Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Flavonoids Derived from *Abelmoschus esculentus* Attenuates UV-B Induced Cell Damage in Human Dermal Fibroblasts Through Nrf2-ARE Pathway

Juilee Patwardhan, Purvi Bhatt

Department of Biological Sciences, Sunandan Divatia School of Science, NMIMS (Deemed-to-be) University, Mumbai, Maharashtra, India

Submitted: 27-11-2015 Revised: 01-02-2016 Published: 11-05-2016

ABSTRACT

Background: Ultraviolet-B (UV-B) radiation is a smaller fraction of the total radiation reaching the Earth but leads to extensive damage to the deoxyribonucleic acid (DNA) and other biomolecules through formation of free radicals altering redox homeostasis of the cell. *Abelmoschus esculentus* (okra) has been known in Ayurveda as antidiabetic, hypolipidemic, demulscent, antispasmodic, diuretic, purgative, etc. **Objective:** The aim of this study is to evaluate the protective effect of flavonoids from *A. esculentus* against UV-B-induced cell damage in human dermal fibroblasts. **Materials and Methods:** UV-B protective activity of ethyl acetate (EA) fraction of okra was studied against UV-B-induced cytotoxicity, antioxidant regulation, oxidative DNA damage, intracellular reactive oxygen species (ROS) generation, apoptotic morphological changes, and regulation of heme oxygenase-1 (HO-1) gene through nuclear factor E2-related factor 2-antioxidant response element (Nrf2-ARE) pathway. **Results:** Flavonoid-rich EA fraction depicted a significant antioxidant potential also showing presence of rutin. Pretreatment of cells with EA fraction (10–30 μg/ml) prevented UV-B-induced cytotoxicity, depletion of endogenous enzymatic antioxidants, oxidative DNA damage, intracellular ROS production, apoptotic changes, and overexpression of Nrf2 and HO-1. **Conclusion:** Our study demonstrated for the 1st time that EA fraction of okra may reduce oxidative stress through Nrf2-ARE pathway as well as through endogenous enzymatic antioxidant system. These results suggested that flavonoids from okra may be considered as potential UV-B protective agents and may also be formulated into herbal sunscreen for topical application.

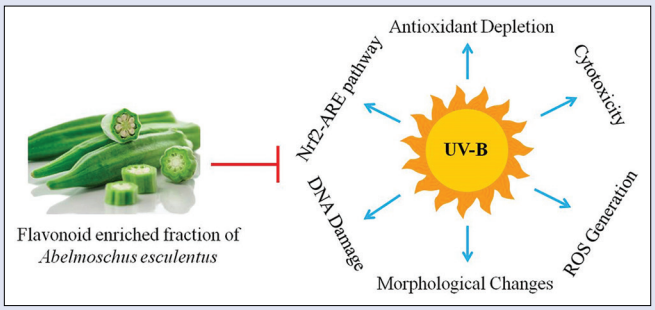
Key words: *Abelmoschus esculentus*, antioxidants, human dermal fibroblasts, Nrf2-ARE Pathway, oxidative stress

SUMMARY

- Flavonoid-enriched ethyl acetate (EA) fraction from *A. esculentus* protected against ultraviolet-B (UV-B)-induced oxidative DNA damage
- EA fraction prevented UV-B-induced cytotoxicity, depletion of endogenous enzymatic antioxidants, and intracellular reactive oxygen species production
- EA fraction could reduce oxidative stress through the Nrf2-ARE Pathway
- EA fraction was found to be nongenotoxic and prevented apoptotic changes.

HIGHLIGHTS

- Flavonoids from *Abelmoschus esculentus* protected from ultraviolet-B-induced damage
- They were capable of reducing oxidative stress through Nrf2-ARE Pathway
- They are nongenotoxic and do not possess mutagenic potential
- Flavonoids from *A. esculentus* can be studied and explored further for its topical application as sunscreen.



Abbreviations used: ABTS: 2,2′-azino-bis-(3-ethylbenzothiazoline -6-sulphonic acid), AO: Acridine orange, ANOVA: Analysis of variance, ARE: Antioxidant response elements, BSA: Bovine serum albumin, CAPE: Caffeic acid phenethyl ester, CAT: Catalase, DCFH-DA: 2′,7′-dichlorofluorescein diacetate, DMEM: Dulbecco's modified eagle's medium, DMSO: dimethyl sulfoxide, DNA: Deoxyribonucleic acid, DPBS: Dulbecco's phosphate-buffered saline, DPPH: 2,2-diphenyl-1-picryl hydrazyl, ECL: Enhanced chemiluminescence, EDTA: Ethylenediaminetetraacetic acid, ELISA: Enzyme-linked immunosorbent assay, EtBr: Ethidium bromide, FBS: Fetal bovine serum, FE Fraction: Flavonoid-enriched fraction, FRAP: Ferric reducing antioxidant power, GPx: Glutathione peroxidase, GR: Glutathione reductase, GST: Glutathione-S-transferase, GSH: Reduced glutathione, GSSG: Oxidized glutathione, HDF: Human dermal fibroblast adult cells, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, HRP: Horseradish peroxidase, HO-1: Heme oxygenase-1, HPTLC: High-performance thin layer chromatography, Keap-1: Kelch-like ECH-associated protein-1, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NaCl: sodium chloride, NFDM: nonfat dry milk, Nrf2: Nuclear factor E2-related factor 2, NQO1: NAD (P) H: Quinine oxidoreductase 1, OH: Hydroxyl ions, PBST: Phosphate-buffered saline with 0.1% tween 20, PCR: Polymerase chain reaction, PMSF: Phenylmethanesulfonyl fluoride, Rf: Retention factor, ROS: Reactive oxygen species, rRNA: Ribosomal ribonucleic acid, SDS: Sodium dodecyl sulfate, SOD: Superoxide dismutase, TLC: Thin layer chromatography, TLC-DPPH: Thin layer chromatography-2,2-diphenyl-1-picryl hydrazyl, UV: Ultraviolet, UV-A: Ultraviolet-A, UV-B: Ultraviolet-B, UV-C: Ultraviolet-C, qPCR: Quantitative polymerase chain reaction

Correspondence:

Dr. Purvi Bhatt,
Department of Biological Sciences,
Sunandan Divatia School of Science,
NMIMS (Deemed-to-be) University, Vile Parle (W),
Mumbai - 400 056, Maharashtra, India.
E-mail: purvi.bhatt@nmims.edu
DOI: 10.4103/0973-1296.182175

Access this article online
Website: www.phcog.com
Quick Response Code:

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Patwardhan J, Bhatt P. Flavonoids derived from *Abelmoschus esculentus* attenuates UV-B Induced cell damage in human dermal fibroblasts through Nrf2-ARE pathway. Phcog Mag 2016;12:S129-38.

INTRODUCTION

Ultraviolet (UV) radiation is a major environmental factor affecting human body in various ways and is divided into three categories such as UV-C (100–280 nm), UV-B (280–315 nm), and UV-A (315–400 nm). UV-B is the smaller fraction of the total radiation reaching the Earth but is most harmful.[1,2] UV-C is totally absorbed by the ozone layer. UV-B radiations penetrate the epidermal and dermal layer of the skin causing sunburn, formation of free radicals, disarrangement of collagen and elastin fibers, immune modulation, etc., UV-B is also genotoxic forming single-strand breaks, thymine dimers, photoproducts, etc., increasing chances of mutations. It reacts with cellular chromophores and photosensitizers forming free radicals, which affect biomolecules such as lipids, proteins, and deoxyribonucleic acid (DNA).[3,4]

UV-B radiations lead to oxidative stress, which alters the redox homeostasis of the cell. Antioxidants are necessary to scavenge the free radicals and further reduce oxidative stress. Endogenous enzymatic and nonenzymatic antioxidants are depleted during oxidative stress, hence provide lesser protection than required. Therefore, exogenous antioxidants as supplements are necessary. Sunscreens and synthetic antioxidants are available but have some limitations on continual use. Hence, natural antioxidants are now studied and developed to be used for protection from oxidative stress.[3,5]

Human body also has various antioxidant defense systems, which are sensitive to oxidative stress, one of which is nuclear factor E2-related factor 2-antioxidant response elements (Nrf2-ARE) pathway. Transcription factor Nrf2 is bound to its inhibitor protein Keap-1, which ensures constant ubiquitination of Nrf2 under normal conditions. During oxidative stress, Nrf2 dissociates from Keap-1 and translocates into the nucleus where it binds to ARE upregulating the downstream antioxidant enzyme and phase II detoxification enzyme genes.[6-8]

Plant polyphenols and flavonoid fractions are studied as natural antioxidants and also as UV-B protectants. They are also studied for topical application to the area of skin requiring protection as oral supplementation of natural antioxidants limits the amount reaching the skin due to biochemical processes.[4] *Abelmoschus esculentus* (Okra) possess a good antioxidant activity and high polyphenolic content[9] and is known in Ayurveda as an antidiabetic,[10] hypolipidemic,[11] demulscent, antispasmodic, diuretic, purgative, etc. Okra is rich in fibers (67.5% - cellulose, 15.4% - hemicellulose, 7.1% - lignin, and 3.4% - pectic matter). Flavonoids such as quercetin and rutin are reported to be present in okra fruit.[12]

UV-B protective activity of polyphenolic compounds such as silymarin,[13] sesamol,[3] ferulic acid,[14] ursolic acid,[15] and epicatechin gallate[16] has been studied extensively. We have also studied the UV-B protective effect of flavonoids from *Eugenia caryophylata*. In the current study, UV-B protective potential of flavonoids from okra was evaluated by studying UV-B-induced cytotoxicity, intracellular reactive oxygen species (ROS) levels, endogenous enzymatic antioxidant levels, DNA damage, apoptotic changes, and heme oxygenase-1 (HO-1) regulation through Nrf2-ARE pathway.

MATERIALS AND METHODS

Chemicals

Human dermal fibroblast adult cells (HDF) (ATCC no. PCS-201-012) from Scientific Research Centre, V.G. Vaze College, Mumbai; Silica Gel 60 F_{254} precoated plates, agarose, goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody from Merck (NJ, USA); Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin-streptomycin, Dulbecco's phosphate-buffered saline (DPBS), trypsin-Ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) from Genetix Biotech (New Delhi, India); 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye, monoclonal antibodies against Nrf2 and HO-1 from Abcam (MA, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Himedia Labs (Mumbai, India); ethidium bromide (EtBr) and acridine orange (AO) from SRL (Mumbai, India); natural product reagent, 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), monoclonal antibody against β-actin, TRI reagent, silymarin from Sigma (St. Louis, MO, USA); cDNA synthesis kit from TAKARA (Shiga, Japan); primers for Nrf2 and HO-1 from Eurofins (Luxembourg, Germany); SYBR green real-time polymerase chain reaction (PCR) master mix from Roche (BASEL, Switzerland); enhanced chemiluminescence (ECL) substrate from Biorad (Berkeley, California); X-ray films from Kodak (NY, USA); and Protease inhibitor cocktail from Amresco (OH, USA). All other chemicals, solvents, and reagents were of analytical grade from S.D. Fine Chemicals (Mumbai, India) and Fisher Inorganic and Aromatic Limited (Mumbai, India).

Preparation of extracts and enrichment of flavonoids

Cold extraction technique using n-hexane, chloroform, and alcohol successively was done to prepare crude extracts.[17] Preliminary analysis depicted the presence of flavonoids in the crude alcoholic extract; hence, it was further used for enrichment of flavonoids.

Crude alcoholic extract (10 g) was dissolved in distilled water (100 ml) and fractioned thrice using equal volume of ethyl acetate (EA).[18] The EA fractions were concentrations and stored in vaccum until further use.

TLC, TLC-DPPH and HPTLC analysis

Detection of flavonoids from the EA fraction, water fraction, and crude alcoholic extract was performed by thin layer chromatography (TLC), TLC-DPPH (TLC-DPPH), and high-performance TLC (HPTLC) analysis using rutin as a standard. Samples were spotted on Silica Gel 60 F_{254} precoated plates and the analysis was carried out using the standardized solvent system of EA:formic acid:glacial acetic acid:methanol (7.5:0.15:0.15:0.9). Plates were derivatized using 1% natural product reagent and observed under UV light at 366 nm.[17-19] 0.1% DPPH was also used as a derivatizing reagent to detect antioxidant potential qualitatively.[20] EA fraction of okra was subjected to HPTLC analysis using DESAGA HPTLC system. The chromatograms were scanned, and the spectra and retention factor (Rf) were recorded using ProQuant software (Informer Technologies, Inc.).

Flavonoid content and antioxidant activity

Flavonoid content of the crude alcoholic extract and EA fraction was determined quantitatively by a previously described method[21] using quercetin as a standard in the concentration range of 10–100 μg/ml. The antioxidant potential of both the crude alcoholic extract and EA fraction were determined by DPPH, ferric reducing antioxidant power, and ABTS assays by previously described methods.[22-24]

EA fraction was further studied in human dermal fibroblast cells.

Cell culture

HDF cells (ATCC No. PCS-201-012) were grown in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 0.1 mg/ml of streptomycin, and 2.5 μg/ml of amphotericin at 37°C, 5% CO_2.

Treatment groups

HDF cells were divided into seven treatment groups for further studies:

Group 1 – Control.
Group 2 – EA fraction treated cells (30 μg/ml).
Group 3 – UV-B-irradiated.
Group 4 – UV-B-irradiated + 5 μg/ml silymarin.
Group 5 – UV-B-irradiated + 10 μg/ml EA fraction.
Group 6 – UV-B-irradiated + 20 μg/ml EA fraction.
Group 7 – UV-B-irradiated + 30 μg/ml EA fraction.

Treatment of human dermal fibroblast adult cells

Cultured HDF cells were treated with EA fraction (10 μg/ml, 20 μg/ml, and 30 μg/ml) and silymarin (5 μg/ml) for 24 h followed by washing the cells with DPBS, covering cells in minimum amount of DMEM and UV-B irradiation. Preliminary cytotoxicity studies were performed by MTT assay to confirm whether these concentrations were nontoxic.

Irradiation procedure

After fraction and silymarin treatment, HDF were UV-B irradiated by a UV-B tube (Sankyo Denki, Japan) with a wavelength range of 280–315 nm and peak at 312 nm. The cells were irradiated at an intensity of 5 mW/cm^2 for 500 s with total UV-B radiation of 2.5 J/cm^2 reducing viability to 50%. After UV-B exposure, cells were incubated at room temperature for 30 min and processed for further experiments.

MTT assay

Cultured HDF cells (5×10^4) were seeded in 96-well plate and treated with EA fraction and silymarin. After 24 h, cells were UV-B-irradiated and incubated for 30 min at room temperature. MTT (5 mg/ml) was added to the cells and incubated at 37°C for 4 h followed by addition of 150 μl dimethyl sulfoxide and measurement of absorbance on an ELISA reader at 570 nm.[25]

Estimation of endogenous antioxidants

Cell lysis buffer containing 50 mM Tris-Cl, 5 mM EDTA, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, 50 mM HEPES, 1 mM PMSF, and 1X protease inhibitor cocktail was used for preparation of cell lysates followed by estimation of proteins using Folin Lowry method. Levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were assayed by previously described methods.[26-29]

Comet assay

HDF cells (1×10^6) were seeded in 6-well plate and then treated with EA fraction and silymarin followed by UV-B irradiation. Comet assay was performed by previously described method.[3] 100 comets of each treatment group were observed at 400× magnification under fluorescent microscope. The comets were analyzed using Casp software version 2.0 (CaspLab.com) and percent head DNA were calculated followed by statistical analysis.

Ethidium bromide/acridine orange staining

After treatment of cultured HDF with EA fraction and UV-B, they were stained using 1:20 diluted mixture of EtBr and AO (100 μg/ml each) and observed at ×400 magnification under a fluorescent microscope.

Quantitation of intracellular reactive oxygen species

After treatment of cultured HDF with EA fraction and UV-B, they were washed and resuspended in DPBS followed by addition of 10 μl DCFH-DA (1 μM) and incubation at 37°C for 45 min. ROS positive cells were measured using flow cytometer at 488 nm laser wavelength and 535 nm detection wavelength.

Ames test

Salmonella typhimurium TA100 was used to determine the mutagenic potential of EA fraction of okra. The strain identification tests (histidine requirement, Rfa mutation (changes bacterial cell wall properties), UVrB mutation (DNA repair), and R-factor assay) were performed to confirm the genotype followed by Ames test which was performed as previously described.[30,31]

qPCR analysis for Nrf2 and HO-1

Expression of Nrf2 and HO-1 was determined by Quantitative PCR (qPCR) analysis using 18S ribosomal ribonucleic acid (rRNA) as internal control. Total RNA extraction was done by TRI reagent followed by cDNA synthesis. SYBR® green real-time PCR master mix was used for the analysis and the primer sequences and standardized reaction conditions for Nrf2, HO-1, and 18S rRNA are given in Tables 1 and 2. Expression of Nrf2 and HO-1 was represented as fold change as compared to expression in UV-B irradiated cells.

Western blot for Nrf2 and HO-1

Cell lysates were prepared and quantified as mentioned above and then electrophoresed and transferred onto nitrocellulose membrane. In case of Nrf2, 5% bovine serum albumin (BSA) was used for blocking the membrane followed by probing with 1:1000 diluted monoclonal Nrf2 antibody and 1:2000 diluted goat anti-mouse HRP-conjugated secondary antibody. Detection was done by ECL and chemiluminescence was recorded on X-ray film. Membrane was stripped using stripping buffer (10% SDS, 0.5M Tris-Cl, β-mercaptoethanol) at 55°C for 30 min and washed 5 times using phosphate-buffered saline. For HO-1, 5% BSA was used for blocking the membrane followed by probing with 1:500 diluted monoclonal HO-1 antibody and 1:2000 diluted goat anti-mouse HRP-conjugated secondary antibody followed by detection. Membrane was restripped and probed for β-actin using 5% nonfat dry milk for blocking, 1:1000 diluted monoclonal β-actin antibody followed by the above given procedure.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software by one-way ANOVA followed by Dunnett's and Tukey's posttest, $^{***}P < 0.001$. Results are expressed as mean ± SD ($n = 3$).

RESULTS

TLC, TLC-DPPH and HPTLC analysis

Flavonoids from EA fraction, water fraction, and crude alcoholic extract of okra were separated by TLC using rutin as the standard. In Figure 1a and b, lanes 1–3 are rutin, crude alcoholic extract, and EA fraction, respectively. Rutin was found to be present in crude alcoholic

Table 1: Primer sequences and product sizes of nuclear factor E2-related factor 2, heme oxygenase-1 and 18S ribosomal ribonucleic acid

Gene	Primer sequence	Product size (bp)
Nrf2	FP: 5'-GGCTACGTTTCAGTCACTTG-3'	180
	RP: 5'-AACTCAGGAATGGATAATAG-3'	
HO-1	FP: 5'-GAGGAGTTGCAGGAGCTGCT-3'	180
	RP: 5'-GAGTGTAAGGACCCATCGGA-3'	
18S rRNA	FP: 5'-GAGTGTAAGGACCCATCGGA-3'	171
	RP: 5'-CCTCCAATGGATCCTCGTTA-3'	

Nrf2: Nuclear factor E2-related factor 2; HO-1: Heme oxygenase-1; rRNA: Ribosomal ribonucleic acid

Table 2: Standardized cycling conditions for nuclear factor E2-related factor 2, heme oxygenase-1 and 18S ribosomal ribonucleic acid

	Nrf2		HO-1		18S rRNA	
Initial denaturation	94°C, 5 min		94°C, 3 min		94°C, 3 min	
Denaturation	94°C, 20 s	35 cycles	94°C, 20 s	40 cycles	94°C, 30 s	30 cycles
Annealing	57°C, 20 s		70°C, 20 s		60°C, 30 s	
Extension	72°C, 20 s		72°C, 20 s		72°C, 30 s	
Final extension	72°C, 7 min		72°C, 7 min		72°C, 5 min	

Nrf2: Nuclear factor E2-related factor 2; HO-1: Heme oxygenase-1; rRNA: Ribosomal ribonucleic acid

extract as well as EA fraction. All the flavonoid bands seen in the crude alcoholic extract were also present in the EA fraction suggesting successful separation. Water fraction showed the presence of blue colored bands which may be duo to some impurities. Flavonoid bands in the fraction were more intense than that in crude alcoholic extract indicating enrichment. TLC-DPPH analysis was done to detect the antioxidant potential qualitatively. The yellow coloration in the EA fraction was more intense than the crude alcoholic extract indicating increased antioxidant potential. Figure 1c and d depicts HPTLC profile and HPTLC spectra of the EA fraction. Four peaks were detected in the spectra and their area, and area (%) and Rf values are given in Table 3. Of the four peaks detected, peak two was confirmed to be rutin.

Flavonoid content and antioxidant activity

Flavonoid content and antioxidant potential of the crude alcoholic extract and EA fraction were determined [Table 4]. EA fraction showed higher flavonoid content and increased antioxidant potential than the crude alcoholic extract confirming successful enrichment of flavonoids. The increased antioxidant potential may be due to higher flavonoid content in EA fraction.

As EA fraction depicted higher flavonoid content and better antioxidant potential than crude alcoholic extract, it was further studied for its UV-B protective potential in human dermal fibroblast cells.

MTT assay

UV-B radiation reduced the cell viability significantly to 50% which was retained by the pretreatment of HDF with EA fraction of okra [Figure 2]. The protective effect against UV-B-induced cell damage and injury was observed to be significant (***$P < 0.001$) in a concentration-dependent manner (10–30 µg/ml).

Estimation of endogenous antioxidants

Levels of the important endogenous enzymatic antioxidants SOD, CAT, GPx, and GR were observed to be significantly reduced which may be due to UV-B-induced excessive generation of free radicals [Figure 3]. EA fraction pretreatment significantly (***$P < 0.001$) retained the levels of all the four enzymes in a concentration-dependent manner (10–30 µg/ml).

Comet assay

Evaluation of protective effect of EA fraction against UV-B-induced DNA damage was done by comet assay [Figure 4]. UV-B radiations induce single-strand breaks in the DNA which resolve out of the cell as fragmented DNA leading to formation of comets and reduction in the percent head DNA of the cell [Figure 4c]. After pretreatment with EA fraction of okra, formation of comets reduced significantly (***$P < 0.001$) also increasing the percent head DNA of the cells indicating protection from UV-B-induced DNA damage. EA fraction treated cells showed absence of comets suggesting that the EA fraction is nongenotoxic.

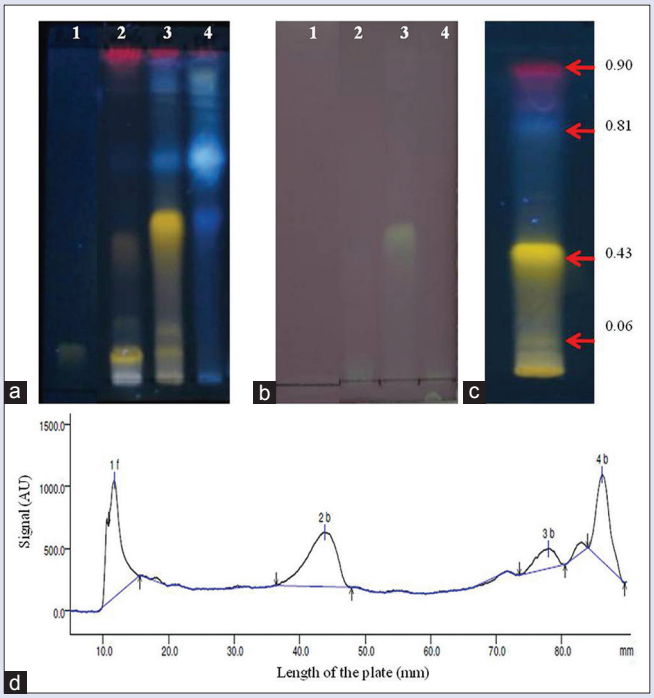


Figure 1: Thin layer chromatography, thin layer chromatography-2,2-diphenyl-1-picryl hydrazyl and high-performance thin layer chromatography analysis. (a) Comparative thin layer chromatography profile of rutin, crude alcoholic extract and ethyl acetate fraction after derivatization with 1% natural product reagent under ultraviolet light at 366 nm, lane 1 - rutin, lane 2 - crude alcoholic extract, lane 3 - ethyl acetate fraction, lane 4 - water fraction; (b) comparative thin layer chromatography-2,2-diphenyl-1-picryl hydrazyl profile of rutin, crude alcoholic extract and ethyl acetate fraction after derivatizing with 0.1% 2,2-diphenyl-1-picryl hydrazyl under visible light, lane 1 - rutin, lane 2 - crude alcoholic extract, lane 3 - ethyl acetate fraction, lane 4 - water fraction; (c) high-performance thin layer chromatography profile of ethyl acetate fraction; (d) high-performance thin layer chromatography densitogram of ethyl acetate fraction at 420 nm

Ethidium bromide/acridine orange staining

UV-B radiations induced loss in cell membrane integrity and also early apoptosis as UV-B irradiated cells appear orange due to uptake of EtBr [Figure 5]. Pretreatment of HDF with 10 µg/ml of EA fraction showed presence of bright spots inside the cell suggesting chromatin condensation and nuclear fragmentation. With the increase in concentration of EA fraction to 30 µg/ml, bright spots disappeared indicating a concentration-dependent protection and restoration of cell membrane integrity.

Quantitation of intracellular reactive oxygen species

UV-B radiations induced excessive formation of intracellular ROS as shown in Figure 6. EA fraction pretreatment showed a significant (***$P < 0.001$) reduction in formation of intracellular ROS in a concentration-dependent manner (10–30 µg/ml). This confirms the free radical scavenging property of EA fraction and also can be correlated to the retained levels of the enzymatic antioxidants.

Ames test

S. typhimurium TA100 strain identity tests were performed to confirm the genotype. It showed presence of colonies on the histidine-positive medium and absence in the histidine-negative medium indicating that the strain is auxotrophic for the amino acid. Along with histidine dependence, the strain also carries an Rfa mutation which decreases the liposaccharide barriers and allows permeability of larger molecules, which was confirmed by observing a clear zone around a disc of crystal violet. The strain also has a compromised DNA repair mechanism which was confirmed by absence of colonies in the UV-irradiated region of the plate. In the R-factor assay, the strain shows resistance to antibiotic ampicillin. These results were in accordance with the genotype of *S. typhimurium* TA100.

All the concentrations of EA fraction of okra (10–100 µg/ml) showed no significant difference in the number of revertant colonies as compared to the negative control indicating that the fraction is unable to induce mutation in the histidine gene [Table 5]. Sodium azide was used as a positive control which showed presence of 4-fold increased number of revertant colonies suggesting that it can induce mutation in the histidine gene making it a wild-type strain.

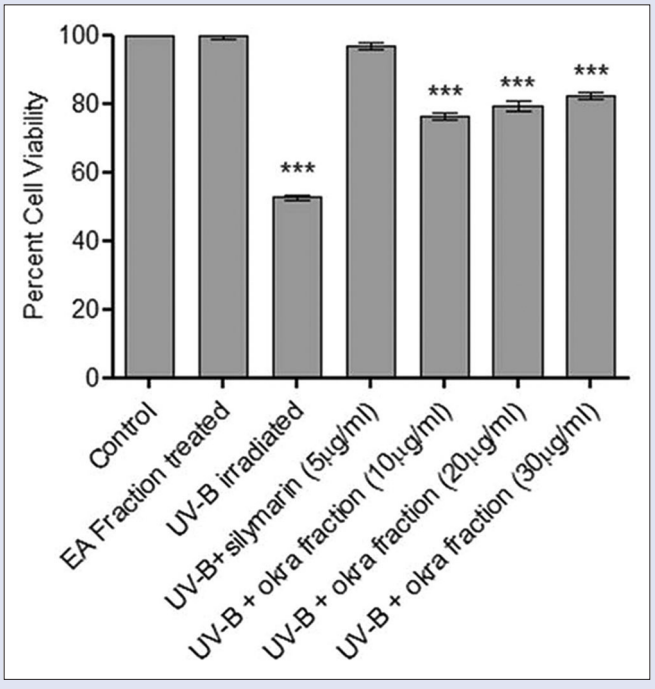


Figure 2: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cytoprotective ability of ethyl acetate fraction. Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, *$P < 0.05$, ***$P < 0.001$

qPCR and western blot analysis for Nrf2 and HO-1

1.8- and 3.3-fold increase in the expression of Nrf2 and HO-1, respectively, was detected in qPCR analysis [Figure 7] in UV-B-irradiated cells which may be possibly due to UV-B-induced oxidative stress. EA fraction pretreatment significantly (***$P < 0.001$) reduced the expression of Nrf2 and HO-1 in a concentration-dependent manner (10–30 µg/ml) which can be correlated to the reduced formation of intracellular ROS, hence, decreasing oxidative stress in the cell. Similar expression pattern was also observed at the protein level in Western blot analysis [Figure 8] suggesting no posttranslational modifications in Nrf2 and HO-1.

DISCUSSION

UV-B radiations penetrate the epidermal and dermal layer of the skin causing various injuries and damages to the cells. They react with photosensitizers forming excessive free radicals leading to aging, immune reactions, sunburns, skin cancer, as well as various systemic diseases. UV-B reacts with DNA directly leading to single-strand breaks, thymine

Table 3: Area, area %, retention factor values and identified compounds detected in the high-performance thin layer chromatography spectra of ethyl acetate fraction after densitometric analysis at 420 nm

Peak	Area	Area (%)	Rf values	Identified compounds
1	1804.7	28.5	0.06	Unknown
2	2269.87	36.0	0.43	Rutin
3	577.59	9.2	0.81	Unknown
4	1655.44	26.2	0.90	Unknown

Rf: Retention factor

Table 4: Flavonoid content and EC_{50} values of crude alcoholic extract and ethyl acetate fraction of *Abelmoschus esculentus*

	Flavonoid content (mg quercetin equivalent/g plant material)	EC_{50} (µg/ml)		
		DPPH assay	ABTS assay	FRAP assay
Crude alcoholic extract	2.3328±0.08	400.46±6.18	5977.3±244.97	1655.22±66.91
EA fraction	3.9082±0.01	87.70±0.90	990.50±18.57	731.77±35.42

All values are expressed as mean±SD for 9 experiments. EA: Ethyl acetate; SD: Standard deviation; DPPH: 2,2-diphenyl-1-picryl hydrazyl; ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); FRAP: Ferric reducing antioxidant power

Table 5: Mutagenic activity of ethyl acetate fraction of *Abelmoschus esculentus* by Ames test

Sample	Number of revertant colonies (mean±SD) (*n*=3)	Comments
Negative control	12±2	Spontaneous revertant colonies
Histidine positive plate	33±4	Confirming genotype
Positive control (sodium azide 1 mg/ml)	54±2	4 fold increase in number of colonies, mutagenic
Okra (10 µg/ml)	12±3	Nonmutagenic
Okra (20 µg/ml)	13±2	Nonmutagenic
Okra (30 µg/ml)	13±2	Nonmutagenic
Okra (40 µg/ml)	12±3	Nonmutagenic
Okra (60 µg/ml)	13±2	Nonmutagenic
Okra (80 µg/ml)	13±3	Nonmutagenic
Okra (100 µg/ml)	14±1	Nonmutagenic

SD: Standard deviation

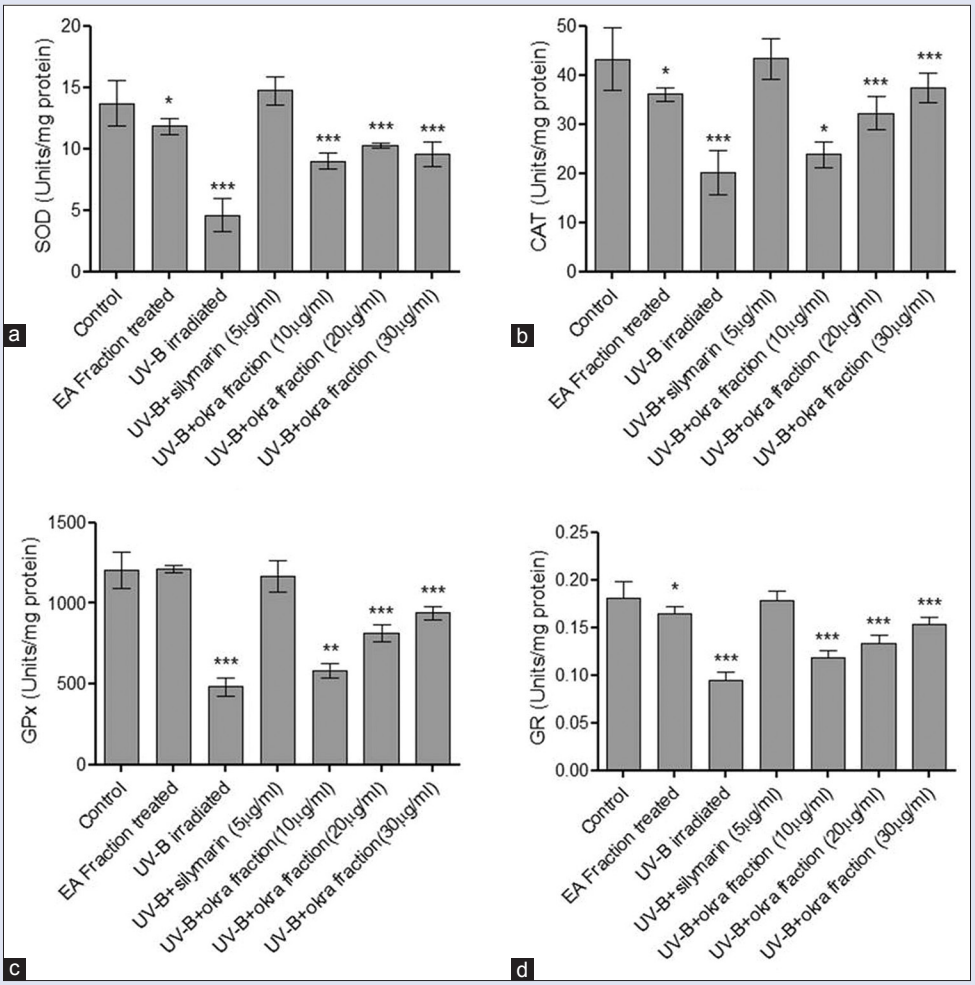


Figure 3: Levels of endogenous enzymatic antioxidants. (a) Superoxide dismutase, (b) catalase, (c) glutathione peroxidase, (d) glutathione reductase. Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

dimers, etc., causing damage and also increasing chances of mutations. Therefore, protection from harmful UV-B radiations is essential.[3,32,33]

Sunscreens and synthetic antioxidants are available commercially for UV protection but are associated with limitations on continual use. They contain chemical compounds such as zinc oxide, titanium dioxide, which are unstable and themselves become free radicals on long exposure to UV radiations. Sunscreens may also block the production of Vitamin D.[34] Hence, use of plant molecules is now regarded as a safer alternative to curb UV-B-induced damage. Plant molecules such as silymarin,[13] sesamol,[3] ursolic acid,[14] ferulic acid,[15] and epicatechin gallate[16] have been successful in protecting against UV-B-induced cell injury and damage. We have also reported a significant UV-B protective potential of flavonoids from *E. caryophylata*. They also showed protection against UV-B-induced cytotoxicity, antioxidant depletion, ROS generation, DNA damage, and apoptotic morphological changes. These flavonoids also reduced oxidative stress in the cell through the Nrf2-ARE pathway maintaining the oxido-redox status of the cell.[35]

Plant molecules can be of various types – flavonoids, alkaloids, tannins, etc. However, flavonoids are considered to be good free radical scavengers exhibiting a better antioxidant potential. EA fraction of okra also showed significantly higher flavonoid content and antioxidant potential than crude alcoholic extract. It also possesses free radical scavenging ability and a reducing potential.

UV-B radiations react with cellular chromophores and photosensitizers forming excessive ROS leading to lipid peroxidation, protein modifications, 8-hydroxyguanine formation, etc., Due to the free radical scavenging property of EA fraction, excessive formation of ROS was prevented. This decreases oxidative stress in the cell also retaining the levels of endogenous enzymatic antioxidant enzymes. There can be various reasons for depletion in levels of endogenous enzymatic antioxidant enzymes – direct absorbance of UV-B radiation, interaction with ROS or the antioxidant recycling mechanisms. Heme group absorbs UV-B radiations and decreases CAT activity; depleted SOD may be due to the formation of superoxide anion and antioxidant recycling mechanism may be the reason for decreased GPx and GR activities.[36,37] EA fraction of okra was observed to be working through the endogenous enzymatic antioxidant system. This fraction may be further useful in diseases caused due to oxidative stress.

UV-B radiations also damaged DNA in HDF cells showing the presence of comets. DNA damage may occur due to direct absorption of UV-B or through ROS.[38] Excessive strand breaks also increase the chances of mutation. EA fraction pretreatment protected against

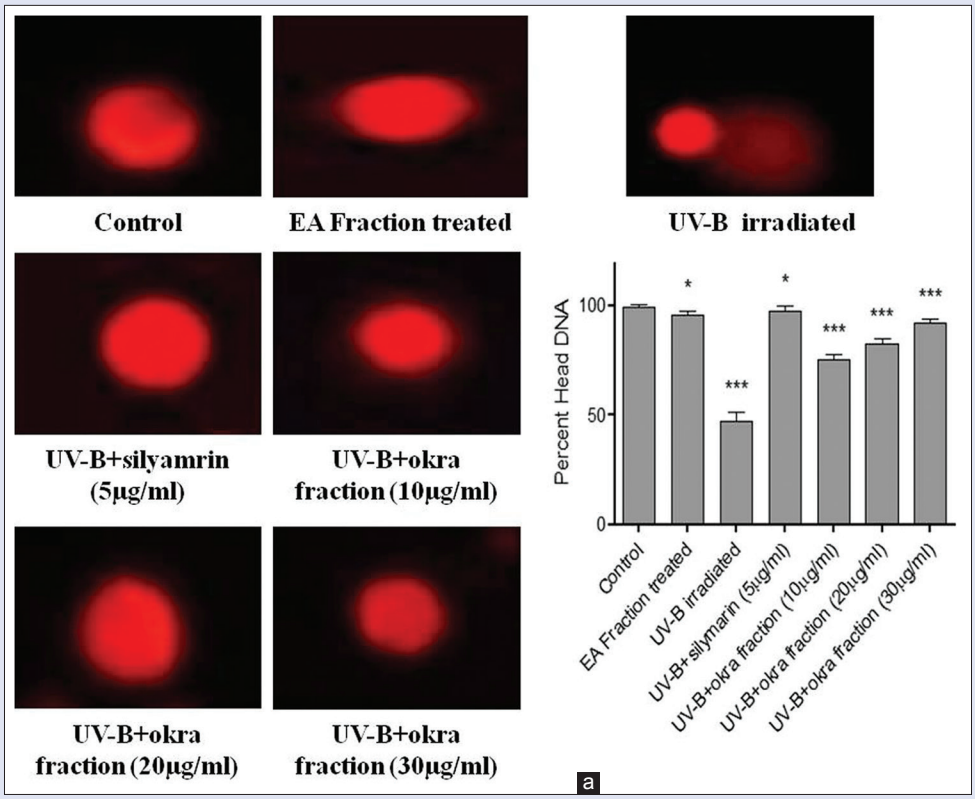


Figure 4: Comet assay for assessment of deoxyribonucleic acid damage. Cells observed under fluorescence microscope, 400×; (a) comparative graph showing percent head deoxyribonucleic acid in all treatment groups. Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, ***$P < 0.001$

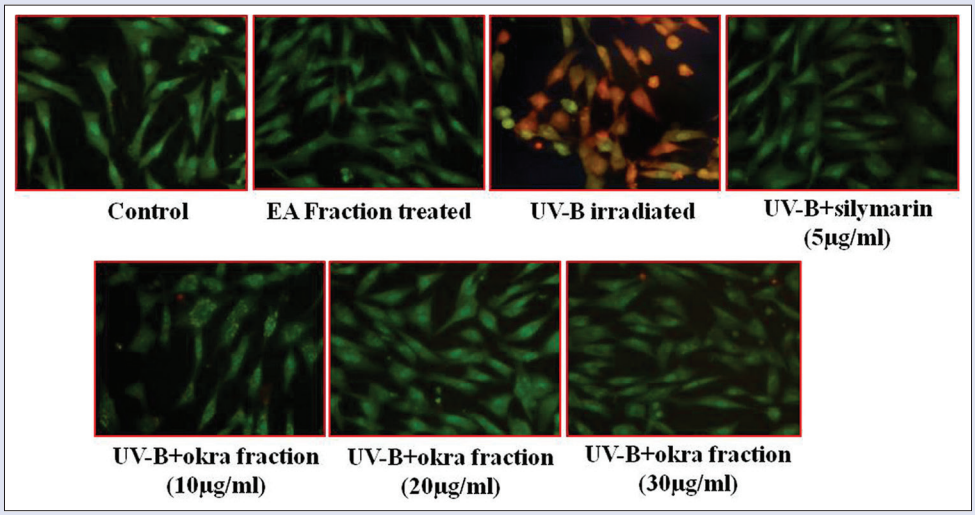


Figure 5: Ethidium bromide/acridine orange staining for detection of apoptotic morphological changes. Cells observed under fluorescence microscope, 400×

UV-B-induced DNA damage which may be due to two reasons – firstly, EA fraction prevented formation of ROS, hence, reducing chances of 8-hydroxyguanine formation; and secondly, EA fraction may be exhibiting a sunscreen effect on DNA preventing direct interaction of UV-B and DNA which will inhibit formation of single-strand breaks, thymine dimers, etc. EA fraction was also observed to be nongenotoxic as it did not lead to DNA damage in the cell. EA fraction also did not show a mutagenic potential till a concentration of 100 µg/ml in *S. typhimurium* TA100 strain.

UV-B radiations also damaged the cell membrane of HDF, which may be due to the lipid peroxidation reactions caused by excessive ROS in the cell. This loss in cell membrane integrity is a characteristic of apoptosis.

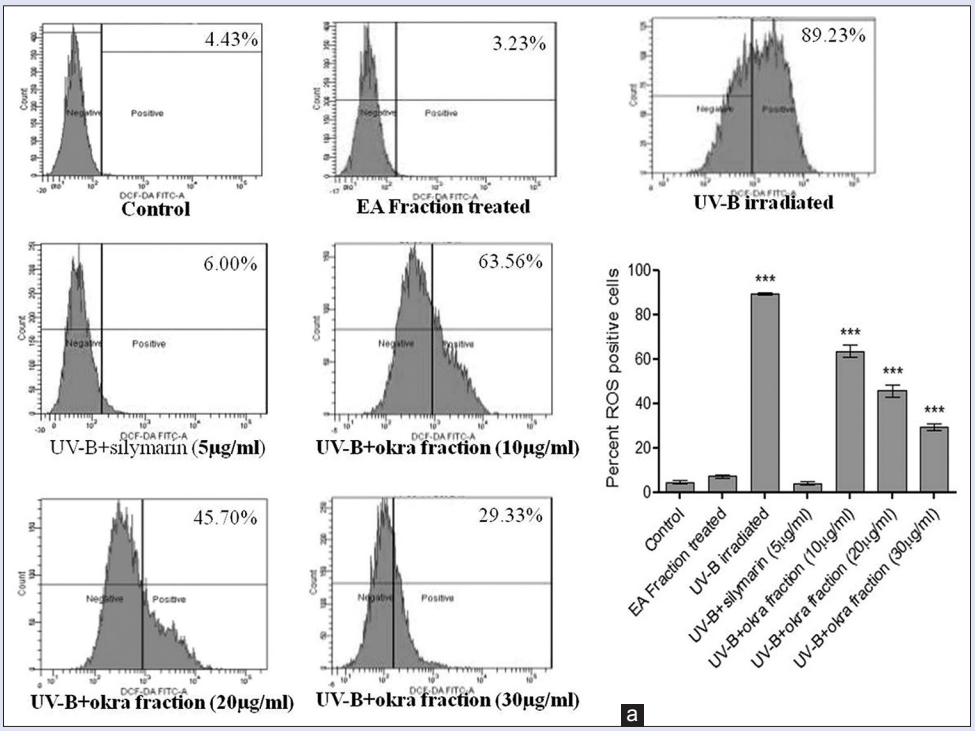


Figure 6: Measurement of intracellular reactive oxygen species by 2′,7′-dichlorofluorescein diacetate using flow cytometry. (a) Comparative graph showing percent reactive oxygen species positive cells in treatment groups. Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

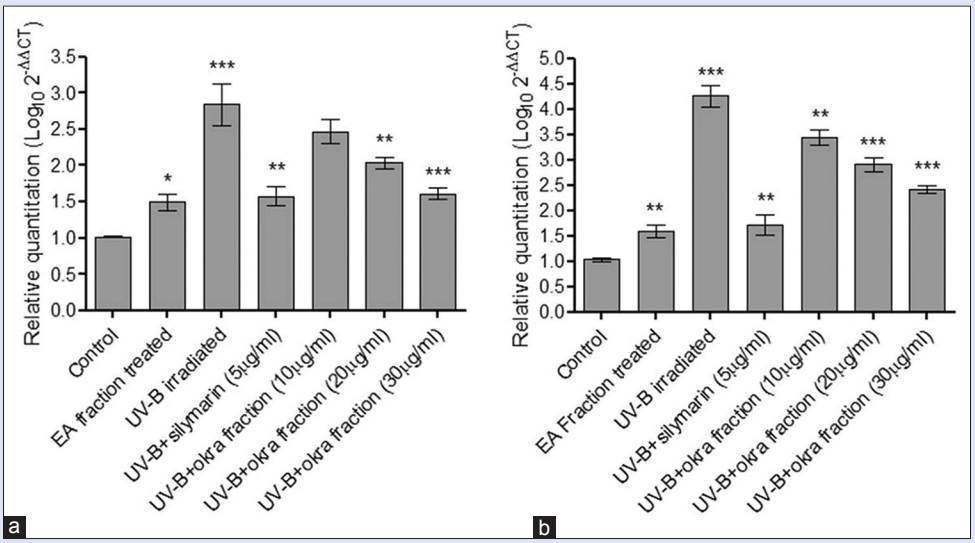


Figure 7: Quantitative polymerase chain reaction analysis for nuclear factor E2-related factor 2 and heme oxygenase-1 expression at mRNA level. (a) Fold change in nuclear factor E2-related factor 2 gene expression, (b) fold change in heme oxygenase-1 gene expression. Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

EA fraction pretreatment reduced the apoptotic morphological changes like chromatin condensation and nuclear fragmentation in a concentration-dependent manner. This may be prevented due to a better redox homeostasis in the cell attained by EA fraction pretreatment.

Nrf2-ARE pathway is important in cellular defence and sensitive towards oxidative stress. It leads to over-expression of antioxidant enzyme and phase II detoxification enzyme genes in presence of oxidative stress as also observed in the qPCR and western blot analysis. EA fraction pretreatment decreased the intracellular ROS formation leading to decreased oxidative stress. Hence, the expression of Nrf2 and HO-1 also decreased in EA fraction pretreated cells.

In the current study, we observed that the EA fraction of okra protected HDF against UV-B-induced cytotoxicity, antioxidant depletion, intracellular ROS, oxidative DNA damage, apoptotic changes and also

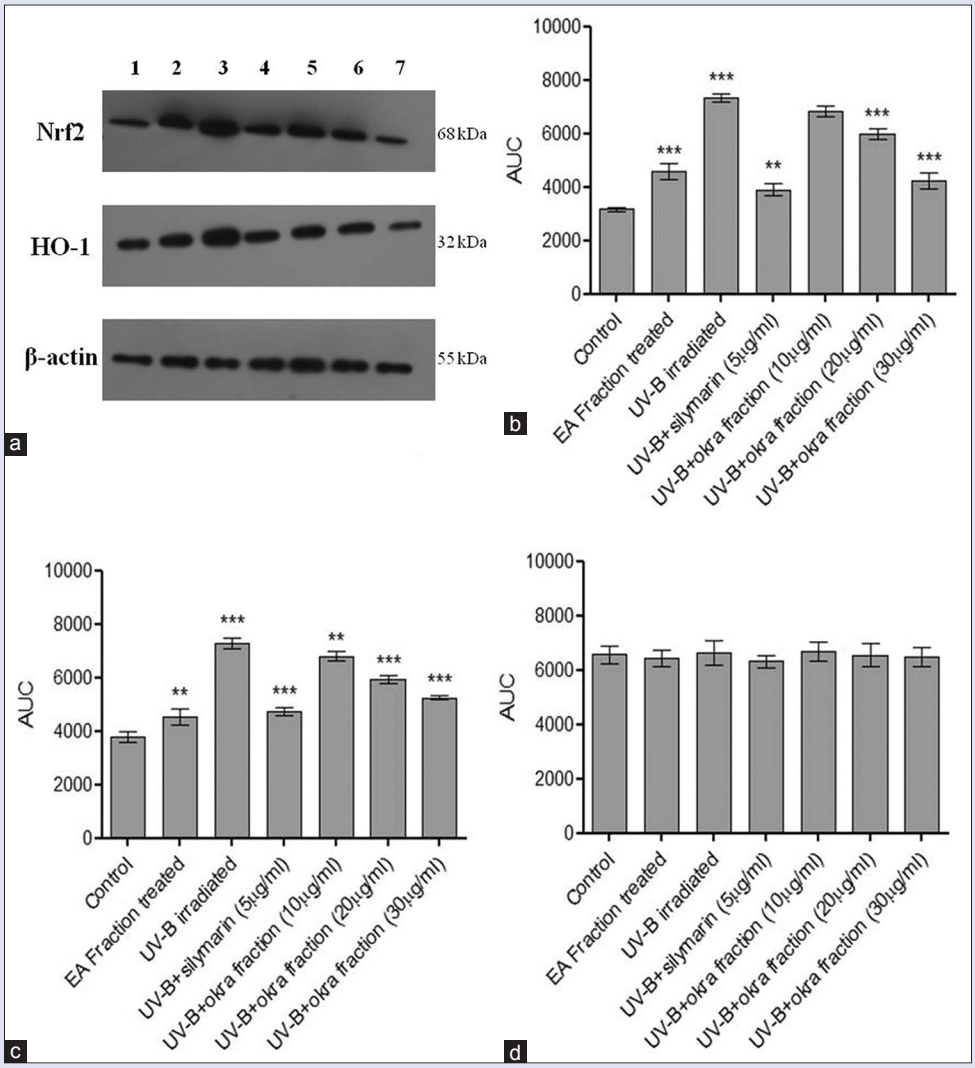


Figure 8: Western blot analysis for nuclear factor E2-related factor 2 and heme oxygenase-1 expression at protein level. (a) Nuclear factor E2-related factor 2, heme oxygenase-1 and β-actin expression where 1 - control, 2 - ethyl acetate fraction treated, 3 - ultraviolet-B irradiated, 4 - ultraviolet-B + silymarin (5 µg/ml), 5 - ultraviolet-B + 10 µg/ml ethyl acetate fraction, 6 - ultraviolet-B + 20 µg/ml ethyl acetate fraction, 7 - +30 µg/ml ethyl acetate fraction; densitometric analysis of nuclear factor E2-related factor 2 (b); heme oxygenase-1 (c); β-actin (d). Results were analysed statistically by GraphPad Prism software version 2.0 using one-way ANOVA followed by Dunnett's and Tukey's posttest, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

reduced oxidative stress through Nrf2-ARE pathway. Taken together, these findings suggest that the flavonoids from okra could potentially be considered as UV-B protectants and can be further studied and developed into a topical formulation against UV-B radiations.

CONCLUSION

In the present study, we have reported protective ability of flavonoids from *A. esculentus* against UV-B-induced cytotoxicity, antioxidant depletion, excessive formation of intracellular ROS, oxidative DNA damage, and apoptotic morphological changes. They were also able to reduce oxidative stress through Nrf2-ARE pathway and maintain the redox homeostasis. Flavonoids from okra can be further explored and developed into an herbal formulation for skin ailments caused due to UV-B radiations.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Afaq F, Adhami VM, Ahmad N, Mukhtar H. Botanical antioxidants for chemoprevention of photocarcinogenesis. Front Biosci 2002;7:d784-92.
2. Katiyar SK. Grape seed proanthocyanidines and skin cancer prevention: Inhibition of oxidative stress and protection of immune system. Mol Nutr Food Res 2008;52 Suppl 1:S71-6.
3. Ramachandran S, Rajendra Prasad N, Karthikeyan S. Sesamol inhibits UVB-induced ROS generation and subsequent oxidative damage in cultured human skin dermal fibroblasts. Arch Dermatol Res 2010;302:733-44.
4. Pinnell SR. Cutaneous photodamage, oxidative stress, and topical antioxidant protection. J Am Acad Dermatol 2003;48:1-19.
5. Black HS, deGruijl FR, Forbes PD, Cleaver JE, Ananthaswamy HN, deFabo EC, *et al.* Photocarcinogenesis: An overview. J Photochem Photobiol B 1997;40:29-47.
6. Ma Q, He X. Molecular basis of electrophilic and oxidative defense: Promises and perils of Nrf2. Pharmacol Rev 2012;64:1055-81.

7. Alrawaiq N, Abdullah A. Dietary phytochemicals activate the redox-sensitive transcription factor Nrf2. Int J Pharm Pharm Sci 2014;6:11-6.
8. Lee JM, Johnson JA. An important role of Nrf2-ARE pathway in the cellular defense mechanism. J Biochem Mol Biol 2004;37:139-43.
9. Ansari NM, Houlihan L, Hussain B, Pieroni A. Antioxidant activity of five vegetables traditionally consumed by South-Asian migrants in Bradford, Yorkshire, UK. Phytother Res 2005;19:907-11.
10. Sabitha V, Ramachandran S, Naveen KR, Panneerselvam K. Investigation of *in vivo* antioxidant property of *Abelmoschus esculentus* (L) moench. fruit seed and peel powders in streptozotocin-induced diabetic rats. J Ayurveda Integr Med 2012;3:188-93.
11. Ngoc TH, Ngoc QN, Van AT, Phung NV. Hypolipidemic effect of extracts from *Abelmoschus esculentus* L. (*Malvaceae*) on tyloxapol-induced hyperlipidemia in mice. Mahidol Univ J Pharm Sci 2008;35:42-6.
12. Adelakun OE, Oyelade OJ, Ade-Omowaye BI, Adeyemi IA, Van de Venter M. Chemical composition and the antioxidative properties of Nigerian Okra Seed (*Abelmoschus esculentus* Moench) Flour. Food Chem Toxicol 2009;47:1123-6.
13. Svobodová A, Psotová J. Natural phenolics in the prevention of UV-induced skin damage-a review. Biomed Pap 2003;147:137-45.
14. Ramachandran S, Prasad NR. Effect of ursolic acid, a triterpenoid antioxidant, on ultraviolet-B radiation-induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes. Chem Biol Interact 2008;176:99-107.
15. Prasad NR, Ramachandran S, Pugalendi KV, Menon VP. Ferulic acid inhibits UV-B–induced oxidative stress in. Human lymphocytes. Nutr Res 2007;27:559-64.
16. Huang CC, Wu WB, Fang JY, Chiang HS, Chen SK, Chen BH, *et al.* (-)-Epicatechin-3-gallate, a green tea polyphenol is a potent agent against UVB-induced damage in HaCaT keratinocytes. Molecules 2007;12:1845-58.
17. Patwardhan J, Pandita N, Bhatt P. Comparative study of anti-oxidant potential of two Indian medicinal plants – *Foeniculum vulgare* and *Eugenia caryophylata.* Int J Pharm Sci Rev Res 2013;21:312-6.
18. Lee GS, Shim H, Lee KM, Kim SH, Yim D, Cheong JH, *et al.* The role of the ethylacetate fraction from hydnocarpi semen in acute inflammation *in vitro* model. Immune Netw 2012;12:291-5.
19. Wagner H, Bladt S. Plant drug analysis – A Thin Layer Chromatography Atlas. 2nd ed. Berlin, Heidelberg: Springer – Verlag; 1996.
20. Sethiya NK, Raja MK, Mishra SH. Antioxidant markers based TLC-DPPH differentiation on four commercialized botanical sources of *Shankhpushpi* (A Medhya Rasayana): A preliminary assessment. J Adv Pharm Technol Res 2013;4:25-30.
21. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 2002;10:178-82.
22. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol 2011;48:412-22.
23. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231-7.
24. Fakruddin M, Mannan KS, Mazumdar RM, Afroz H. Antibacterial, antifungal and antioxidant activities of the ethanol extract of the stem bark of clausena heptaphylla. BMC Complement Altern Med 2012;12:232.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
26. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469-74.
27. Chance B, Herbert D. The enzymesubstrate compounds of bacterial catalase and peroxides. Biochem J 1950;46:402-14.
28. Awasthi YC, Beutler E, Srivastava SK. Purification and properties of human erythrocyte glutathione peroxidase. J Biol Chem 1975;250:5144-9.
29. Goldberg DM, Spooner RJ. Oxidoreductases acting on groups other than CHOH glutathione reductase. In: Bergmeyer HU, Bergmeyer J, Grassl M, editors. Methods of Enzymatic Analysis. Weinheim: Verlag Chemie; 1983. p. 258-65.
30. Magesh V, Raman D, Pudupalayam KT. Genotoxicity studies of dry extract of *Boswellia serrata.* Trop J Pharm Res 2008;7:1129-35.
31. Sundaram SG, Vijayalakshmi M, Nema RK. Antimutagenicity of ethanol extract of *Derris brevipes.* J Chem Pharm Res 2010;2:598-603.
32. Patlolla A, Knighten B, Tchounwou P. Multi-walled carbon nanotubes induce cytotoxicity, genotoxicity and apoptosis in normal human dermal fibroblast cells. Ethn Dis 2010;20 1 Suppl 1:65-72.
33. Johnson JA, Johnson DA, Kraft AD, Calkins MJ, Jakel RJ, Vargas MR, *et al.* The Nrf2-ARE pathway: An indicator and modulator of oxidative stress in neurodegeneration. Ann N Y Acad Sci 2008;1147:61-9.
34. Ambothi K, Nagarajan RP. Ferulic acid prevents UV-B radiation induced oxidative damage in human dermal fibroblasts. Int J Nutr Pharmacol Neurol Dis 2014;4:203-13.
35. Patwardhan J, Bhatt P. Ultraviolet-B protective effect of flavonoids from *Eugenia Caryophylata* on human dermal fibroblast cells. Pharmacogn Mag 2015;11:397-406.
36. Rai R, Shanmuga SC, Srinivas C. Update on photoprotection. Indian J Dermatol 2012;57:335-42.
37. Lü JM, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: Experimental approaches and model systems. J Cell Mol Med 2010;14:840-60.
38. Shindo Y, Witt E, Han D, Tzeng B, Aziz T, Nguyen L, *et al.* Recovery of antioxidants and reduction in lipid hydroperoxides in murine epidermis and dermis after acute ultraviolet radiation exposure. Photodermatol Photoimmunol Photomed 1994;10:183-91.

Dr. Purvi Bhatt

ABOUT AUTHOR

Dr. Purvi Bhatt is an Assistant Professor in the Department of Biological Sciences, SDSOS, NMIMS, Mumbai. Her areas of specialization are Biochemistry, Reproductive Biology, Cancer Biology. She is also actively involved in teaching, training postgraduate students and guiding students for their doctoral research projects in the fields of phytochemistry, cancer biology and nanotechnology.