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ORIGINAL ARTICLE

The Cytoprotective Benefits of a Turmeric, Quercetin, and Rosemary Blend through Activation of the Oxidative Stress Pathway

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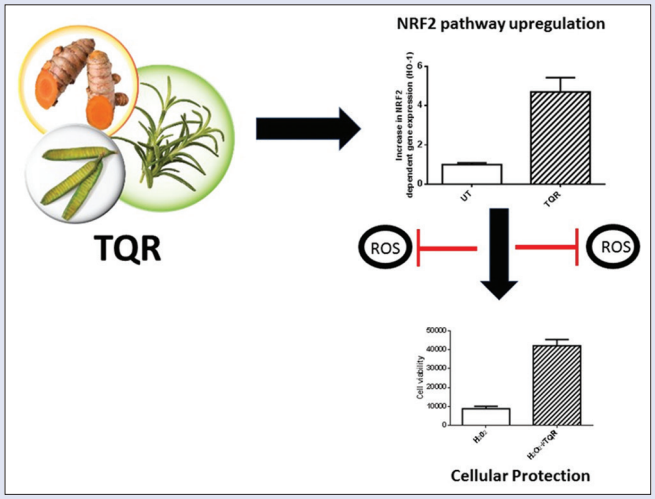
ABSTRACT

Background: An imbalance between oxidative and reductive processes within cells can result in oxidative stress leading to a decrease in cellular survival. Use of natural products with antioxidant properties may reduce this oxidative stress. **Objective:** The objective of the study is to determine whether a natural product blend of turmeric, pagoda tree seed pod (quercetin), and rosemary (TQR) extracts can protect human liver cells from oxidative stress-induced cytotoxicity. **Materials and Methods:** HepG2 cells were treated with a blend of botanical extracts of TQR (1:3:5, w: w:w) and expression of genes downstream of nuclear factor (erythroid-derived 2)-like 2 (NRF2) activation was measured. We also measured the ability of the extract blend to protect DNA and lipids from oxidative stress by measuring via the comet assay and 8-isoprostane production, respectively. Finally, we measured the effect of the extract blend on HepG2 cell protection against oxidative stress-induced cytotoxicity. **Results:** We provide evidence that the TQR blend activates the NRF2 pathway leading to DNA protection, a decrease in lipid peroxidation, and whole cell protection against oxidative stress. **Conclusion:** These results suggest that consuming a blend of TQR at the ratio of 1:3:5 could provide benefits against environmental stressors that increase exposure to reactive oxygen species.

Key words: 8-Isoprostane, glutamate-cysteine ligase modifier subunit, heme oxygenase I, nuclear factor (erythroid-derived 2)-like 2, turmeric, quercetin, rosemary

SUMMARY

- The aim of this study was to show the beneficial effect of the botanical blend consisting of turmeric, pagoda tree seed pod (quercetin), and rosemary (TQR) extract on human liver cells. TQR botanical blend protects human liver cells against oxidative stress by preventing DNA damage, isoprostane formation, and cellular necrosis. This beneficial effect potentially may be due to nuclear factor (erythroid-derived 2)-like 2 pathway activation.



Abbreviation used: NRF2: Nuclear factor (erythroid-derived 2)-like 2; GCLM: Glutamate-cysteine ligase modifier subunit; HO-1: Heme oxygenase I; TQR: Turmeric, quercetin, rosemary.

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INTRODUCTION

Age-related pathologies are thought to occur as a consequence of an imbalance in the cellular redox environment.[1] When this imbalance results in an oxidative environment, oxidative stress causing DNA, lipid, and protein modifications leads to a decrease in cellular survival.[2-4] In living cells, equilibrium between oxidation and reduction is maintained via regulation of antioxidant enzymes.[5] Expression of antioxidant enzymes such as heme oxygenase 1 (HO-1), glutamate-cysteine ligase modifier subunit (GCLM), NADPH: quinone oxidoreductase (NQO-1), and many others increase during oxidative stress in order to maintain cellular redox homeostasis.[2,5,6]

In recent years, the biological pathway regulating the expression of antioxidant enzymes has been studied extensively.[6-9] It is now clear that the expression of these enzymes is regulated by the transcription factor nuclear factor erythroid-2-related factor 2 (NRF2).[10-13] Various studies in humans and mice have shown that stimulating the NRF2 pathway results in cytoprotection against oxidative stress.[10,14-16] One of the earliest studies showing the importance of the NRF2 pathway-utilized NRF2 knockout mice and showed that these mice were more susceptible to acetaminophen compared to wild type.[17] Dimethyl fumarate, a drug for relapsing multiple sclerosis, has been shown to work in part by inducing

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NRF2.[18] Recently, Kubben *et al.*[19] have shown in the premature aging disorder Hutchinson–Gilford progeria syndrome that a mutant form of Lamin A sequesters NRF2 leading to subnuclear mislocalization. This results in impaired NRF2 activity and hence chronic oxidative stress resulting in premature aging.

Consuming fruits and vegetables regularly has been shown to be beneficial for maintaining optimal health, and studies have shown that some of their health benefits come from priming the NRF2 pathway.[20-22] It has further been observed that consumption of cruciferous vegetables is associated with a lowered risk of cancer development[23] and that the compound sulforaphane, which is present in these vegetables, activates NRF2 and adapts cells against cancer.[24] Based on this wealth of information, it can be inferred that activation of NRF2 is an adaptive mechanism against environmental and oxidative stress and that fruits and vegetables are a good source to activate this pathway due to the presence of various phytochemicals found in them.[25-27]

The initial goal for this study was to screen for botanicals that stimulate the NRF2-mediated antioxidant defense pathway.[28] Turmeric, pagoda tree seed pod (quercetin), and rosemary (TQR) were three botanical extracts that showed activity in a NRF2-dependent luciferase reporter assay.[28] There have been numerous studies on turmeric (*Curcuma longa*) due to its use in ethnobotanical medicine for many years in India and Southeast Asia.[29] Turmeric has been shown to have anti-inflammatory, antioxidant, wound healing, and anticarcinogenic function.[30] Notably, curcumin, the main component of turmeric, is a hormetic agent, stimulatory at low doses and inhibitory at high doses.

Quercetin is one of the most abundant dietary flavonoids. It is found in fruits, green leafy vegetables, and in many seeds.[31] This plant-derived compound has been shown to have a variety of health benefits such as anticancer, anti-inflammatory, vasodilatory effects, antiobesity, and antioxidant.[31] Rosemary (*Rosmarinus officinalis*) is one of the species in the genus *Rosmarinus* and is native to the temperate countries of the Mediterranean region.[32] Rosemary has been widely used not only in cooking but also in traditional medicine being used to prevent and cure colds, rheumatism, muscle pains, and joints. It is one of the most popular sources of natural bioactive compounds, as this plant exerts various pharmacological activities such as antibacterial, antidiabetic, and anti-inflammatory.[32]

Liver cells have a very critical function to filter the blood coming from the digestive tract before sending it to the rest of the body. In addition, they play a major role in the detoxification of harmful chemicals. Protection of the liver against reactive oxygen species (ROS) toxicity is important for the proper functioning of the whole organism. Hence, we wanted to test the protective effect of our botanical blend against liver toxicity. To do so, we used HepG2 cells as they are frequently used as an *in vitro* alternative to primary human hepatocytes.[33]

MATERIALS AND METHODS

Botanical extracts and reagents

Details of the botanical authentication can be found in a previous publication.[28] Briefly, Turmeric (*C. longa* Linn; *Zingiberaceae*) rhizome extract (Batch# OCL3EG1301C01) standardized to 85% total curcuminoids was purchased from Verdure Sciences (Noblesville, IN); Pagoda Tree (*Styphnolobium japonicum* (L) Schott, syn. *Sophora japonica* L; *Fabaceae*) seed pod extract (Batch#0100019904), standardized to 95% anhydrous Quercetin, was purchased from Novel Ingredient Services (East Hanover, NJ); and Rosemary (*R. officinalis* L; *Lamiaceae*) leaf extract (Batch# 610036338), standardized to 6% rosmarinic acid, was from Naturex (South Hackensack, NJ). All extracts were authenticated through vendor certification, examination of high-performance liquid chromatography chromatograms for phytochemical content and comparison to published reports.

Chromatographic analysis of botanical extracts

Quercetin (99.5%), rosmarinic acid (99.4%), and curcumin (99.0%) reference standards were obtained from the United States Pharmacopeia. Standards were dissolved in a methanol: DMSO (4:1) solution and covered a linear range of approximately 6–500 ppm. Total curcuminoids were calculated as the sum of bisdemethoxycurcumin, demethoxycurcumin, and curcumin. Botanical extract samples were prepared for UPLC analysis by adding approximately 0.4 g of rosemary extract, 0.1 g of pagoda tree seed pod extract, or 0.1 g of turmeric extract to 60 mL of a methanol: DMSO (4:1) solution, sonicating for 30 min and diluting the samples to a final volume of 100 mL. The turmeric and pagoda tree extracts were further diluted 1:3.33 with solvent. Samples were mixed, filtered using a 0.22-µm GV-PVDF Millipore syringe filter (Millipore Corp.) and directly injected into the UPLC system. Chromatographic separation was performed on a Waters Acquity H-Class UPLC equipped with an Acquity eλ photodiode array detector, monitoring at 280 nm (Waters Corp.). The column used was a Waters UPLC HSS T3, 1.8 µm, 2.1 mm × 100 mm. The mobile phases used were: (A) 0.2% o-phosphoric acid in water (v/v), (B) methanol, and (C) acetonitrile. The ternary mobile phase gradient for analysis was as follows: initial, 60% A, 25% B, 15% C; 3.2 min, 45% A, 35% B, 20% C; 4.8 min, 20% A, 40% B, 40% C; 5.8 min, 10% A, 45% B, 45% C; 5.81–7.0 min, 50% B, 50% C; and 7.01–10.0 min, 60% A, 25% B, 15% C. The column temperature was 25°C and the sample temperature was 20°C. UPLC figure is provided in the supplementary material.

Cell culture reagents

HepG2 cells were purchased from ATCC (Manassas, VA) and were cultured in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS) in a humidified, 5% CO_2 atmosphere at 37°C. Tissue culture reagents were purchased from Mediatech (Manassas, VA), except for FBS which was from HyClone (Logan, UT, USA). All incubation steps were in a humidified, 5% CO_2 atmosphere at 37°C, unless otherwise noted.

Real-time-quantitative polymerase chain reaction

HepG2 cells were plated at 5 × 10^5 cells/well in 2 mL of MEM-10% FBS in a six-well plate and incubated for 18 h at 5% CO_2 and 37°C. The media was replaced with MEM-1% FBS, treatments were added and cells were incubated for 4 h at 5% CO_2 and 37°C. RNA was harvested using Qiashredder columns and RNeasy kits (Qiagen, Valencia, CA). cDNA synthesis was completed using iScript reverse transcription supermix (Bio-Rad, Hercules, CA), with 5 min at 25°C for priming, 30 min at 42°C for reverse transcription, and 5 min at 85°C for real-time (RT) inactivation. Quantitative polymerase chain reaction (qPCR) reactions were completed using Evagreen Ssofast qPCR supermix (Bio-Rad, Hercules, CA), with 30 s at 95°C for enzyme activation, followed by 40 cycles of 5 s at 95°C for denaturation and 5 s at 58°C for annealing/extension. All steps were on the BioRad CFX96 RT system and C1000 thermal cycler. Primers were purchased from Qiagen (Valencia, CA), GCLM (QT00038710), HO-1 (QT00092645), NRF2 (QT00027384), and Actin (QT01680476).

Comet assay

HepG2 cells were plated at 1.5 × 10^5 cells/well in 500 µl of MEM-10% FBS in 24-well plate and incubated for 24 h at 5% CO_2 and 37°C. The media was aspirated and the cells were treated for 60 min with extracts at the concentrations described in Figure 1. Subsequent to treatment, the

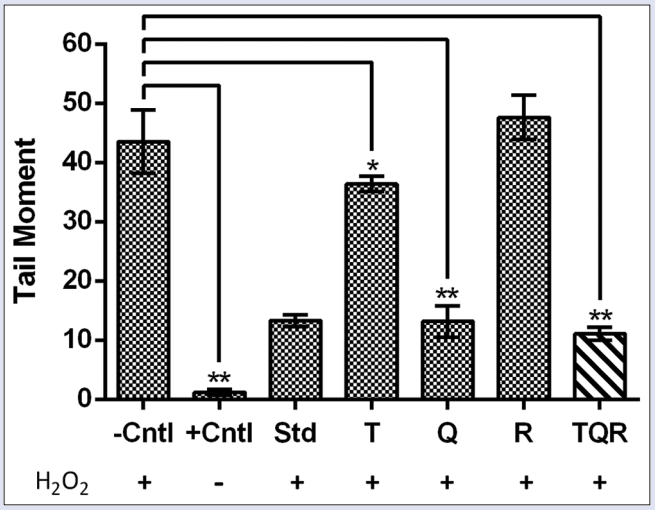

Figure 1: The turmeric, pagoda tree seed pod (quercetin), and rosemary blend protects cellular DNA against oxidative stress damage. Cells were treated with the turmeric, pagoda tree seed pod (quercetin), and rosemary blend at 200 µg/mL and T (22.2 µg/mL), Q (66.6 µg/mL), and R (111 µg/mL) for 1 h and then challenged with 50 µM H_2O_2. DNA damage was measured by the alkaline version of the comet assay. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ when compared to the stressed control (the cells treated with only H_2O_2) by ANOVA using GraphPad Prism 6.00. +Cntl: Cells are only treated with H_2O_2. –Cntl: cells are untreated with either H_2O_2 or turmeric, pagoda tree seed pod (quercetin), and rosemary. Std: Positive control (Quercetin Pharmaceutical Secondary from Sigma-Aldrich St. Louis, MO, USA)

cells were washed 1 time with phosphate-buffered saline (PBS). Fresh media with 500 µM hydrogen peroxide (H_2O_2) was added to the cells and incubated at 37°C for 20 min. Cells were washed one time with PBS and then 100 µl of trypsin was added and incubated at 37°C for 5 min to remove the cells from the plates. Cells were observed under the microscope to confirm their detachment. 400 µl of MEM-10% FBS was added to neutralize the trypsin. The cell suspension was centrifuged at 400 ×*g* for 4 min, and the pelleted cells were resuspended in 200 µl PBS. Next, 20 µl of the cell suspension was added to 200 µl of low melting agarose, mixed and 30 µl of that was spread over each well of a two-well Comet Slide from Trevigen (Gaithersburg, MD) and incubated for 10 min at 4°C to harden. The cells were lysed by submerging the slides in lysis solution (200 mL) for 1 h at 4°C. The lysis solution was removed, and the slides were incubated in unwinding solution (200 mL) in the dark at room temperature for an additional 1 h. The slides were then electrophoresed for 15 min at 21V in a cold electrophoresis apparatus from Trevigen (Gaithersburg, MD). Once electrophoresis was completed, the slides were washed twice in water and once in 70% ethanol and dried. Slides were stained with SYBR green dye and imaged on a Nikon Eclipse 800, fluorescence microscope and scored using the Comet IV software from Perceptive Instruments, V4.3, (Bury St Edmunds UK). All the buffers used were identical to that described by Collins and Azqueta[34] All the reagents were purchased from Trevigen (Gaithersburg, MD), except SYBR green, which was purchased from Thermo Fisher Scientific (Waltham, MA).

ELISA for 8-isoprostane

HepG2 cells were plated at 2.5×10^4 cells/well in 100 µL of MEM-10% FBS in a 96-well plate and incubated for 18 h at 5% CO_2 and 37°C. The media was then replaced with MEM-1% FBS. Samples were prepared as stock solutions at 50 mg/mL in 70% DMSO and diluted in MEM-1% FBS media to the treatment concentrations. The cells were incubated with the samples for 4 h after which 200 µM H_2O_2 was added and the cells were further incubated for 24 h. Culture supernatants were analyzed for the presence of 8-isoprotane using a commercially available ELISA kit (Cayman #516351, Ann Arbor MI).

Cell viability

HepG2 cells were plated at 3×10^4 cells/well in 200 µL of MEM-10% FBS in a 96-well plate and grown overnight. The next day, sample extracts were prepared as stock solutions of 100 mg/mL in 70% DMSO and diluted in MEM-10% FBS to the treatment concentrations. The cells were treated with the extracts as described in the figure legend for 24 h. Subsequent to this, the media was removed and replaced with fresh media (MEM-10%FBS) containing 5 mM H_2O_2 and treated for 4 h. After the H_2O_2 treatment, the HepG2 cells were lysed and viability was measured using the CellTiter-Glo© reagent (Promega, Madison, WI, PR-G7571) according to the protocol provided by Promega on an M5 Spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical methods

All experiments were performed in triplicate. Data were expressed as mean ± SD and were analyzed by one-way ANOVA analysis using the statistical software package GraphPad Prism 6 software, (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Activation of nuclear factor (erythroid-derived 2)-like 2 pathway by turmeric, pagoda tree seed pod (quercetin), and rosemary

As has been previously reported by our group, turmeric and pagoda tree seed pod extract (quercetin) activated a NRF2-antioxidant response element (ARE) luciferase reporter construct in HepG2 cells and that a blend of TQR (1:3:5, w: w:w) activated this response to a greater extent than was expected.[28] As a follow-up from our previous publication,[28] cytoprotective benefits of this blend were investigated in the human liver cell line, HepG2, in relation to the NRF2 pathway. Expression of genes under NRF2 regulation, such as HO-1 and GCLM,[35] was assessed after treatment of HepG2 cells with turmeric (2.8 µg/mL), quercetin (8.3 µg/mL), rosemary (13.9 µg/mL), and the TQR blend (25 µg/mL). Concentrations were aligned with previous work,[28] and the individual concentrations were chosen according to the 1:3:5 ratio of the blend. The cells were treated for 4 h, and subsequently, total RNA was isolated, cDNA synthesized, and qPCR carried out. As shown in Figure 2a, the TQR blend stimulated GCLM gene expression by nearly five-fold and was statistically significant ($P < 0.01$) when compared to untreated cells. The individual ingredients TQR also stimulated GCLM gene expression. Similarly, HO-1 gene expression [Figure 2b] was stimulated five-fold by the TQR blend. This increase was statistically significant ($P < 0.01$) when compared to untreated cells. Turmeric also stimulated HO-1 gene expression significantly ($P < 0.01$) but not to the level stimulated by the TQR blend.

Protection of DNA against oxidative stress by turmeric, pagoda tree seed pod (quercetin), and rosemary

Once we established that the TQR blend induced expression of genes under the control of the NRF2 pathway, we focused on the cytoprotective benefits resulting from NRF2 activation.

Since it is known that oxidative stress damages cellular macromolecules such as DNA and lipids,[36] the comet assay was utilized to test the TQR

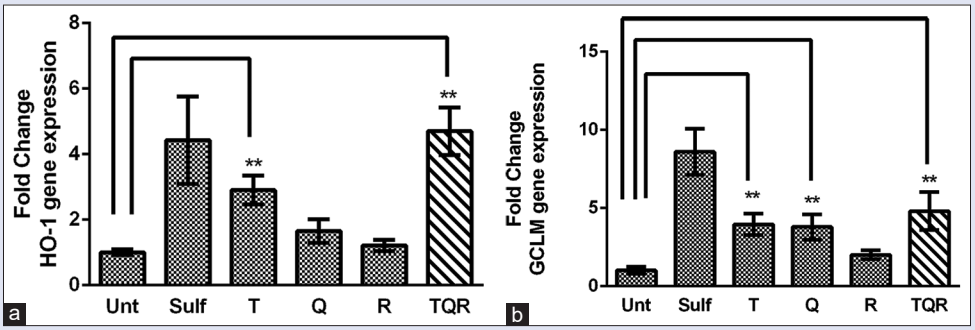

Figure 2: Effect of the turmeric, pagoda tree seed pod (quercetin), and rosemary blend (25 µg/mL) on gene expression of glutamate-cysteine ligase modifier subunit and heme oxygenase I in HepG2 cells after 4 h of treatment. Sulforaphane was used as positive control. (a) The gene expression of glutamate-cysteine ligase modifier subunit after treatment with the turmeric, pagoda tree seed pod (quercetin), and rosemary blend and turmeric (2.8 µg/mL), quercetin (8.4 µg/mL) (quercetin), and rosemary (13.8 µg/mL) alone at the concentrations found in the 25 µg/mL turmeric, pagoda tree seed pod (quercetin), and rosemary blend. (b) The gene expression of heme oxygenase I after treatment with turmeric, pagoda tree seed pod (quercetin) and rosemary and its constituents as described above. $^{**}P \leq 0.01$ when compared to Unt control by ANOVA using GraphPad Prism 6.00. Sulf: Sulforaphane, Unt: Untreated cells

blend for its ability to protect against oxidative DNA damage. As shown in Figure 1, the TQR blend showed statistically significant ($P < 0.01$) DNA protection against an oxidative challenge. In addition, turmeric and quercetin also showed significant DNA protection ability, $P < 0.05$, $P < 0.01$, respectively, among the individual ingredients.

Turmeric, pagoda tree seed pod (quercetin), and rosemary protects against lipid oxidation in HepG2 cells

The TQR blend was also tested for its ability to prevent lipid oxidation. Isoprostanes are formed by the free radical catalyzed oxidation of fatty acids, and they have been shown to be a reliable biomarker for oxidative stress in both animal and human clinical tests.[37,38] Therefore, the ability of the TQR blend to reduce the formation of isoprostanes when cells were exposed to an oxidative challenge was tested. HepG2 cells were treated with 12.5 µg/ml of the TQR blend and Figure 3 shows that TQR protected the cells from forming isoprostane, this protection was significant ($P < 0.01$). showing that the botanical blend protected the cells from oxidative lipid damage. Quercetin also showed significant protection (P<0.01).

Turmeric, pagoda tree seed pod (quercetin), and rosemary protects HepG2 cells against oxidative stress

Finally, whole cell protection against oxidative stress was tested. In Figure 4, we show that the TQR blend at both 25 and 12.5 µg/ml showed statistically significant ($P < 0.01$) protection of HepG2 cells against an oxidative challenge as measured by cellular viability. Turmeric and quercetin also showed significant protection. However, TQR blend showed the highest cellular protection compared to individual botanical extracts in the blend against oxidative insult.

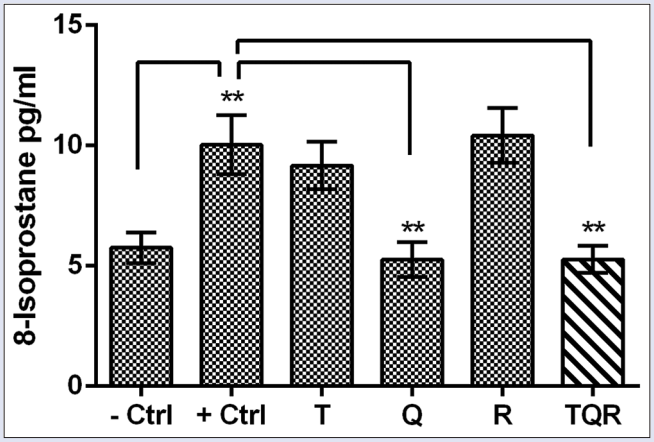

Figure 3: Turmeric, pagoda tree seed pod (quercetin), and rosemary blend protects HepG2 cells from phospholipid damage due to oxidative stress as shown by a decrease in the cellular concentration of 8-isoprostane. HepG2 cells were treated with turmeric, pagoda tree seed pod (quercetin), and rosemary (12.5 µg/mL) and the individual extracts of turmeric (1.4 µg/mL), quercetin (4.2 µg/mL), and rosemary (6.9 µg/mL) for 4 h and then further treated with H_2O_2 for 24 h. 8-isoprostane was measured using ELISA. $^{**}P \leq 0.01$ when compared with untreated (0ug/mL) by ANOVA using Graphpad prism 6.00. –Ctrl: Untreated HepG2 cells. +Ctrl: HepG2 cells treated with H_2O_2, but not pretreated with turmeric, pagoda tree seed pod (quercetin), and rosemary

DISCUSSION

ROS in physiological concentrations has important functions in intracellular signaling, pathogen infection and metabolism within cells, and whole organisms.[39] However, overproduction of ROS has been implicated in many chronic and degenerative diseases such as cancer and neurodegenerative and respiratory disorders. Increased levels of ROS have been shown to damage protein, DNA, and lipids, which leads to impaired cellular function and ultimately to chronic and degenerative diseases associated with aging.[39,40] Therefore, maintaining a normal ROS level is critical. In the case of overproduction of ROS, antioxidants act as natural neutralizers. One of the best ways to increase antioxidants is through the regular consumption of fruits and vegetables,[41,42] as this has shown to provide benefits against environmental and oxidative stressors.[21,43]

One of the primary regulators involved in the maintenance of redox levels in cells is NRF2, which in unstressed conditions, is restricted from inducing antioxidant and cytoprotective genes by cytoplasmic Keap1-mediated ubiquitination and additional negative regulators present in the nucleus.[8,10,13,44] Cellular stress leads to the release of NRF2 from Keap1, translocation to the nucleus, and the formation of a heterodimer complex with small Maf proteins. This complex binds to the ARE and stimulates the expression of antioxidant and detoxification

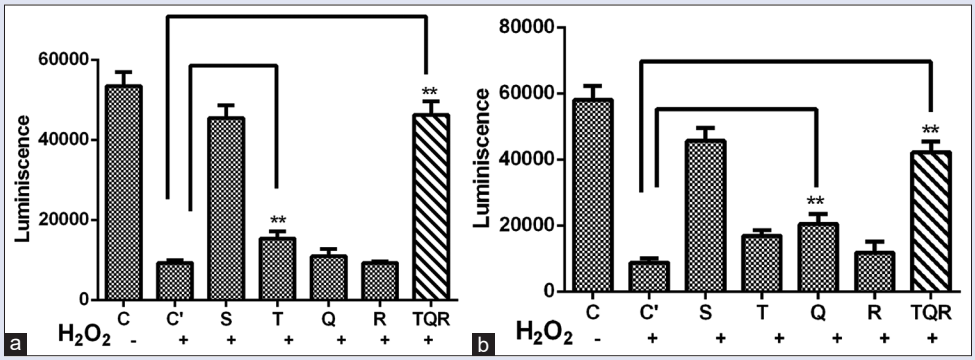

Figure 4: The turmeric, pagoda tree seed pod (quercetin), and rosemary blend protect HepG2 cells from cellular toxicity mediated by oxidative stress. Pretreatment of HepG2 cells in (a): Turmeric, pagoda tree seed pod (quercetin), and rosemary (12.5 µg/mL) and individual extracts turmeric (1.4 µg/mL), quercetin (4.2 µg/mL), and rosemary (6.9 µg/mL); in (b): Turmeric, pagoda tree seed pod (quercetin), and rosemary (25 µg/mL) and individual extracts turmeric (2.8 µg/mL), quercetin (8.4 µg/mL), and rosemary (13.8 µg/mL) for 24 h protects cellular viability when challenged for 4 h with 5 mM H_2O_2. S: 10 µM sulforaphane, positive control, C`: Challenged control, C: Untreated control $^{**}P \leq 0.01$ when compared to the challenged control (c`) by ANOVA using GraphPad prism 6.00

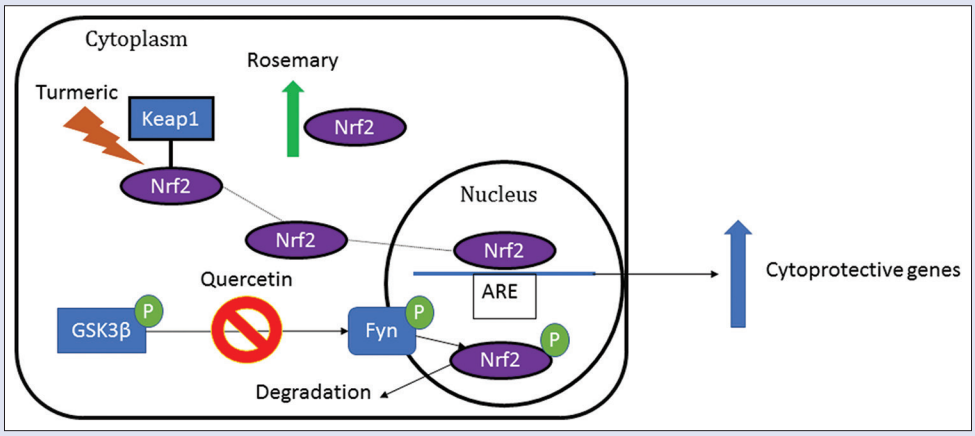

Figure 5: Pathways that may be modulated by turmeric, quercetin, and rosemary

enzymes.[13] Phytochemicals have been shown to induce changes in the NRF2-pathway by multiple mechanisms, enabling it to bind to the ARE promoter sequence to induce antioxidant gene expression.[45-47]

In this manuscript, the blend of botanical extracts consisting of TQR (1:3:5, w:w:w), shown previously to synergistically activate the NRF2 pathway,[28] has been further investigated to show cellular benefits in the presence of ROS. Evidence is provided for TQR activation of NRF2 pathway by showing that the blend activates the genes HO-I and GCLM subunit that are regulated by NRF2. Next, the positive consequences of NRF2 pathway activation by TQR are shown. Data are provided to show DNA and lipid protection capability against oxidative stress as well as evidence for whole cell protection against oxidative toxicity.

Various components of TQR act through different and complementary mechanisms of action to increase NRF2 activity and thereby increase downstream cytoprotective proteins. Curcumin in turmeric extract contains Michael acceptor groups that could modify cysteine sulfhydryls in the Keap1, causing the dissociation between Keap1 and NRF2.[48] Treatment with pagoda tree pod seed decreased the expression of GSK3 β, a kinase responsible for phosphorylating Fyn, a protein that shuttles NRF2 out of the nucleus for degradation. Decreasing the nuclear export leads to a buildup of nuclear NRF2.[49,50] Finally, carnosol, a component of rosemary has been shown to upregulate translation of NRF2 protein[51] A summary of these activities is presented in Figure 5.

CONCLUSION

The botanical blend (TQR) protected human liver cells against oxidative stress by preventing DNA damage, isoprostane formation, and cellular necrosis. This may be due to activation of NRF2 pathway.

Acknowledgements

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Nil.

Conflict of interest

There are no conflicts of interest.

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