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Ameliorative Role of Jambolan against Ocular Toxicities of Anticancer Drug Capecitabine in male rats

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A B S T R A C T

Capecitabine (Xeloda, Roche) is a pro- anticancer drug, that is enzymatically converted to 5-fluorouracil in the body. In the present work, we evaluate the ameliorated role of the Jambolan fruit-extract against the ocular toxicities of the used drug. Forty male Wistar albino rats (*Rattus norvegicus*) weighing approximately 120 g were used. Animals were divided into four groups; control (saline-treated), Jambolan-treatment (400mg/kg, of fruit components), capicitabine-treatment (40 mg /Kg body weight for 30 days) and capicitabine and Jambolan-treatment.Daily oral treatment were carried out for 30 days. At the end of treatment, the animals were sacrificed and their livers and kidney were incised and processed for histopathological investigation as well as flow cytometric analysis for apoptosis. The present findings revealed that the pro-anticancer drug capecitabine-treatment possessed damage of corneal epithelium and deterioration of stramal tissues as well as early pattern of cataractous lenses and retinopathy. Jambolan-treatment showed a marked amelioration of the ocular toxicities. Finally the authors concluded that the patient undergoing chemotherapeutic treatment must consumed fruits and vegetable rich in antioxidants to ameliorate the deleterious of drug toxicities.

Introduction

The wide application of chemotherapeutic agents has resulted in ocular toxicities in patients. Consequently, the adverse ocular side effects secondary to these antineoplastic agents includes a broad spectrum of disorders reflecting the unique anatomical, physiological and biochemical features of the eye (Singh and Singh, 2012). Systemic chemotherapy can lead to a variety of ocular complications, such as cataract, macular edema, retinopathy, and optic neuropathy (Al-Tweigeri *et al.*, 1996; Schmid *et al.*, 2006). Although bulbar perforation with orbital cellulitis has been reported in an immunocompromised patient, corneal perforation has not been documented in patients undergoing systemic chemotherapy (Cavallaro *et al.*, 1999). Anticancer agents such as 5-flurouracil, deoxycoformycin, and

tamoxifen have been known to cause corneal toxicity (Lochhead *et al.,* 2003; Gorin *et al.,* 1998).

Capecitabine (CPC), an oral antineoplastic and immunosuppressive agent, is a prodrug that is converted to 5-fluorouracil (5-FU) in vivo (Roche, Pharmaceuticals: Xeloda. Product information. http://www. rocheusa. com /products/xeloda/, 2005). CPC is most commonly administered as a chemotherapeutic agent in a variety of human cancers (Waikhom *et al.,* 2000). Eye irritation with corneal deposits has been reported with the use of CPC in humans. However, the histopathology of CPC corneal toxicity has not been described (Roche, Pharmaceuticals: Xeloda. Product information. http://www.rocheusa.com/ products/xeloda) (Fraunfelder and Fraunfelder, 2001).

Zarfoss *et al.* (2007) reported superficial keratitis in capecitabine-treated dogs, characterized by multifocal geographic epithelial erosions and rapid, superficial corneal epithelial pigmentation. Over the subsequent weeks, both dogs developed unilateral corneal neovascularization and associated mild corneal edema. The mechanism of action of 5-FU and CPC ocular toxicity is not fully understood. In humans, fluorouracil can be measured in tears after intravenous injection. It has been shown in humans that the effects of CPC vary greatly, an effect that may relate to individual susceptibility, drug dosage, or to enzymatic deficiencies that promote serious adverse systemic effects (Reigner *et al.,* 2001). In both these dogs and in human patients, ocular toxicity of CPC is ameliorated by the discontinuation of the drug (Waikhom *et al.,* 2000). Further study is needed to investigate the presence of CPC in tears, the mechanism of toxicity, the presence/involvement of limbal stem cells, and possible treatments to prevent or ameliorate CPC toxicity.

The present work aimed to illustrate the role of capaicitabine in inducing the histopathological changes in the ocular regions and the role of the jumblan fruits in ameliorating the drastic pathological alterations.

Materials and Methods

Drug and applied dose-treatment

Capecitabine (Xeloda, Roche) is an orally-administered chemotherapeutic agent used in the treatment of numerous cancers. It is a pro-drug, that is enzymatically converted to 5-fluorouracil in the body. The therapeutic dose (40 mg/kg body weight in 0.4 mL saline solution orally administered for one month) of this drug for rat was calculated according to Paget and Barnes (1964). The chosen dose was nearly comparable to the human effective therapeutic dose (ETD). The applied dose emulsified in saline solution and orally administered daily for one month.

Experimental work

Forty Wistar albino rats (*Rattus norvegicus*) weighing approximately 120 g were used in the present study. All rats were kept under good ventilation and aerated room. Excess standard diet was supplied *ad libitum* during the experimental period. They were allowed free access to water. Animals were divided into four groups. The first served as control, the second received Jambolan-treatment (400mg/kg, of fruit components oral doses in saline solution) (n=10). The third group received the capicitabine-treatment. The fourth group received both capicitabine and jambolan fruit-treatment for 30 days.

At the end of treatment, both control and experimental groups were sacrificed by light anesthesia with chloroform. Their eye globes were separated and dissected to separate the retina, lens and cornea and subjected for the following investigations.

Histological investigations

Retina, lens and cornea of both control and experimental groups were incised immediately, fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated in ascending grades of ethyl alcohol, cleared in xylol, nd mounted in molten pararplast 58−62 °C. Serial 5 µm thick sections were cut and stained with Haematoxylin and eosin (H&E), examined under bright field light microscopy, and photographed.

Flow cytometric analysis of cell cycle apoptosis

DNA ploidy and apoptosis were analyzed using fluorescence activated cell sorting (FACS) flow cytometer (Becton Dickinson, Sunnyvale, CA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. FL1 (FITC) signals were detected through a 530/30 nm band-pass filter; FL2 (PI) signals were detected through a 585/42 nm band-pass filter. A total of 20 000 events were recorded in list mode and analyzed using the Cell Quest Pro software (Becton Dickinson) at Mansoura University Hospital. The cell populations were gated assuming the linear forward scatter (FSC) and side scatter (SSC) properties. Biopsies from retina of studied animals were taken, and cell suspension was prepared with Tris-EDTA buffer (pH 74) (Sigma-Aldrich Co.). Cell suspension was fixed in ice-cold 96−100% ethanol (Sigma) at 4 °C overnight, centrifuged at 1500 rpm for 10 min, and then resuspended in PBS containing 50 µg/mL propidium iodide (PI) (Sigma-Aldrich Co.). The cells were incubated at 37 °C for 30 min before analysis by flow cytometry. PI fluorescence excitation at 512 nm, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Apoptosis was indicated by the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle.

Results

Cornea

At light microscopic level, both control and Jambolan-treatment showed normal arrangement of five layers of the cornea. The stratified squamous non keratinized layer appeared with its basal layer formed of columnar cells, intermediate layers formed of polygonal cells and superficial layers formed of squamous cells. The corneal epithelium appeared resting on a uniform basement membrane underneath it was the Bowman's layer. The corneal stroma consists of regularly organized collagen fibrils parrallel to each other. Flattened fibrocytes (keratocytes) are located between the layers of collagen fibres. The regularity of the collagen fibres are acount for the transparency of the cornea. Descemet’s membrane appeared beneath the stroma and it was covered by Descemet’s endothelium (Fig.1 A).

In experimental group-treated with the anticancer drug capecitabine, there is a detected abnormal corneal surface with characteristic irregular evaginated bleb structure. The corneal epithelia become severely desquamated with almost remaining one basal epithelial cells and degenerated superficial layer with abundant cytoplasmic vacuoles. Basal cells contained vacuolated cytoplasm, indicating edematous change. Many of the epithelial cells with either vesicular vacuolar degenerated nuclei

or pyknotic cell death. The anterior region of stroma showed degenerated corneal stroma associated with leukocytic infiltration. (Fig.1 A).

In experimental group-treated with the anticancer drug capecitabine and received phytotherapy -treatment with jubolan, there is a marked amelioration of the corneal epithelium with missing of the previous detected pathological alteration. The stroma appears of normal architecture structure (Fig.1A2).

Lens

In control Jambolan-treatment, the cornea consists of a lens capsule, the subcapsular epithelium and lens fibres. The lens capsule is a thick, elastic basal lamina. Underneath the epithelial cells arranged in one cell layer thick underlying in a basal lamina. The lens fibres appeared normally perpendicularly oriented (Fig.1B).

In capecitabine-treated group, there was a detected thinning of lens capsule and deterioration of lens epithelium. The underlying lens epithelium become degenerated and formed a honey-comb pattern structure suspected to be early phase of post-capsular cataract (Fig.1B1).

In anticancer drugs treated with Jambolan, there was a marked amelioration of the lens structure including lens capsule and epithelium, however the previously detected necrotic spots in anticancer-treament, become invaded by sparse distribution of inflammatory cells suspected to regenerate the lens fiber (Fig. 1B2).

Retina

Histologically, the retina is divided into 10 cell layers from inner to outer: pigmented epithelium, rod and cone processes of the photoreceptor cells, outer limiting membrane, outer nuclear cell layer, outer plexiform layer, inner nuclear cells, inner plexiform layer, ganglion cell layer, afferent fibres layer, and inner limiting membrane. In control, the retina is composed of normarly oriented cell layers. The ganglion cells arranged in one to two cell-layered thick; the outer nuclear layer attained a considerably thickening. The photoreceptors were clearly differentiated. The pigmented epithelial layer was regularly arranged as a single layer of cells with a prominent basal lamina (Fig. 1C).

In anticancer-treated group, there was a considerable reduction of retinal layers associated with invasion of blood vessels. The nerve fibres and ganglion cells were markedly deteriorated and showed apparent thinning. There was a detected deterioration of the outer plexiform layer and massive reduced thickness of the inner one with characteristic spongiform pattern structure. There was a marked decrease of neural cell densities of both inner and outer nuclear layers. Photoreceptors showed marked fragility and swollen (Fig. 1C1).

In anticancer-treated group received Jambolan-treatment, there was a considerable amelioration of the retinal picture except mild change in the photoreceptor layer (Fig, 1 C2).

Flow cytometry of retinal cell cycle

From table (1), and Fig. 4, there was a considerable increase of M1 (subG1 apoptsis) in capecitacin-treated retina and a decrease in the other cell cycle phases (M2, M3, and M4) comparing with the control and jambolan-treatment. On the other hand, capicitacin intoxication and jambolan-treatment exhibited marked amelioration by decrease the incidence of apoptosis of M1.

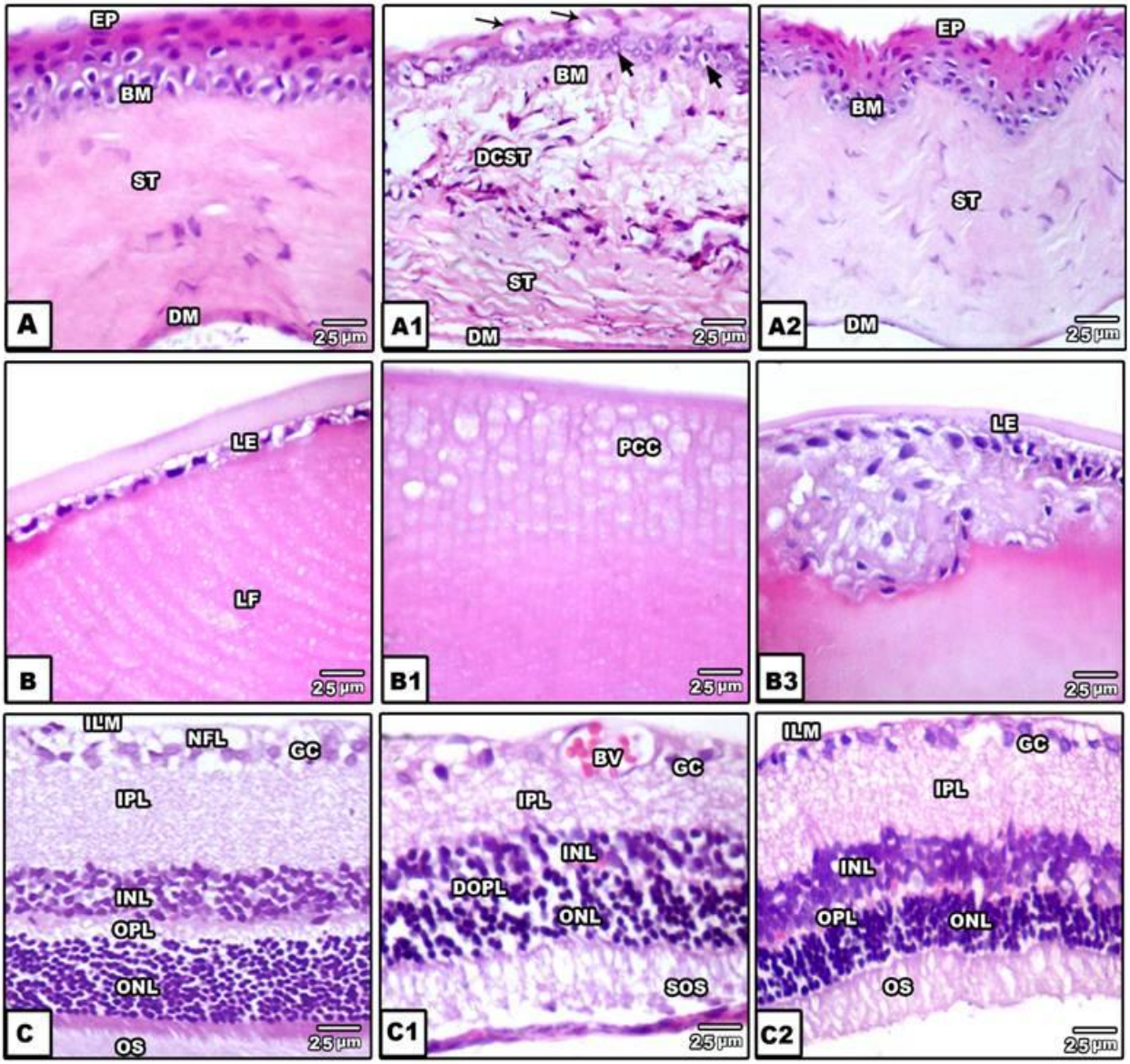


Fig.1(A-C2) Photomicrographs of cornea (A-A2),lens (B-B2) and retina (C-C2).A. Control cornea showing normal epithelium, stroma and endothelium; A1. Capecitabine-treatment showing degenerated epithelium and stroma; A2. Combined Capecitabine and Jambolan-treatment showing improement of histologic picture; B. Normal lens; B1. Capecitabine-treatment showing degeneration of lens fibre forming primary post-capsular cataract; B2. Combined Capecitabine and Jambolan-treatment showing newly formed lens epithelium cells a sign of recovery; C. normal retina; C1. Capecitabine-treatment showing neovascularization, rduction of nuclear cells and fragility of photoreceptors; C2. Combined Capecitabine and Jambolan-treatment showing amelioration. HX-E.

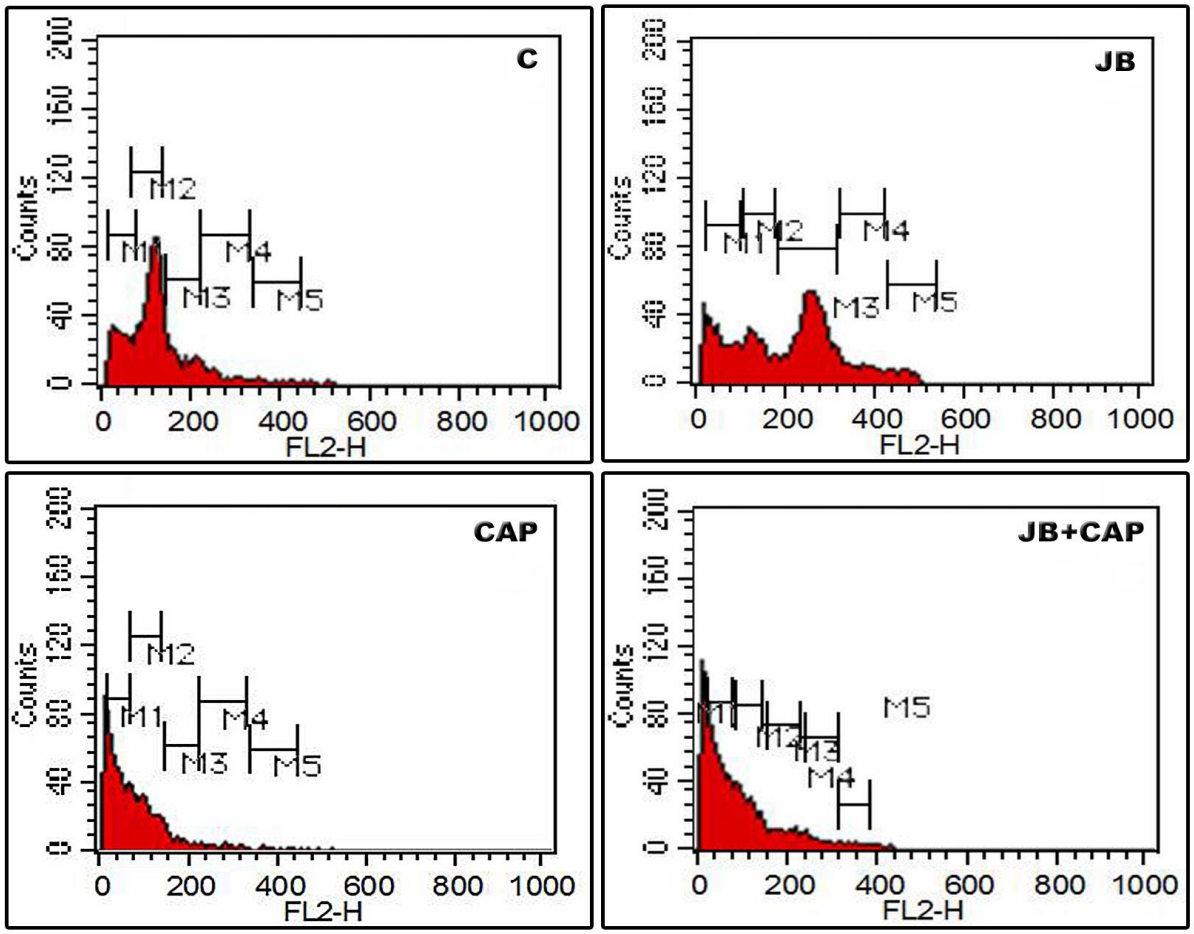
Abbreviations; BM, Basemen membrane,BV, Blood vessel, DCST, degenerated connective stromal tissue, DM, Descement membrane,DOPL, Degenerated outer plexiform layer, EP, Epidermis, GC, Ganglion cell, ILM, Inner limiting membrane, INL, inner nuclear layer, IPL, Inner plexiform layer, LE, Lenticular epithelium, LF, Lens fibre, NFL, Nerve fibre layer, ONL, Outer nuclear layer, OPL, Outer plexiform layer, OS, Outer segment,SOS, Swelling outer segment, PCC,Post-capsular cataract

Table.1 Flowcytometry of cell cycle of retina of capecitabine intoxicated rat and treated with Jambolan

	Control	CAP-Treatment	JB.-treatment	CAP.& JB-treatment
M_1 (sub-G0/G1 apoptosis)	12.6±2.7	64.9±4.6*	19.5±1.7**	28.8±2.3*
M_2 (G0/1 phase)	31.0±1.8	26.8±2.1**	41.5±3.5*	25.4±2.3**
M_3 (S phase)	7.9±0.6	4.77±0.2**	30.3±2.3*	18.2±1.7*
M_4 (G2/M phase)	3.6±0.1	2.8±0.4**	14.9±1.7*	20.8±1.6*

Each replicate represent the M±SE. n=5. Significance at P 0.05.CAP, capecitabine, JB, Jambolan.

Fig.2 Flowcytometry of rettinal cells. C&JB. Control and Jambolan-treatment showing normal range of cell cycle. CAP. Capecitabine-treatment showing increased average of apoptosis. CAP&JB. Combined Capecitabine and Jambolan-treatment showing reduced cell damage



Discussion

The corneal epithelium represents the periphery contact corneal region which is characterized by their dynamic turnover due to the sustained proliferation of basal epithelial cells. These basal cells are then displaced outward by the next generation of mitotic cells, and eventually they are lost by desquamation (Pajoohesh-Ganji and Stepp, 2005, Pellegrini *et al.*, 2009). The mechanisms that regulate corneal proliferation under normal physiological conditions and in disease states are multifactorial and complex.

These findings revealed that the anticancer drug capecitabine-treatment led to abnormal corneal surface with characteristic irregular evaginated bleb structure. The corneal epithelia become severely desquamated with almost remaining vacuolar and degenerated and abundant epithelium with pyknotic nuclei. The anterior region of stroma showed marked degeneration with leukocytic infiltration.

Similar findings of corneal damage was induced by N-ethyl-Nnitrosourea (ENU) in mice treated with 600 mg/kg group for 7 days (Yoshizawa *et al.*, 2011).

Also, the applied drug-treatment led to marked deterioration of lens epithelium. The underlying lens epithelium become degenerated and formed a honey-comb pattern structure suspected to be early phase of post-capsular cataract.

Similar studies have been reported with other different inducers. Chemically induced cataract formation in animals has been well documented (Tsubura *et al.*, 2005). Cataracts can be rapidly induced in premature rats with the alkylating chemical *N*-methyl-*N*nitrosourea (MNU) (Miki and Tsubura, 2000). When a single *i.p.* injection of 100 mg/kg MNU was given to 0-, 5-, 10-, and 15-day-old male and female Sprague-Dawley rats, gross lens opacity was recognized 7 days later in 0-day-old MNU-treated rats, but not in the suspected developmental ages.

In addition, capecitabine-treatment led to neovascularization, comparative reduction of retinal neuronals in the ganglion and nuclear layers. There was a detected deterioration of the outer plexiform layer and massive reduced thickness of the inner one with characteristic spongiform pattern structure. The photoreceptors showed marked fragility and swollen.

Retinal dysplasia is induced when neonatal rats or mice receive more than 3 mg/kg of cisplatin (Yang *et al.*, 2000). Vigabatrin, gabapentin, sildenafil, tamoxifen, isotretinoin, interferon and omeprazole were found to induce retinopathy especially of the retinal pigmented epithelium (Nencini *et al.*, 2008).

The observed ocular toxicities post-drug treated was confirmed by flowcytometric analysis which reveals a considerable increase of M1 (subG1 apoptosis) in capecitacin-treated retina. Alkylating agents were found to induce DNA adduct formation of target cells, followed by cell death or gene mutation (Doak *et al.*, 2007). *N*-Methyl-*N*-nitrosourea (MNU), an alkylating agent was recorded to cause DNA adduct formation in photoreceptor nuclei, followed by down-regulation of Bcl-2, up-regulation of Bax, and activation of caspase families, followed by retinal degeneration (Yoshizawa and Tsubura, 2005).

On the other hand Jambolan-treament of rat subjected to the anticancer-treatment was found to ameliorate the corneal, lens and retinal damage as well as resulted the degree of apoptic cell death. The amelioration of

the ocular disease as a result of jambolan fruits-extract treatment attributed to the antioxidant capacity including alpha-glucosidase and alpha-amylase inhibitory activities as well as Cyanidin, quercetin, ellagic acid (EA), proanthocyanidins, phenolic content catechin and also high-ascorbic acid (Correia *et al.*, 2012).

The in vitro ability of *Pothomorphe umbellata* ethanolic crude extract to inhibit matrix metalloproteinase (MMP) in normal cornea and in cornea after alkali injury (Barros *et al.*,2005). *Centella asiatica* is a traditional herbal medicine was found to show significant enhancement of migration rate corneal epithelial wound healing in in vitro (Ruszymah *et al.*, 2012). Similar findings of ameliorated cataractous lenses by doxorubicin carried out by feeding rats on 5 g/day hazelnut, a maturally occuring antoxidant (Bayer *et al.*, 2005). El-Sayyad *et al.* (2011) reported amelioration of cataractous lenses and retinopathy induced by diabetes and hypercholesterolemia by administration of *Morus alba* leaves extract.

Finally the authors concluded that the patient undergoing chemotherapeutic treatment must consumed fruits and vegetable rich in antioxidants to ameliorate the deleterious of drug toxicities.

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