Cytotoxicity/Genotoxicity of Natural Dyes in Comparison with Synthetic Extracted from Egyptian Cotton Clothes

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Abstract: The present study was aimed to evaluate the toxicological effects of natural dyes (hibiscus, turmeric and henna) in comparison with synthetic ones (red, olivaceous and yellow) extracted from some cotton clothes distributed in Egyptian local markets. Fish liver cells homogenate and human lymphocytes cultures used as *in vitro* biological model systems instead of intact animals. Dyes purified extracts were prepared from cotton clothes samples and exposure to fish liver cells homogenate which incubated at 27 ^{0}C (5% CO_2 extension) for 24 hrs. The obtained data indicated that some cytotoxic effects such lysosomes (NR assay) and mitochondria (MTT assay) dysfunction as well as cell wall membrane integrity (CV assay) were observed on the liver cells at the lower concentrations of tested synthetic dyes extracts compared with little effects induced by the naturals dyes extracts. According to midpoint cytotoxicity values, the sequence of tested dyes extract for the different cytotoxicity assays were red > yellow > olivaceous > henna > hibiscus > turmeric. Data for DNA damage detected by comet assay in human lymphocytes culture indicated that synthetic dye clothes extracts significantly (p<0.01) increased the percentage of total damaged spots while little effects induced by natural dye extracts. In conclusion, synthetic dyes extracted from clothes distributed in Egyptian local markets could be constituted real threaten to human health through inducing many cyctotoxic and mutagenic effects. Therefore, natural dyes such as hibiscus, turmeric and henna recommended to be used in textile dying technology instead of the synthetic ones.
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1. Introduction

Primitive dyeing techniques included sticking plants to fabric or rubbing crushed pigments into cloth. With the time, dyeing techniques became more complicated and techniques using natural dyes extracted from different fruits, vegetables and other plants, which were boiled into the fabric and gave light and water fastness, were developed. For example, some of the well known ancient natural dyes include madder, a red dye made from the roots of the *Rubia tinctorum*, blue indigo from the leaves of *Indigofera tinctoria*, yellow from the stigmas of the saffron plant, and dogwood, an extract of pulp of the dogwood tree. A bright red called cochineal was obtained from an insect native to Mexico. All these produced high-quality dark colours. Until the mid-19th century all dyestuffs were made from natural materials, mainly vegetable and animal matter (Foulds, 1989).

In recent years, dyeing of textiles is a complex and specialized science. Nearly all dyestuffs are now produced from synthetic compounds. This means that costs have been greatly reduced and certain application and wear characteristics have been greatly enhanced. On the other side, epidemiological studies indicated that exposure of human to dyes from wearing of some clothes appears to be an important risk factor for cancer, phototoxicity, photoallergy etc. Many authors found that dyed clothes contain potentially dangerous amounts of toxic and carcinogenic chemicals such as organic amines (bezidine and its derivatives) and formalin (Beaudouin *et al*., 1995; Nahal and Lim, 1995; CAMAG, 1999; Fassold *et al.,* 1999).They are usually use in manufacturing of phenolic resins, artificial silk and cellulose esters, intermediates for the benzidine yellow pigments, and in the manufacturing of the azo dyes which using in a large scale through textiles production. Fassold *et al.,* (1999) reported that products such as textiles, leather goods, furs, paper, cardboard, mineral oil products, and textile paint of every price class are suspected to contain azo-dyes. Exposure of consumers to azo-dyes takes place only if the dye or pigment migrates from the substrate to the human skin. Also, in reference to textile related contact dermatitis, Hatch and Maibach classify 49 dyes as contact allergens (CAMAG, 1999). Two thirds of these are dispersing dyes. They are used for dying textile made of acetate, polyester, polyamide, polyacrylonitrile, PVC, and polyurethane by immersion or by printing. Textiles having direct skin contact such as underwear, stockings, bathing suits, shirts, pants, baby's and children's clothing as well as lines and sleeping bags are getting attention. In a safety

point of view, many authors and academic centers back to pay attention for using natural dyeing of textiles. Additionally, many practitioners of the craft of natural dying (i.e. using naturally occurring sources of dye) maintain that natural dyes have a far superior aesthetic quality which is much more pleasing to the eye.

In the study presented herein, samples of lab natural dying of cotton clothes with hibiscus (*Hibiscus cannabinus*) , turmeric (*Curcuma longa*) and henna (*Lawsonia inermis*) in contrast with synthetic dying samples collecting from the Egyptian local markets were subjected to toxicological evaluation studies. Recently simple, rapid and economical *in vitro* techniques developed in our previous studies were used (Elhassaneen, 1996, 2000, 2001, 2002 and 2009).

2. Materials and Methods

2.1. Materials

Hibiscus (*Hibiscus cannabinus*), turmeric (*Curcuma longa*) and henna (*Lawsonia inermis*) powder used for dyeing processing were obtained from the local markets of Ataba Square, Cairo. Crude fabrics (100 % cotton) were obtained as a donation from Nasr Company for spinning and weaving, El-Mehala El-Kobra, Gharbia Governorate. Mordant, Copper sulphate, was purchased from El-Gomhoryia Company for Drugs and Chemicals Trading, Shobra, Cairo.

2.1.1. Textile samples:

A total of eighteen samples of cotton fabrics ready dyeing with synthetic red, olivaceous and yellow dyes (six samples per each) were collected at random from the supermarkets of Shibin El-Kom City, Minoufiya Governorate, Egypt (Photo 1). All samples were purchased within about one week, transported to the laboratory then used for dyes extraction.

Photo 1: Samples of lab natural dying cotton fabrics and synthetic dying cotton clothes collected from Egyptian markets.

2.2. Methods

Dyeing process:

Simplest form of dyeing a textile material with natural dyes (100g/l) and mordant, copper sulphate (20g/l) was performed in Nasr Company for Spinning and Weaving, El-Mehala El-Kobra, Gharbia Governorate, Egypt according to the method mentioned by Foulds (1989). Pad-thermosol machine (Switzerland) was used in dyeing process and the operation conditions were adjusted as follow: preparation temperature 110 ^{0}C; pressure, 10 bar and velocity, 2 m/min; roasted temp., 160 ^{0}C; pressure, 10 bar and velocity, 3 m/min.

Extraction:

Cotton clothes sample (100 g) were extracted with 80% aqueous ethanol (750 ml) on an orbital shaker for 120 min at 70 ^{0}C. The mixture was subsequently filtered (Whatman No. 5) on a Buchner funnel, and the filtrate was assayed for toxicity assays.

Cytotoxicity assays of the textile dye extracts using liver cell homogenate as an *in vitro* biological model system

Fish:

Cat fish (*Claris spp.*), 220±20 g, were collected from the Nile River, Egypt by arrangement with some fisherman's and transported to the laboratory in 10-gallon plastic trash cans. Fish were held outside the laboratory in 20-liter sinks including Nile River water with two aerators pushed oxygen at a flow rate of 1 L/min. Fish were fed a daily ration of 2% of their body weight/day (Basic Flake, Aquarium design, Union City, CA, USA) and the amount adjusted every two weeks. The water temperature adjusted in the range 18-25 ^{0}C by using a heating system inside the Aquarius. The photoperiod was 14-h light and 10-h dark. No diseases were observed in experimental fish.

Preparation of Cat fish liver homogenate:

Cat fish liver cells homogenate were isolated according to the method mentioned by Elhassaneen (1996) with some few modifications. Briefly, Cat fish were anesthetized in tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO) and weight, length, and sex was recorded. Livers were excised to a 60 x 15 mm petri dish (Baxter Healthcare Corp., McGaw Park, IL) containing Hank's Balanced Salt Solution (HBSS; Sigma Chemical Co.). Other tissues unless livers were cut away and the HBSS were removed. The livers were minced with a sterilize scissors and resuspended in RPMI-1640 (Sigma Chemical Co.) adjusted to 330 mOs/kg and supplemented with 25 mM HEPES buffer, 2 mM L(+)glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal Calf serum (FCS; all from Sigma Chemical Co.) to give a concentration (10 mg protein/ml).

Experimental design:

Liver homogenate of Cat fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 tissue culture plate. 100 µl of FCS/RPMI-1640 medium was added to each well. Seven ten fold dilutions of dyes textile extracts were prepared and 50 µl of different dilutes were added to each well and incubated at 27 °C for 24 hrs in 5% Co_2 tension. The plates were prepared for different cytotocxicity assays.

Cytotoxicity assays

Neutral red (NR) assay:

According to the method described by of Borenfreund and Puerner (1984). The medium was removed gently then, 0.2 ml of NR stain/media (containing 50 µg NR/ml) were added to each well. The plates were returned to the incubator for 3 hrs to allow for uptake of the stain into the lysosomes of viable uninjured cells. The stain/media were removed and the cells were washed with a mixture of 1% formaldehyde-1% $CaCl_2$ about three times or until no more NR stain comes off. 0.2 ml of a 1% acetic acid-50% ethanol mixture were added to each well to extract the dye. Thereafter, the plates were remained still 10 min at room temperature and agitated briefly on a microtitre-plate shaker for 15-seconds. The absorbance of the dye extract was measured by using MR-5000 microtiter plate reader at a wavelength of 490 nm.

Methyl tetrazollium (MTT) assay:

The MTT assay was described by Borenfreund *et al.* (1988), as follow: a medium (0.1 ml) was discarded and 0.1 ml of MTT-containing medium was added to each well. The plates were covered with foil and incubated at 27^{o}C for 3 hrs. The supernatant was discarded from the wells and 0.1 ml of a solution of 1N HCl-isopropanol was added to extract and solubilize the formazan. The plates were standed up 10 min at room temperature and agitated briefly on a microtitre-plate shaker for 5 seconds. The optical density of the dye extracted of each well was measured by using MR-5000 microtiter plate reader at wavelength 490 nm.

Crystal violet (CV) assay:

The CV assay was described by **Saotome *et al.*, (1989)** as follow: 40 µl of 50% formalin were added to each well and left for one hour. The plates were rinsed under a gentle flow tap water and 200 µl of crystal violet (0.0625%) were added to each well and standed up 5 min at room temperature. The stain was removed and the plates were washed with tap and deionized

water, and dried. The absorbance were measured by using microtiter plate reader MR-5000 at a wavelength of 540 nm.

Measuring of the genotoxicity affects of the textile dye extracts against the Human lymphocyte cells

Preparation of Human lymphocyte cells:

Human lymphocyte cells were isolated according to **Boyum (1968)** from whole blood by ficoll separating solution (Sigma Co.); and the cells were washed by medium of TGD of DNA. The isolated human lymphocytes were incubated with biogenic amine extracts in TGD of DNA and comet assay medium for 2 hrs; the viability of the cells was determined by trypan blue 1 ml (2 x 10^6 lymphocytes) from the stock of suspended cells in medium was transferred to 1.5 ml Ependorf tube and completed to 1.5 ml with the medium. Viability of treated cells was measured after 2 hrs of treatment with biogenic amine extracts. The treated cells (0.7 ml) were transferred into 15 ml falcon tube for comet assay.

The comet assay or single cell gel electrophoresis:

This technique permits the detection of single strand breaks and alkali-labile site such as mentioned by **Singh *et al.* (1988)** and modification was done by **Hassab- Elnabi (1996).**

Electrophoresis and staining:

100 µl of 0.5 % ultra pure agarose (BRL Co.) dissolved in Ca^{2+} and Mg^{2+} -free. PBS buffer was added to clean microscope slides, immediately covered with cover slips and kept for 1 to 2 min at – 12 ^{0}C to solidify. Cover slips were removed and the pellet of treated cells were suspended in 100 µl 0.7 % low melting agarose and added to the slides. The slides were covered again with cover slips and kept at – 12 ^{0}C for 1 min to solidify the low melting agarose. Then the cover slips were removed and a top layer of 100 µl of 0.5 % ultra pure agarose was added and the slides were again kept cold at -12 ^{0}C for 1 min. After removal of the cover slips, the slides were immersed in a jar containing cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % *N*-lauroylsacosine, pH 10; 1 % Triton X-100 and 10 % DMSO; All chemicals from Sigma Co.). The slides were kept at 4 ^{0}C for at least 1 h. After lysis, the slides were placed on a horizontal electrophoresis box. The unit was filled with a freshly made alkaline buffer (300 mM NaOH and 12 mM EDTA, pH 13) to a level of 0.25 cm above the slides. The cells were exposed to alkali for 20 min to allow for DNA unwinding and expression of alkali-labile sites. For electrophoresis of the DNA, an electric current of 25 V (0.86 V/cm) and 300 mA was applied for 30 min. Alkali and electrophorsis were performed in an ice bath. All of these steps were conducted under dim light to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were placed horizontally and *tris* buffer (0.4 M *Tris*, pH 7.5, Sigma Co.) was added to neutralize the excess alkali. The slides were allowed to sit for 5 min. Finally, 100 µl ethidium bromide (20 µg/ml; Sigma Co.) was added to each slide, covered with cover slips and stored at 4 ^{0}C for 4 days in moist environment (plate III).

Damage scoring:

Scoring was performed according to **Hassab-Elnabi (1996)**. Examinations were done with a fluorescent microscope equipped with an excitation filter (510 nm) and barrier filter (590 nm). The migration was evaluated by observing and measuring the nuclear DNA, where the rounded spot of DNA was considered as a normal DNA spot, while migrated nuclear DNA, which migrates towards the anode, appears as comet spot and considered as damaged DNA spot (plat III A, B. C). One thousand spots of DNA were examined and classified into three types of spots: normal spots (plate III A), when the spot has a round shape; damaged spots, when the length of the migrated fragments is less or equals to the diameter of the basal nuclear DNA, (plate III B); the third one is strongly damaged spots, whereas, the length of the comet is more than the diameter of the basal nuclear DNA (plate III C).

Statistics:

All experiments of cytotoxicity were performed at least three times, using four wells for each concentration of tested agent. Data for the dose-response cytotoxicity curves were presented as the arithmetic mean ± SD. Comparative cytotoxicity of tested toxicant compounds i.e. the concentration of toxicant needed to reduce absorbance of the NR and MTT by 10% (NR_{90}, MTT_{90} and CV_{90} values) and by 50% (NR_{50}, MTT_{50} and CV_{50}values) were computed by linear regression analysis of the data as percentage of control versus the logarithmic concentration of the toxicant.

3. Results and Discussion

Cytotoxicity assay

Studying the potential cytotoxic effects of tested natural dyes in comparison with synthetic extracted from Egyptian clothes towards fish liver homogenate:

Catfish liver cells homogenate were used as a biological experimental tool for studying the potential toxic effects of some natural dyes in comparison with synthetic extracted from clothes distributed in Egyptian local markets. For cytotoxic effects, NR, MTT, and CV assays which determined the lysosomes activity, mitochondrial activity, and cell membrane integrity of liver cells, the data were standardized by expressing absorbance data in the presence of each dye extract as a percentage of that in the control medium. Tables (1-3)

and Figure (1) represent typical NR, MTT and CV assays in catfish liver cells homogenate exposed to six dyes extract including natural (hibiscus, turmeric and henna) and synthetic (red, olivaceous and yellow clothes). The absorbance measurements of assays (as % of control) were 41.10- 108.23, 44.16- 111.56, 37.52-104.20, 17.98-92.98, 22.15-96.35 and 20.26-91.76 (for NR); 43.30-106.55, 48.51-114.40, 41.42-107.98, 19.65-91.20, 29.89-97.05 and 21.08-92.10 (for MTT); and 48.88-109.70, 50.78-108.45, 42.99-103.55, 24.66-93.89, 34.71-96.38 and 24.17-93.00 (for CV) for the previous extracts, respectively. The highest adverse cytotoxic effects were recorded for synthetic dye clothes extract i.e. red dye followed by olivaceous and yellow, respectively. A little effect induced by the all natural dye extracts. Also, it could be easily concluded that, NR assay is more sensitive to all tested dyes extracts under this investigation than others assays. These data are in agreement with that obtained by Elhassaneen (1996 and 2001) who studied the cytotoxic effects of some organic and inorganic toxic chemicals by using isolated fish hepatocytes and found that the absorbance measurements for the NR assay is more sensitive than MTT and CV assays.

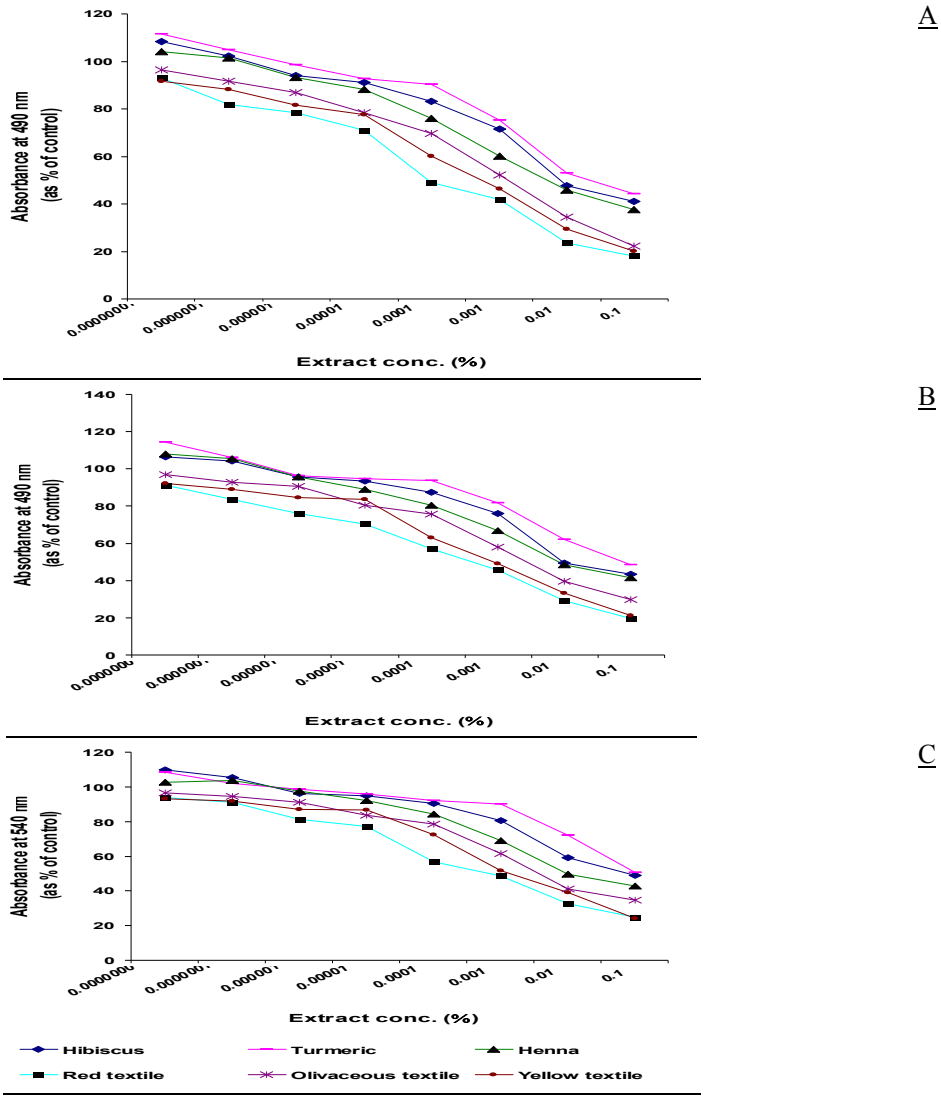

Figure 1: Cytotoxicity of natural dyes in comparison with synthetic extracted from Egyptian cotton clothes as determined by A- neutral red (NR), B- methyl tetrazollium (MTT) and C- crystal violet (CV) assays*

* Liver homogenate of cat fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 tissue culture plate. 100 µl of RPMI-1640/FCS growth medium was added to each well. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each well and incubated at 27 °C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for (NR, MTT and CV) assays such as described in material and methods. Each value represents the mean of three replicates.

Studying the initial and midpoint toxicity of the tested natural dyes in comparison with synthetic extracted from Egyptian clothes towards fish liver homogenate: To analyze the cytotoxicity data it was necessary to determine the exposure concentration to tested dyes extract causing initial toxicity (NR_{90}, MTT_{90} and CV_{90} values) and those causing midpoint toxicity (NR_{50}, MTT_{50} and CV_{50} values). Such data (Table 4) were necessary to distinguish and/or for comparison amongst the all tested extracts. For example, the midpoint cytotoxicity value for turmeric dye extract was recorded lowest values for NR, MTT and CV followed by hibiscus, henna, olivecoeous, yellow and red clothes. Therefore, the natural dye extracts (turmeric, hibiscus and henna) were recorded lower cytotoxicity values compared with the synthetic dyes clothes extract (red, olivecoeous and yellow). According to these data, the sequence of tested dyes extract for the different cytotoxicity assays were red > yellow > olivaceous > henna > hibiscus > turmeric.

From the above-mentioned data it could be noticed that, amongst tested clothes dyes extract the synthetic ones induced many cytotoxic effects in liver homogenate at low concentrations. These cytotoxic effects include lysosomes and mitochondria dysfunction as well as cell wall membrane integrity, which assayed by NR, MTT, and CV assays. There are some variations between the sensitivity of different cytotoxic assays. These Variations may be resulted from the difference of the idea, which each assay based on. For example, the lysosomes activity assay is based on the uptake of neutral red (NR), a supravital dye, and its accumulation in the lysosomes of viable uninjured cells (Borenfreund and Puerner, 1984). While, the mitochondria activity assay is based on the reduction of soluble yellow tetrazolium salt (MTT) to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase (Mosmann, 1983). So, the lower sensitivity of the mitochondria in tested chemical may be due to the poor solubility of the MTT formazan product and/or lower amount of tetrazolium salt reduced by mitochondria.

In general, by using three cytotoxic testing i.e. NR, MTT, and CV, we were able to demonstrate two different toxic responses as a consequence of exposure to synthetic dye clothes extracts (see Figur1). The first type of response was the inhibition of cell division, which characterized by stabilization or slightly increases the initial count of cultured cells even with increasing the concentration of extract. This type of response may be due to the cellular dysfunction or damage (Kocan *et al.,* 1985). The second type of response was the cytotoxicity or cell death, which could be characterized by the decreasing of the growth assay with the increasing of the concentration of tested extracts. Regarding the natural dyes extract one more response has been recorded which called "hormesis". It means increasing occurs in cells number over the controls at low concentrations of the tested substance but the toxic effect does not manifest itself until a higher critical dose level is reached (Laughlin *et al*., 1981). Previous studies of Kocan *et al.* (1985) and Elhassaneen *et al.* (1997) demonstrated that cytotoxicity can be resulted from cells dying and/or inhibition of cell proliferation. All of the present data are in accordance with that obtained by Elhassaneen, (1996); Elhassaneen *et al.,* (1997) and Elhassaneen, (2001) when fish isolated liver cells were exposed to paper industry effluent, pesticides, and heavy metals, respectively.

Genototoxicity assay

The mutagenic (DNA damage detection by comet assay) potential of natural and synthetic dye clothes extracts in human lymphocyte cultures: Table (5) and Figures (2-3) shows the percentages of normal and migrated spots of DNA of natural and synthetic dyes extracted from clothes distributed in the Egyptian local markets. The tested dilutions (10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1}) of natural and synthetic dye clothes extracts increased the percentage of total damaged spots (plate III) with values of 98.20, 95.17, 94.87, 93.23, 91.22, 88.43, 85.62 and 83.20 (for red dye); 98.96, 96.04, 95.96, 95.17, 92.11, 91.17, 90.06 and 90.82 (for olivaceous dye); and 98.01, 96.01, 95.19, 94.03, 92.40, 90.55, 88.37 and 88.03 (for yellow dye), respectively when compared with control samples. A very little effects induced by natural dye extracts (hibiscus, turmeric and henna) especially at the high concentrations. These values were found to be highly significant ($p<0.001$) and dose dependent.

All of these data are in accordance with that obtained by many previous investigations. For example, Hassab-Elnabi (1996) used human lymphocytes in studying the antigenotoxic effect of propolis and cloves with lead nitrate as a heavy metal. The comet assay has already been used in many studies to assess DNA damage and repair induced by various agents in a variety of cells *in vitro* and *in vivo* (Fairbairn *et al*., 1995 and Tice, 1995). The test has widespread applications in genotoxicity testing, DNA damage and repair studies, environmental biomonitoring, and human pollution monitoring (Speit and Hartmann, 2000). A broad spectrum of DNA-damaging agents' causes increased DNA migration in the comet assay: UV and ionizing radiation, hydrogen peroxide and other radical-forming chemicals, alkylating agents, biogenic amines, polycyclic aromatic hydrocarbons, and other adduct-forming compounds, radiomimetic chemicals, and various metals (Tice, 1995; and Hassab-Elnabi, 1996; Ragab, 2003).

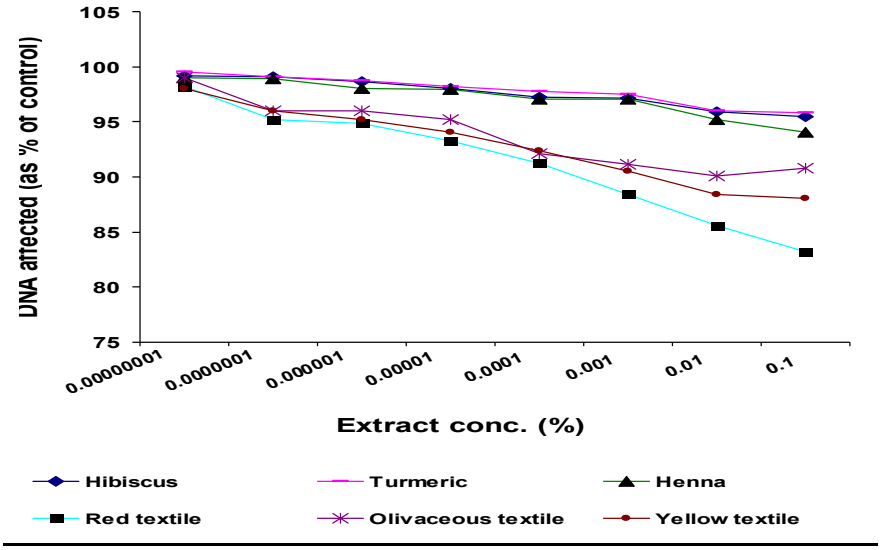

Figure 2: Comet assay of DNA-lymphocytes (as % of control) as affected by natural dyes in comparison with synthetic extracted from Egyptian cotton clothes

* Lymphocytes of human were seeded at 100 cells per tissue culture flask. 25 ml of RPMI-1640/FCS growth medium was added to each flask. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each flask and incubated at 27 °C for 4 h in the presence of 5% Co_2 tension. The flasks were prepared for comet assay (DNA, as % of control) such as described in material and methods. Each value represents the mean of three replicates.

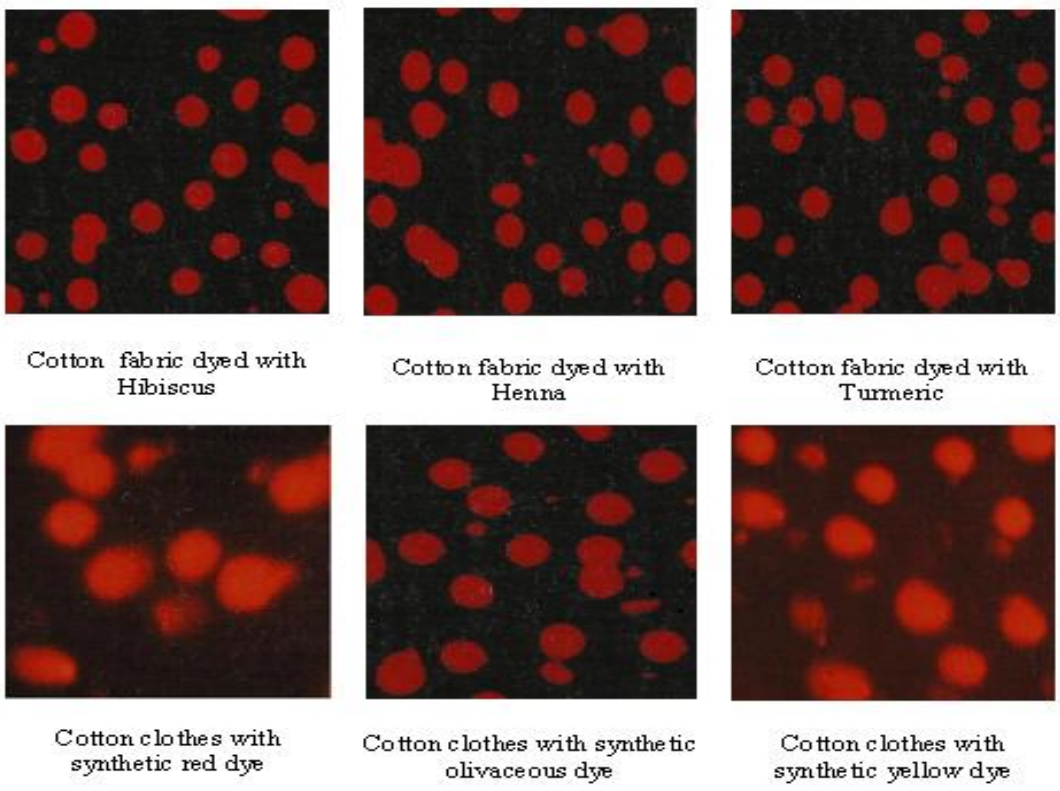

Figure 3: Comet assay of DNA-lymphocytes as affected by lab natural dying cotton fabrics in comparison with synthetic extracted from Egyptian cotton clothes

Table 1: Cytotoxicity of natural dyes in comparison with synthetic extracted from Egyptian clothes as determined by neutral red (NR) assay*

Dye extracts conc. (%)	Hibiscus		Turmeric		Henna	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.191	108.23	1.227	111.56	1.146	104.20
0.0000001	1.125	102.30	1.154	104.87	1.116	101.44
0.000001	1.035	94.09	1.085	98.65	1.025	93.20
0.00001	1.003	91.21	1.021	92.82	0.972	88.32
0.0001	0.916	83.23	0.994	90.40	0.837	76.11
0.001	0.786	71.44	0.828	75.29	0.662	60.18
0.01	0.524	47.61	0.584	53.10	0.504	45.80
0.1	0.452	41.10	0.486	44.16	0.413	37.52
Dye extracts conc. (%)	Red clothes		Olivaceous clothes		Yellow clothes	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.023	92.98	1.060	96.35	1.009	91.76
0.0000001	0.900	81.83	1.007	91.54	0.970	88.17
0.000001	0.863	78.44	0.957	87.00	0.897	81.50
0.00001	0.782	71.07	0.861	78.30	0.853	77.50
0.0001	0.540	49.11	0.766	69.63	0.662	60.20
0.001	0.460	41.84	0.574	52.22	0.509	46.29
0.01	0.258	23.45	0.379	34.46	0.324	29.44
0.1	0.198	17.98	0.244	22.15	0.223	20.26

* Liver homogenate of cat fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 tissue culture plate. 100 µl of RPMI-1640/FCS growth medium was added to each well. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each well and incubated at 27 °C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for (NR) assay such as described in material and methods. ** Each value represents the mean of three replicates.

Table 2: Cytotoxicity of natural dyes in comparison with synthetic extracted from Egyptian clothes as determined by methyl tetrazollium (MTT) assay*

Dye extracts conc. (%)	Hibiscus		Turmeric		Henna	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.172	106.55	1.258	114.40	1.188	107.98
0.0000001	1.147	104.31	1.166	105.96	1.160	105.44
0.000001	1.051	95.50	1.058	96.15	1.053	95.77
0.00001	1.027	93.38	1.040	94.55	0.979	89.03
0.0001	0.963	87.54	1.031	93.76	0.885	80.41
0.001	0.837	76.11	0.898	81.62	0.736	66.90
0.01	0.544	49.44	0.684	62.19	0.534	48.50
0.1	0.476	43.30	0.534	48.51	0.456	41.42
Dye extracts conc. (%)	Red textile		Olivaceous textile		Yellow textile	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.003	91.20	1.068	97.05	1.013	92.10
0.0000001	0.919	83.56	1.019	92.67	0.981	89.15
0.000001	0.836	76.01	0.996	90.59	0.930	84.51
0.00001	0.774	70.35	0.886	80.54	0.919	83.59
0.0001	0.626	56.90	0.834	75.82	0.695	63.19
0.001	0.503	45.72	0.637	57.90	0.539	49.03
0.01	0.320	29.05	0.435	39.55	0.365	33.17
0.1	0.216	19.65	0.329	29.89	0.232	21.08

* Liver homogenate of cat fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 tissue culture plate. 100 µl of RPMI-1640/FCS growth medium was added to each well. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each well and incubated at 27 °C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for (MTT) assay such as described in material and methods. ** Each value represents the mean of three replicates.

Table 3. Cytotoxicity of natural dyes in comparison with synthetic extracted from Egyptian clothes as determined by crystal violet (CV) assay*

Dye extracts conc. (%)	Hibiscus		Turmeric		Henna	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.207	109.70	1.193	108.45	1.129	102.66
0.0000001	1.159	105.40	1.122	101.99	1.139	103.55
0.000001	1.059	96.23	1.084	98.51	1.072	97.43
0.00001	1.042	94.72	1.053	95.70	1.014	92.15
0.0001	0.996	90.55	1.014	92.20	0.926	84.21
0.001	0.885	80.42	0.992	90.19	0.758	68.90
0.01	0.651	59.14	0.794	72.20	0.545	49.52
0.1	0.538	48.88	0.559	50.78	0.473	42.99
Dye extracts conc. (%)	Red textile		Olivaceous textile		Yellow textile	
	Absorbance	**% of control**	Absorbance	**% of control**	Absorbance	**% of control**
0.00000001	1.033	93.89	1.060	96.38	1.023	93.00
0.0000001	1.001	90.98	1.041	94.66	1.011	91.93
0.000001	0.892	81.11	1.002	91.11	0.958	87.05
0.00001	0.850	77.30	0.921	83.72	0.953	86.66
0.0001	0.626	56.90	0.864	78.56	0.797	72.41
0.001	0.534	48.55	0.675	61.40	0.567	51.56
0.01	0.360	32.76	0.452	41.06	0.429	39.03
0.1	0.271	24.66	0.382	34.71	0.266	24.17

* Liver homogenate of fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 tissue culture plate. 100 µl of RPMI-1640/FCS growth medium was added to each well. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each well and incubated at 27 °C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for (CV) assay such as described in material and methods. ** Each value represents the mean of three replicates.

Table 4: Initial and midpoint toxicity of natural dyes in comparison with synthetic extracted from Egyptian clothes towards cat fish liver homogenate (in %)

Compounds	NR assay		MTT assay	
	NR_{90} *	NR_{50} **	MTT_{90} *	MTT_{50} **
Hibiscus	$1.0x10^{-5}$	$1.0x10^{-2}$	$1.45x10^{-4}$	$1.0x10^{-2}$
Turmeric	$1.0x10^{-4}$	$5.5x10^{-2}$	$3.25x10^{-4}$	$1.0x10^{-1}$
Henna	$5.5x10^{-6}$	$7.75x10^{-3}$	$7.75x10^{-6}$	$1.0x10^{-2}$
Red textile	$2.8x10^{-8}$	$1.0x10^{-4}$	$1.9x10^{-8}$	$5.5x10^{-4}$
Olivaceous textile	$1.0x10^{-7}$	$1.0x10^{-3}$	$1.0x10^{-6}$	$5.5x10^{-3}$
Yellow textile	$1.0x10^{-8}$	$5.5x10^{-4}$	$6.4x10^{-8}$	$1.0x10^{-4}$

Compounds	CV assay	
	CV_{90} *	CV_{50} **
Hibiscus	$1.0x10^{-4}$	$1.0x10^{-1}$
Turmeric	$1.0x10^{-3}$	$1.0x10^{-1}$
Henna	$3.25x10^{-5}$	$1.0x10^{-2}$
Red textile	$1.0x10^{-7}$	$1.53x10^{-3}$
Olivaceous textile	$1.08x10^{-5}$	$1.1x10^{-2}$
Yellow textile	$1.08x10^{-6}$	$1.0x10^{-3}$

* Initial toxicity: mean concentrations of herb extract required to reduce absorbance by 10 % to initial toxicity (NR_{90}, MTT_{90}, CV_{90},GA_{90} and PA_{90}).

** Mid toxicity: mean concentrations of herb extract required to reduce absorbance by 50 % to initial toxicity (NR_{50}, MTT_{50}, CV_{50},GA_{50} and PA_{50}).

Table 5. Comet assay of DNA-lymphocytes (as % of control) as affected by natural dyes in comparison with synthetic extracted from Egyptian clothes

Dye extracts conc. (%)	Hibiscus	Turmeric	Henna	Red textile	Olivaceous textile	Yellow textile
0.00000001	99.21 ±0.74	99.56 ±0.33	99.04 ±0.32	98.20 ±0.08	98.96 ±0.54	98.01 ±1.02
0.0000001	99.12 ±0.61	99.07 ±0.21	98.90 ±0.45	95.17 ±0.56	96.04 ±0.78	96.01 ±0.99
0.000001	98.65 ±0.28	98.77 ±0.70	98.07 ±0.33	94.87 ±0.09	95.96 ±0.65	95.19 ±0.67
0.00001	98.05 ±0.59	98.20 ±0.53	97.94 ±0.42	93.23 ±0.89	95.17 ±0.55	94.03 ±0.45
0.0001	97.26 ±1.01	97.74 ±0.32	97.10 ±0.74	91.22 ±1.08	92.11 ±1.04	92.40 ±0.65
0.001	97.14 ±0.67	97.54 ±1.11	97.04 ±0.41	88.43 ±2.06	91.17 ±1.23	90.55 ±1.11
0.01	95.88 ±0.58	96.04 ±0.77	95.24 ±0.83	85.62 ±1.54	90.06 ±1.09	88.37 ±1.06
0.1	95.44 ±0.22	95.80 ±1.27	94.05 ±0.91	83.20 ±0.87	90.82 ±1.11	88.03 ±0.80

* Lymphocytes of human were seeded at 100 cells per tissue culture flask. 25 ml of RPMI-1640/FCS growth medium was added to each flask. Eight ten fold dilutions of each dye extract were prepared and 100 µl of different dilutes were added to each flask and incubated at 27 °C for 4 h in the presence of 5% Co_2 tension. The flasks were prepared for comet assay (DNA, as % of control) such as described in material and methods. Each value represents the mean of three replicates ± SD.

4. Conclusion

The present investigation proved that fish liver homogenate and human lymphocytes culture can be used successively as a simple and inexpensive *in vitro* biological model for monitoring and/or assess the adverse effects of synthetic clothes dye extract. In addition to, synthetic dyes extracted from clothes distributed in Egyptian local markets constitute real threaten to human health through inducing many toxic and mutagenic effects in liver cells. For toxicological point of view, the research and academic centers need to pay more attentions in the future for using the natural dyes instead of the synthetic ones in textile dying technology.

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