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Cytotoxicological evaluation of semi-purified extracts of some dye yielding plants of the Kashmir Valley on Normal Intestinal Cell Line (IEC-6) by MTT assay

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ABSTRACT

Plant extracts are widely used in many fields and there is a need to evaluate their cytotoxic effect to determine their non-cytotoxic concentration at which they can be used in a safe manner. Keeping this in view, the present study was designed to evaluate the *in vitro* toxicity of *Celosia argentia* L. var plumosa (Cockscomb), *Calendula officinalis* L. (Pot Marigold), *Indigofera heterantha* Wall. (Himalayan Indigo) and *Rubia cordifolia* L. (Indian Madder) on Normal Intestinal Cell Line (IEC-6) by MTT assay to test their feasibility for natural edible dye extraction. The experimental material, comprised of inflorescence of *Celosia argentia* L. var plumose, petals of the two varieties of *Calendula officinalis* L., leaves of *Indigofera heterantha* Wall. and leaves and roots of the *Rubia cordifolia* L. Cell line was exposed to 1, 4, 16, 64 and 256µg/ml concentrations of plant extracts for 24, 48, and 72hr at 37ºC. Results revealed that both the varieties of *Calendula officinalis* L. var. Gitana Orange and Gitana Yellow did not show any cytotoxic effect on IEC-6 cell line while as *Celosia argentia* L. var *plumose*, *Indigofera heterantha* Wall. and *Rubia cordifolia* L. showed cytotoxicity. From the present study it was concluded that the extracts of the both varieties of *Calendula officinalis* L. var. Gitana Orange and Gitana Yellow extracts are non-toxic in nature, thus can be utilized for the extraction of natural edible dye while as the extracts of *Celosia argentia* L. var *plumose*, *Indigofera heterantha* Wall. and *Rubia cordifolia* L. had potent *in vitro* cytotoxic activity thus they cannot be used for extraction of natural edible food colour. However, to better evaluate the cytotoxic effect of these plant extracts, *in vivo* experiments on laboratory animal followed by histological analysis should be done.

Keywords: Cytotoxicity, *Celosia argentia* L., *Calendula officinalis* L., *Indigofera heterantha* Wall., *Rubia cordifolia* L.

INTRODUCTION

Many of the herbs and spices used by humans to season food yield useful medicinal compounds [1, 2, 3, 4]. Herbs are staging a comeback and the herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to humans and environment. In India, drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda since ancient times. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Dyes stuff are produced over 700,000 tons annually estimated from more than 100,000 commercially available dyes [5] and applied in many different industries, including the textiles, paper, cosmetic, leather, food and pharmaceutical industries. Artificial food dyes impair and disrupt the behavior of the children [6], further dye manufacturing industry emits volatile organic compounds (VOCs), nitrogen oxides (NOx), hydrogen chloride (HCl), and sulfur oxides (SOx) [7]. From this perspective, the present study was designed to evaluate the *in vitro* toxicity of *Celosia argentia* L. var plumosa, *Calendula officinalis* L., *Indigofera heterantha* Wall. and *Rubia cordifolia* L. on Normal Intestinal Cell Line (IEC-6) by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to test their feasibility for natural edible dye extraction and to evaluate their cytotoxic effect to determine their non-cytotoxic concentration at which they can be used in a safe manner. The MTT assay is a colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. MTT (a yellow tetrazole), is reduced to purple formazan in living cells [8]. A solubilization solution (usually dimethyl sulfoxide), is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. These reductions take place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. The experimental material, comprised of inflorescence of *Celosia argentia* L. var plumose, petals of the two varieties of

Calendula officinalis L., leaves of *Indigofera heterantha* Wall. and leaves and roots of the *Rubia cordifolia* L. *Celosia argentia* L. var plumose is an annual erect herb cultivated in lawns as an ornamental, *Calendula officinalis* L. is an annual erect herb belonging to the *Asteraceae* family with hispidly pubescent, corymbosely branched stem cultivated in lawns, *Indigofera heterantha* Wall. is a small copiously branched shrub belonging to the family *Fabaceae* often forming dense scrubs found in forests and dry slopes and *Rubia cordifolia* L. is a perennial herbaceous climber/creeper belonging to the *Rubiaceae* family which grows near shady and moist places.

MATERIALS AND METHODS

Collection of plant material

Fresh plant samples (*Celosia argentia* L. var plumosa, *Calendula officinalis* L., *Indigofera heterantha* Wall. and *Rubia cordifolia* L.) collected from different sites of Kashmir Valley were identified by [9] in conformity with recent literature and were air dried and homogenized to a fine powder with the help of a mixer grinder. The powdered material was then used for extraction of dyes.

Preparation of plant extracts

1 g of dried plant sample was ground in 10 ml of acetone. The supernatant was collected and filtered with the help of Whatman No. 1 filter paper. Filtrate was allowed to evaporate till completely dry. The extract was dissolved in DMSO (dimethyl sulfoxide) and kept in sterile air tight eppendorf tube at 4°C till further use.

Cell lines and culture

Rat intestinal epithelial cell lines obtained from NCL, PUNE were cultured as mono layers according to instructions provided by the National Chemical Laboratory. Rats intestinal epithelial cell line IEC-6 was cultured in DMEM (Dulbecco's Modified Eagle Medium) media (Sigma- Aldrich) supplemented with Insulin containing 10% heat-inactivated fetal bovine serum (FBS) obtained from Gibco-BRL, USA. Cell lines were maintained as monolayer cultures in a humidified 5% CO_2 incubator (Hereaus 6220, Germany) at 37°C in 75 cm^3 tissue culture flask (Falcon, USA).

The cell lines were passaged twice weekly in fresh medium. When cells reached 75-85% confluence, the medium was removed and cells washed with phosphate-buffer saline (PBS). The cells were then treated with Trypsin-EDTA (Gibco-BRL, USA) to dislodge single cells. Fresh medium was added to inactivate the Trypsin-EDTA. All the suspension was collected in 15 ml centrifuge tube and centrifuged at 350Xg for 5 min. Supernatant were discarded and pellet was re-suspended in complete fresh medium. Cells were counted by hemocytometer. One lakh cells were seeded in 75 cm^3 culture flasks with fresh medium containing 10% FBS.

Extract dilution

Stock concentrations of the plant extracts were diluted in media to obtain 1 and 5mg/ml. For the initial screening of extracts 1.0, 4.0, 16, 64 and 256µg/ml range of doses was prepared from 1mg/ml.

Assessment of plant extracts cytotoxicity by MTT assay

Cell line (IEC-6) was plated in 48 well plates (15000cells/well). Next day plated cells were treated with a dosage of 1.0, 4.0, 16, 64 and 256 µg/ml for three different time points 24, 48 and 72h and incubated in 5.0% CO_2 incubator at 37 ºC. Three hour prior to completion of desired time point the MTT (Gibco-BRL, USA) was added in 1:10 ratio and then kept in CO_2 incubation chamber. After the completion of desired time point (24, 48 and 72 h) media was discarded and 400 µl DMSO (Sigma, USA) was added to dissolve the formazan crystal and plate was read in ELISA (Enzyme-linked immunosorbent assay) reader after 30 min at 550 and 660 nm wavelength.

RESULTS

MTT Assay of plant extracts on the Intestinal Epithelial cell line IEC-6 revealed no significant cytotoxic effect at all the time points i.e, 24, 48 and 72h due to 1, 4, 16 and 64 µg/ml concentrations of *Celosia argentia* L. var plumose and *Indigofera heterantha* Wall. *Celosia argentia* L. var plumosewas slightly toxic whereas *Indigofera heterantha* Wall. was toxic due to 256 µg/ml concentration (Table-1; Fig. 1 and 2).

No cytotoxicity effect on IEC-6 cell line was recorded due to *Rubia cordifolia* L. leaf and root extract both at all-time points due to1ug/ml concentration whereas due to rest concentrations (4, 16, 64 and 256µg/ml) cell death was recorded. Thus both leaf as well as root extract of Indian Madder are toxic in nature (Table-1; Fig. 3 and 4).

Calendula officinalis L. var. Gitana Orange and Gitana Yellow did not show any cytotoxic effect on IEC-6 cell line neither at 24h nor at 48 and 72h due to 1, 4, 16, 64 and 256 µg/ml concentration (Table-1; Fig. 5 and 6).

Table 1: Cytotoxic effect of plant extracts against intestinal epithelial cell line IEC-6 by MTT assay

Concentrations (µg ml^{-1})	Cell viability 2%)		
	24 hr.	48 hr.	72 hr.
A) Cockscomb (*Celosia argentia* L. var *plumose*)			
0 (Control)	100	100	100
1	98.28	102.31	103.01
4	97.45	99.52	101.38
16	93.19	94.79	99.2
64	92.37	93.30	96.48
256	88.73	89.18	92.79

B)	**Himalayan Indigo (*Indigofera heterantha* Wall.)**			
	0 (Control)	100	100	100
	1	97.78	117.42	106.05
	4	97.34	111.61	104.45
	16	94.28	102.89	97.22
	64	94.02	99.16	92.57
	256	75.87	95.57	81.75
C)	**Indian Madder (*Rubia cordifolia* L.)**			
	i) Leaf			
	0 (Control)	100	100	100
	1	100.52	98.75	92.08
	4	97.27	95.52	86.93
	16	95.26	88.16	73.63
	64	71.12	61.33	56.33
	256	63.25	52.11	49.96
Concentrations		**Cell viability (%)**		
		24 hr.	**48 hr.**	**72 hr.**
	ii) Root			
	0 (Control)	100	100	100
	1	91.67	123.92	106.92
	4	86.61	111.73	106.65
	16	81.21	92.12	91.46
	64	71.39	81.52	74.53
	256	70.79	77.58	69.37
D)	**Pot Marigold (*Calendula offcinales* L.)**			
	i) Var. Gitana orange			
	0 (Control)	100	100	100
	1	111.12	108.03	111.94
	4	108.04	106.06	110.28
	16	107.69	104.04	108.85
	64	107.19	102.28	106.47
	256	104.30	102.19	104.54
	ii) Var. Gitana yellow			
	0 (Control)	100	100	100
	1	105.65	114.13	114.84
	4	102.68	111.04	113.48
	16	100.8	102.87	103.57
	64	96.48	98.51	102.74
	256	92.47	93.67	101.53

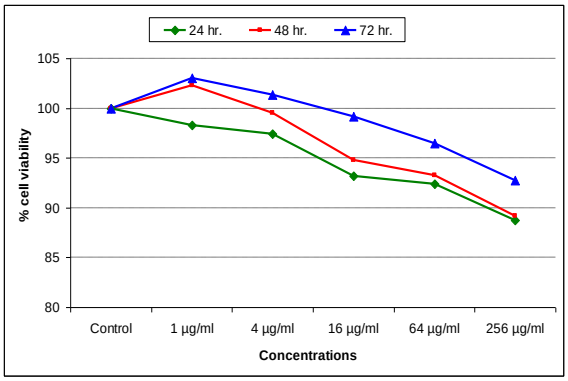

Figure 1: Cytotoxic effect of Cockscomb (*Celosia argentia* L. var *plumose*) against intestinal epithelial cell line IEC-6 by MTT assay

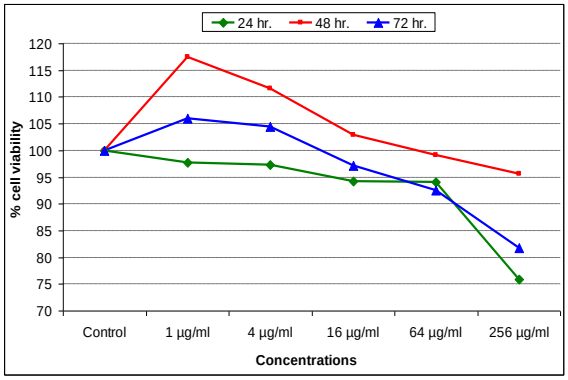

Figure 2: Cytotoxic effect of Himalayan Indigo (*Indigofera heterantha* Wall.) against intestinal epithelial cell line IEC-6 by MTT assay

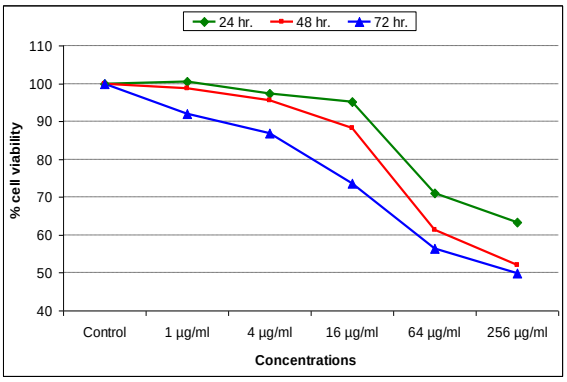

Figure 3: Cytotoxic effect of Indian Madder (*Rubia cordifolia* L.) leaf extract against intestinal epithelial cell line IEC-6 by MTT assay

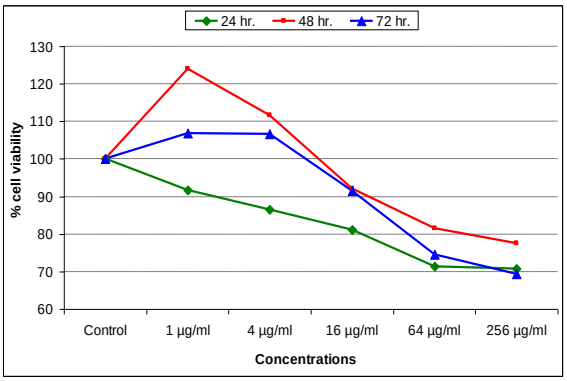

Figure 4: Cytotoxic effect of Indian Madder (*Rubia cordifolia* L.) root extract against intestinal epithelial cell line IEC-6 by MTT assay

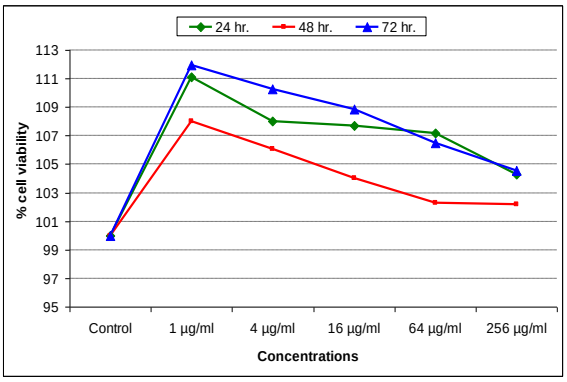

Figure 5: Cytotoxic effect of Pot Marigold (*Calendula officinalis* L. var Gitana orange) against intestinal epithelial cell line IEC-6 by MTT assay

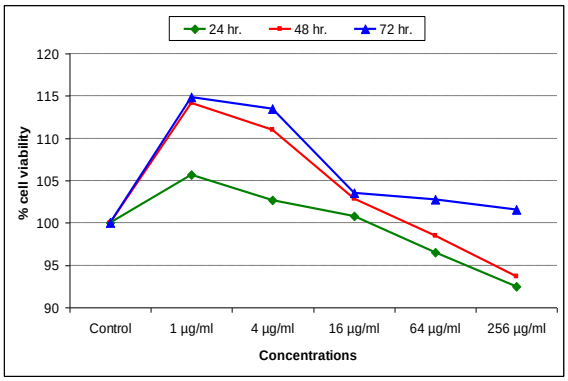

Figure 5: Cytotoxic effect of Pot Marigold (*Calendula officinalis* L. var Gitana yellow) against intestinal epithelial cell line IEC-6 by MTT assay

DISCUSSION

MTT Assay of plant extracts on the Intestinal Epithelial cell line IEC-6 revealed no significant cytotoxic effect at all the time points i.e., 24, 48 and 72 h due to 1, 4, 16 and 64 µg/ml concentrations of *Celosia argentia* L. var plumose and *Indigofera heterantha* Wall. though low to moderate phytotoxicity has been reported [10], yet the seeds of Himalayan Indigo is used in hilly areas for treatment of various diseases like gastrointestinal disorders and abdominal pain [10].

Rubia cordifolia L. is popular all over the world for its medicinal use in skin diseases like eczema, dermatitis, skin ulcers, etc. The plant's roots contain an organic compound called Alizarin, which gives its red colour to a textile. The roots of *Rubia cordifolia* L. are also the source of a medicine used in Ayurveda. The plant is a constituent of many Ayurveda drugs like, Septilin, Rumalaya and Herbinol. Roots are credited with tonic, astringent, antidysenteric, antiseptic and deobstruent properties. They are used in rheumatism and form an ingredient of several Ayurveda preparations. Roots are said to be active against *Staphylococcus aureus* and are made into a paste for application into ulcers, inflammations and skin troubles. A decoction of leaves and stems is used as a vermifuge. In present study no cytotoxicity effect on IEC-6 cell line was recorded due to *Rubia cordifolia* L. leaf and root extract both at all time points due to1ug/ml concentration whereas due to rest concentrations cell death was recorded. Thus both leaf as well as root extract of Indian Madder are toxic in nature. The significant decrease in the wound area due to *Rubia cordifolia* L. in mice has been reported [11]. Further, *Rubia cordifolia* L. root extract has the antimicrobial activity, thus indicating its usage as alternative to the toxic chemicals used in food preservation [12].

Calendula officinalis L. flower extract is used to cure inflammatory and infectious diseases, for wound healing and even cancer due to its therapeutic properties or toxic effects, many of which can be attributed to the presence of flavonols. The tincture from flowers of *Calendula officinalis* L. is used internally for gastritis and menstrual difficulties [13]. *Calendula officinalis* L. var. Gitana Orange and Gitana Yellow did not show any cytotoxic effect on IEC-6 cell line, neither at 24h nor at 48 and 72h. In present study both the varieties showed no significant toxicity effect up to 256 µg/ml reflecting its safety upto this level.

CONCLUSSION

The extracts of the both varieties of *Calendula officinalis* L. var. Gitana Orange and Gitana Yellow extracts are non-toxic in nature, thus can be utilized for the extraction of natural edible dye while as the extracts of *Celosia argentia* L. var *plumose*, *Indigofera heterantha* Wall. and *Rubia cordifolia* L. had potent *in vitro* cytotoxic activity thus they cannot be used for extraction of natural edible food colour. However, to better evaluate the cytotoxic effect of these plant extracts, *in vivo* experiments on laboratory animal followed by histological analysis should be done.

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