

Although much work has been done on the pharmacological properties of indigo, very few studies have evaluated its genotoxic potential [9,10] or that of extracts from species belonging to the genus *Indigofera* (*I. truxillensis* and *I. suffruticosa*) [11].

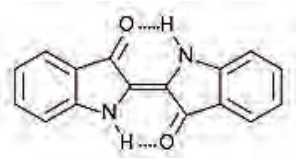

Figure 1: Chemical structure of indigo, 2,2'-Bis(2,3-dihydro-3-oxoindolyliden ($C_{16}H_{10}N_2O_2$) [CAS Reg. # 482-89-3].

With regard to indigo mutagenicity, the data are controversial. Herbert *et al*. [9] observed no statistically significant increase in micronucleus frequency in the bone marrow of mice treated with natural indigo by oral gavage or an increase of mutagenicity towards strains TA1538 and TA98 in the *Salmonella*/microsome test with metabolic activation. On the other hand, Jongen and Alink [10] investigated the mutagenic potential of two natural and seven synthetic commercial indigo dye products and observed that the natural products showed no mutagenicity in *S. typhimurium* strains TA98 and TA100.

As regards *I. truxillensis* and *I. suffruticosa* extracts, mutagenic activity was observed in the *Salmonella*/microsome mutagenicity test for methanol extracts, and the authors suggested that the alkaloid indigo was the main compound responsible for this effect [11].

The aim of the present study was to evaluate, in an *in vitro* laboratory approach, the possible genotoxicity of *Indigofera tinctoria* leaf extracts (indigo) by evaluating chromosomal damage (*i.e.* micronucleus test) in the human liver HepG2 cell line.

Micronuclei (MN) appear in the cytoplasm of interphasic cells as small additional nuclei, smaller than the main nuclei (1/20 to 1/3), when the daughter cells separate. MN generate during the anaphase from acentric chromosome fragments or whole chromosomes that are left behind during mitotic cellular division and, consequently, are excluded from both of the daughter nuclei. The formation of MN in dividing cells is the result of chromosome breakage or chromosome malsegregation due to spindle dysfunction produced by either clastogen or aneugen agents, respectively. Thus, MN provide a measure of both chromosome damage and chromosome loss and this biomarker is considered to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome aberrations analysis [12].

Genotoxicity testing has been performed on the HepG2 cell line (ATCC HB 8065). The HepG2 cell line is an excellent model to investigate toxicity of drugs, since it retains many of the morphological characteristics of the liver parenchymal cells from which they originate [13] and present endogenous bioactivation capacity expressing phase I and phase II enzymes involved in the activation and/or detoxification of xenobiotics [14,15]. The use of metabolically competent cells, such as HepG2, in genotoxicity testing is considered to be closer to the *in vivo* situation as the addition of an exogenous metabolic activation system (*i.e.* S9-mix) is not needed [16].

At the end of the experiments cell viability, as evaluated with the fluorochrome-mediated viability test by simultaneous staining with fluorescein diacetate and propidium iodide, was always found to be over 90% (data not shown).

The MN frequencies in HepG2 cells, following 24 h *in vitro* treatment with various amounts of indigo naturalis prepared from *Indigofera tinctoria* leaves, are shown in Tables 1 and 2. Neither aqueous nor DMSO solutions induced an increase in the MN frequency relative to negative controls (untreated cells). Statistical analysis did not show any statistically significant difference, for either solution, compared to the respective negative control. The positive controls showed the expected significant increase (p<0.001).

To investigate the possible influence of *in vitro* treatment with indigo naturalis solutions on cell cycle and speed of replication of HepG2 cells, the mitotic index (MI) and the nuclear division index (NDI) were evaluated (Tables 1 and 2). Neither of the two considered indices showed a possible interference (negative or positive) on the proliferation properties of HepG2 cells in the range of the doses tested.

Table 1: Frequency of micronuclei (MN) in binucleated cells (MN/1,000 cells), mitotic index (MI) and nuclear division index (NDI) in HepG2 cells treated *in vitro* (24 h) with different concentrations of indigo naturalis (from *Indigofera tinctoria* leaves), in aqueous solution. Data reported as the experimental mean values ± standard deviation.

Indigo naturalis aqueous solutions (µg/mL)	MN	MI	NDI
25	12.5 ± 0.7	0.47±0.09	1.49±0.01
50	13.7 ± 4.4	0.47±0.02	1.48±0.01
100	12.0 ± 4.2	0.49±0.02	1.51±0.02
250	12.3 ± 1.7	0.47±0.03	1.48±0.03
500	13.5 ± 4.1	0.45±0.01	1.46±0.01
800	13.0 ± 1.4	0.35±0.05	1.42±0.04
1,000	15.0 ± 4.2	0.48±0.02	1.47±0.03
Controls (–)[a]	14.8 ± 2.9	0.48±0.02	1.49±0.02
(+)[a]	36.7 ± 2.9	0.47±0.01	1.45±0.02

[a] Negative control: MEM; positive control: B(a)P 1.6 µg/mL.

Table 2: Frequency of micronuclei (MN) in binucleated cells (MN/1,000 cells), mitotic index (MI) and nuclear division index (NDI) in HepG2 cells treated *in vitro* (24 h) with different concentrations of indigo naturalis (from *Indigofera tinctoria* leaves), in DMSO. Data reported as the experimental mean values ± standard deviation.

Indigo naturalis DMSO solutions (µg/mL)	MN	MI	NDI
25	9.8 ± 0.9	0.47±0.05	1.49±0.06
50	11.5 ± 3.3	0.47±0.01	1.49±0.06
100	12.7 ± 2.5	0.42±0.01	1.42±0.02
250	14.8 ± 3.5	0.40±0.01	1.42±0.01
500	10.3 ± 2.5	0.43±0.01	1.45±0.01
800	14.0 ± 3.2	0.43±0.05	1.45±0.06
1,000	16.3 ± 3.4	0.46±0.02	1.48±0.01
Controls (–)[a]	12.5 ± 2.1	0.55±0.06	1.56±0.06
(+)[a]	37.5 ± 3.5	0.45±0.02	1.46±0.01

[a] Negative (vehicle) control: DMSO 5 µl/mL; positive control: B(a)P 1.6 µg/mL.

Studies on the mutagenic/genotoxic effects of indigo naturalis have rarely been found in the literature and to our knowledge there are no studies that have tested indigo genotoxicity in human cell lines. The negative results obtained in this *in vitro* approach are in agreement with the results of the only *in vivo* study that evaluated the clastogenic potential of indigo naturalis by the MN test in the bone marrow of male mice [9].

In conclusion, even though the results of this study are largely negative in terms of genotoxicity, for a final decision it seems to be necessary to conduct further research, particularly *in vivo*, in animal models, to exclude any genotoxic risk to the consumer.

Experimental

Chemicals and Media: Indigo naturalis powder (from leaves) was of commercial quality. All reagents used were of analytical grade. Acetic acid, dimethyl sulfoxide (DMSO), Giemsa stain solution, methanol, potassium chloride (KCl), disodium phosphate (Na_2HPO_4), monobasic potassium phosphate (KH_2PO_4) and sodium hydroxide (NaOH) were purchased from Carlo Erba Reagenti Srl, Milan, Italy. Benzo[a]pyrene (B[a]P), cytochalasin-B, fluorescein-diacetate (FDA) and propidium iodide (PI), were obtained from Sigma-Aldrich Srl, Milan, Italy. Gibco Eagle's Minimum Essential Medium (MEM), fetal bovine serum (FBS), *L*-glutamine, antibiotics, sodium pyruvate, and Dulbecco's phosphate-buffered saline, pH 7.4 (PBS), were purchased from Invitrogen Srl, Milan, Italy. Conventional microscope slides and coverslips were supplied by Knittel-Glaser, Braunschweig, Germany. Eukitt was from O. Kindler GmbH, Freiburg, Germany. Distilled water was used throughout the experiments.

Sample preparation: Indigo naturalis powder (produced by shredding the leaves of *Indigofera tinctoria*) was purchased from an herbalist's shop.

The solutions used in this experimental approach were obtained by dissolving commercial powder in MEM at concentrations of 5 mg/mL (aqueous solution) and in DMSO at concentrations of 100 mg/mL (DMSO solution). For the procedure, to ensure optimal solubilization of the *Indigofera tinctoria* powder, the solutions were subjected to sonication. Prior to use, the solutions were filtered to remove the coarse woody debris.

Cell cultures: Human HepG2 cells were obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Brescia, Italy. The cells were grown as monolayer cultures, in 25 cm^2 tissue flasks, in MEM with the addition of 10% FBS, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 0.1 mg/mL streptomycin. HepG2 subcultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cell stocks were routinely frozen and stored in liquid nitrogen (N_2).

Cell treatment and micronucleus test: HepG2 cells were cultured in 6-well tissue culture plates (Orange Scientific, Belgium) inoculated with 5 mL of complete MEM containing 1×10^6 cells per well. The overall culture time was 72 h throughout the experiments. After 24 h the cells were maintained in complete MEM, the culture medium was replaced by fresh complete growth medium containing different concentrations of the natural indigo solutions (25 to 1,000 µg/mL in MEM and in DMSO), and the cells were incubated further for 24 h. Positive and negative controls were included in each experiment. The model mutagen B[a]P (1.6 µg/mL) was used as positive control.

The cytokinesis-block micronucleus (CBMN) test was performed following the original method [17], with minor modifications. After the *in vitro* treatment was performed the medium was replaced by fresh MEM containing cytochalasin B (final concentration: 3 µg/mL) to inhibit cell division after mitosis. The cells were then incubated for the final 24 h period. After completing the treatments, the cells were washed twice with 2 mL PBS, harvested by trypsinization (300 µl of 0.05% trypsin-EDTA, 5 min) and centrifuged for 5 min at 105×*g*. The pellets were then resuspended in hypotonic solution (3 mL of 0.56% KCl) at room temperature and fixed with 3 mL of Carnoy's reagent (methanol:glacial acetic acid - 5:1 v/v). The cell suspensions were centrifuged again for 5 min at 105×*g* and resuspended in 6 mL of fixative. Next, the tubes were centrifuged for 5 min, the supernatant discarded and the cell suspensions poured onto pre-cleaned frosted microscope slides. After drying, the slides were stained with 2% Giemsa in phosphate buffer (0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4, pH 6.8) for 8 min, washed with distilled water, air-dried and finally mounted with Eukitt. Cells were examined for MN at 400× magnification according to established criteria [18]. MN were scored in 1,000 binucleated cells (BNC) for any concentration of each repeated experiment. Two independent experiments were performed.

Furthermore, to investigate the impact of the tested extracts on cell proliferation, the mitotic index (MI) and nuclear division index (NDI) were determined. The MI was calculated by the ratio of binucleated and polynucleated cells on a total number of 1,000 cells. The NDI was calculated for each experimental point using the formula [19]:

$$\text{NDI} = \frac{[1\times N_1]+[2\times N_2]+[3\times N_3]+[4\times N_4]}{N}$$

where N_1–N_4 represent the numbers of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

The results for MN, MI and NDI were expressed as the mean ± standard deviation of duplicate determinations from independent experiments.

Fluorochrome-mediated viability test: At the end of the treatments, cytotoxic effects were evaluated in culture aliquots by simultaneous staining with FDA and PI [20]. Dye working solutions were prepared immediately prior to use by adding 20 µl of 5 mg/mL

FDA (in acetone) to 5 mL of PBS, or 20 µl of 20 µg/mL PI to 1 mL of PBS. Subsequently, 100 µl of 20 µg/mL FDA and 30 µl of 0.4 µg/mL PI were added directly to 200 µl of each cell suspension. The cells were then stained for 3 min at room temperature. Cell counts were performed using ten-chamber disposable microscope slides provided with a haemocytometer-like counting grid (Kova, Hycor Biomedical, USA). After the counting chamber was loaded, the cells were examined with a standard fluorescence microscope (Dialux 20, Leitz, Germany) equipped with epi-illumination provided by a 50 W high-pressure mercury lamp (HBO 50, Osram, Germany). Viable cells fluoresced green, whereas dead cells were indicated by orange-stained nuclei.

References

[1] Pawlak K, Puchalska M, Miszczak A, Rosłoniec E, Jarosz M. (**2006*)*** Blue natural organic dyestuffs from textile dyeing to mural painting. Separation and characterization of coloring matters present in elderberry, logwood and indigo. *Journal of Mass Spectrometry*, ***41***, 613-622.

[2] Lin YK, Leu YL, Huang TH, Wu YH, Chung PJ, Su Pang JH, Hwang TL. (**2009**) Anti-inflammatory effects of the extract of indigo naturalis in human neutrophils. *Journal of Ethnopharmacology*, ***125***, 51-58.

[3] Kirtikar KR, Basu BD. (**1987**) *Indian Medicinal Plants* (2nd edn). Reviewed by Blatter E, Caius JF, Mhaskar KS (Eds). Lalit Mohan Basu, Allahabad, India.

[4] Singh B, Saxena AK, Chandan BK, Bhardwaj V, Gupta VN, Suri OP, Handa SS. (**2001**) Hepatoprotective activity of indigtone a bioactive fraction from *Indigofera tinctoria* Linn. *Phytotherapy Research*, ***15***, 294-297.

[5] Kirtikar KR, Basu BD. (**2000**) *Indigofera tinctoria* Linn. Indian Medicinal Plants, (3rd edn). Reviewed by Blatter E, Caicus JF, Mhaskar KS (Eds). Sri Satguru Publications, Delhi, India.

[6] Liang H, Jin Z. (**1997**) Development of medicinal chemistry in China. In *Comprehensive Medicinal Chemistry*, Vol. ***1***, Kennewell PD (Ed). Pergamon Press, Oxford UK, 99–125.

[7] Lin YK, Chang CJ, Chang YC, Wong WR, Chang SC, Pang JH. (**2008**) Clinical assessment of patients with recalcitrant psoriasis in a randomized, observer-blind, vehicle-controlled trial using indigo naturalis. *Archives of Dermatology*, ***144***, 1457-1464.

[8] Lin YK, Leu YL, Yang SH, Chen HW, Wang CT, Pang JH. (**2009**) Anti-psoriatic effects of indigo naturalis on the proliferation and differentiation of keratinocytes with indirubin as the active component. *Journal of Dermatological Science*, ***54***, 168-174.

[9] Hesbert A, Bottin MC, de Ceaurriz J, Protois JC, Cavelier C. (**1984**) Testing natural indigo for genotoxicity. *Toxicology Letters*, ***21***, 119-125.

[10] Jongen WM, Alink GM. (**1982**) Enzyme-mediated mutagenicity in *Salmonella typhimurium* of contaminants of synthetic indigo products. *Food and Chemical Toxicology*, ***20***, 917-920.

[11] Calvo TR, Cardoso CR, da Silva Moura AC, Dos Santos LC, Colus IM, Vilegas W, Varanda EA. (**2009**) Mutagenic Activity of *Indigofera truxillensis* and *I. suffruticosa* Aerial Parts. *Evidence-Based Complementary and Alternative Medicine*, (on-line journal).

[12] Fenech M. (**1997**) The advantages and disadvantages of the cytokinesis-block micronucleus method. *Mutation Research*, ***392***, 11-18.

[13] Knowles BB, Howe CC, Aden DP. (**1980**) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*, ***209***, 497-499.

[14] Diamond L, Kruszewski F, Aden DP, Knowles BB, Baird WM. (**1980**) Metabolic activation of benzo[a]pyrene by a human hepatoma cell line. *Carcinogenesis*, ***1***, 871-875.

[15] Sassa S, Sugita O, Galbraith RA, Kappas A. (**1987**) Drug metabolism by the human hepatoma cell, Hep G2. *Biochemical and Biophysical Research Communications*, ***143***, 52-57.

[16] Knasmüller S, Mersch-Sundermann V, Kevekordes S, Darroudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ. (**1998**) Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. *Toxicology*, ***198***, 315-328.

[17] Fenech M. (**2000**) The *in vitro* micronucleus technique. *Mutation Research*, ***455***, 81-95.

[18] Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirchner S, Lorge E, Morita T, Norppa H, Surrallés J, Vanhauwaert A, Wakata A. (**2003**) Report from the *in vitro* micronucleus assay working group. *Mutation Research*, ***540***, 153-163.

[19] Eastmond DA, Tucker JD. (**1989**) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environmental and Molecular Mutagenesis*, ***13***, 34-43.

[20] Jones KH, Senft JA. (**1985**) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *Journal of Histochemistry and Cytochemistry*, ***33***, 77-79.