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ARTICLE

In Vitro Evaluation of Antioxidants of Fruit Extract of *Momordica charantia* L. on Fibroblasts and Keratinocytes

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The antioxidant activity of the total aqueous extract (TAE) and total phenolic extract (TPE) of *Momordica charantia* fruits was assayed by radical-scavenging methods and cytoprotective effects on hydrogen peroxide (H_2O_2)- and hypoxanthin−xanthin oxidase (HX−XO)-induced damage to rat cardiac fibroblasts (RCFs), NIH 3T3, and keratinocyte (A431). Cell viability was monitored by a 3-[4,5-dimethyltriazol-2-yl]-2,5-diphenyltretrazolium (MTT) assay. For fibroblasts, TPE at 200 and 300 μg/mL showed maximum and consistent cytoprotection against oxidants. The extract at 50 μg/mL also had significant and slightly protective effects on fibroblasts against H_2O_2- and HX−XO-induced damage, respectively. RCF was more tolerant toward the damage. For keratinocytes, a dose-dependent relationship of oxidant toxicity was only seen with H_2O_2 but the protective action of the extract correlated with oxidant dosage. At 200 and 300 μg/mL TPE, cytoprotection was dose-dependent against oxidants. Extracts had no effect on HX−XO toxicity at 50 μg/mL. Pretreatment with both the extracts did not show any cytoprotection.

KEYWORDS: Rat cardiac fibroblasts; NIH 3T3; A431; *M. charantia* fruit; oxidative damage; cytoprotection; radical scavenging

INTRODUCTION

Momordica charantia L. (bitter melon, bitter gourd, or karela in Hindi) has long been regarded as a food and medicinal plant. It is a plant native to the semi-tropical climate of China, India, Asia, and Africa, bears fruits that are used as medicinal herbs for anti-HIV, anti-ulcer, anti-inflammatory, anti-leukemic, anti-microbial, anti-diabetic, antioxidants, and anti-tumor, to name a few (*1*−*5*), and is one of the most promising alternative medicines for the diseases.

Oxidants and antioxidants play very important roles in tissue repairs and various metabolic disorders, such as diabetes, because persistent hyperglycemia may cause a high production of free radicals. Free radicals are known to be the major cause of other diseases, including aging, coronary heart disease, inflammation, stroke, cancer, and various chronic and degenerative disorders (*6*, *7*). Antioxidants, on the other hand, significantly prevent tissue damage and stimulate tissue regeneration. Reactive oxygen species (ROSs) include free radicals, such as superoxide anion and hydroxyl radical, and other nonradical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen, can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes (*8*, *9*). The tissue injury caused by ROSs may include DNA damage (*9*, *10*), protein damage (*11*), and oxidation of important enzymes (*12*) in the human body. These events could consequently lead to the occurrence of various free-radical-related diseases.

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Recently, natural foods and food-derived antioxidants, such as vitamins and phenolic phytochemicals, have received growing attention, because they are known to function as chemopreventive agents against oxidative damage and are considered beneficial for human health, decreasing the risk of degenerative diseases by the reduction of oxidative stress and inhibition of macromolecular oxidation (*13*, *14*).

The bitter gourd, a commonly used vegetable reported to have antioxidant activities, is not yet authenticated by employing cell models. The purpose of this present study was to investigate the antioxidant activity of phenolic compounds of bitter gourd fruits using cell-based assays and to evaluate the same using different *in vitro* chemical methods. Aqueous and phenolic extracts of *M. charantia* L. fruit were prepared according to traditional food and medical practices in an attempt to make systematic comparisons among their antioxidant activities and identify the extracts with high antioxidant activity.

MATERIALS AND METHODS

Chemicals and Reagents. All cell-culture supplements were purchased from Life Technologies, Carlsbad, CA. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, ethylenediaminetetraacetic acid (EDTA), and phenolic acids [tannic acid, gallic acid, and (+)-catechin] were obtained from Fluka (Neu-Ulm, Germany). All other chemicals and organic solvents used were of the highest analytical grade.

Plant Material. The fruits of *M. charantia* L. were collected from a local vegetable market, Chennai, Tamil Nadu, India, and authenticated by a pharmacognosy expert, Head of the Department of Botany, CLRI, Chennai, India, before subjecting it to extraction and phytochemical charecterization. The voucher specimens (MC1BPT) were identified and stored.

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Preparation of the Fruit Extract. A total of 0.5 kg of fresh fruit was chopped into small pieces and then homogenized in a commercial blender, followed by shade drying. The extracts prepared from the homogenized dried fruit powder were made by boiling in distilled water for 5 min. The ratio between the sample and extraction medium was 1:20. The resulted mixture was then centrifuged at 5000 rpm, and the supernatant was dried under reduced pressure (45−50 °C) to obtain total aqueous extract (TAE).

Phenolics Extraction. The phenolic compounds in investigated samples were extracted using the little modified procedure described previously (*15*). Each sample (5 g) was mixed with 50 mL of methanol/HCl (100:1, v/v); the mixture was then centrifuged at 5000 rpm; and supernatant was evaporated to dryness under reduced pressure (45−50 °C). The residue was redissolved in 25 mL of water/ethanol (80:20, v/v) and extracted 4 times with 25 mL of ethyl acetate. The organic fractions were combined, dried for 30−40 min with anhydrous sodium sulfate, filtered through a Whatman-40 filter, and evaporated to dryness under vacuum (45−50 °C) to obtain total phenolic extract (TPE). The residue was redissolved in 5 mL of methanol/water (50:50, v/v) and filtered through a 0.45 μm filter before injection (20 μL) into the high-performance liquid chromatography (HPLC) aperture.

Determination of Total Phenolic Content (TPC). The TPC was determined using the Folin−Ciocalteu reagent (*16*). The reaction mixture contained 1 mL of extract, 0.5 mL of the Folin−Ciocalteu reagent, 3 mL of 20% sodium carbonate, and 10 mL of distilled water. After 2 h of reaction at 37 °C, the absorbance at 765 nm was measured and used to calculate the phenolic content, using gallic acid as a standard. The TPC was then expressed as gallic acid equivalents (GAE) in mg/g of dry sample.

HPLC Analysis of Phenolic Compounds. HPLC analysis was performed with a C-18 Inertsil ODS-3 column (5 μm particle, 4.6 × 250 mm inner diameter). The composition of solvents and used gradient elution conditions were described previously (*15*). The solvent system used was a gradient of mobile phase A containing 3% acetic acid in water; solution B contained a mixture of 3% acetic acid, 25% acetonitrile, and 72% water. The following gradient was used: 0−3 min, from 100% A to 30% A and 70% B with a flow rate of 1 mL/min; 3−6 min, from 30% A and 70% B to 20% A and 80% B with a flow rate of 1 mL/min; 6−9 min, from 20% A and 80% B to 15% A and 85% B with a flow rate of 1.2 mL/min; 9−12 min, from 15% A and 85% B to 10% A and 90% B with a flow rate of 1.2 mL/min; and 12−15 min, 10% A and 90% B with a flow rate of 1.2 mL/min. The UV detection wavelength was 278 nm, and the sample injection volume was 20 μL at a 40 °C column temperature.

Hydroxyl Radical-Scavenging Activity. Hydroxyl radical-scavenging activity was determined by the previously described method (*17*). A solution of 0.2 mL of $FeSO_4 \cdot 7H_2O$ (10 mM) and EDTA (10 mM) was prepared in a screw-capped test tube, and 0.2 mL of 2-deoxyribose solution (10 mM), the sample solution (TAE), and phosphate buffer (pH 7.4, 0.1 M) were added to give a total volume of 1.8 mL. Finally, 200 μL of H_2O_2 solution (10 mM) was added to this reaction mixture, and then the whole mixture was incubated at 37 °C for 4 h. After the incubation, 1 mL each of a trichloroacetic acid solution (2.8%) and a thiobarbituric acid solution (1.0%) were added to the reaction mixture, the whole mixture was boiled for 10 min and cooled in ice, and its absorbance was measured at 520 nm. The •OH scavenging activity was calculated as the inhibition rate of 2-deoxyribose.

$$\text{•OH scavenging activity } (\%) = [1 - (A_s - A_o)/(A_c - A_o)] \times 100$$

where A_o is the absorbance with no treatment, A_c is the absorbance of the treated control, and A_s is the absorbance of the treated sample.

DPPH Radical-Scavenging Activity. The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the previously described method (*18*). TAE (0.1 mL) was added to 3 mL of 0.001 M DPPH in methanol. Absorbance at 517 nm was determined after 30 min, and the percent inhibition (quenching) of activity was calculated as $[(A_o - A_e)/A_o] \times 100$ (where A_o is the absorbance without extract and A_e is the absorbance with extract).

Cell Culture. To observe the protective effect of extracts on the damage induce by H_2O_2 and hypoxanthine−xanthine oxidase (HX−XO), rat cardiac fibroblasts (RCFs), NIH 3T3 fibroblasts, and keratinocyte A341 cell lines were used. Different concentrations of the extracts (TAE and TPE) were employed to observe their protective effects on the oxidant-induced damage.

Isolation and Culture of Cardiac Fibroblasts. Cardiac cells were obtained from neonate rats using standard collagenase digestion methods. Cardiac fibroblasts isolation from myocytes and subsequent preparation were performed as described previously (*19*). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (50 units/mL), and streptomycin (50 μg/mL) at 37 °C, humidified with 5% CO_2. These preparations contained more than 95% cardiac fibroblasts as determined by morphological appearance and immunocytochemical staining. Fibroblasts were used between passages 3 and 4 for the studies.

Culture of NIH 3T3 and A431 Cell Lines. The two cell lines were obtained from the National Center for Cells Science (NCCS), Pune, India, and the cultures were maintained in DMEM with 10% FCS supplemented with penicillin (120 units/mL), streptomycin (75 mg/mL), gentamycin (160 mg/mL), and amphotericin B (3 mg/mL) at 37 °C, humidified with 5% CO_2.

Experimental Design for Antioxidant Effects on Cells. To assess the protective effects of the extracts on cells, the previously described methods (*20*, *21*) were employed to induce damage by H_2O_2 and HX−XO. Rat cadiac fibroblasts, NIH 3T3 fibroblasts, or epidermal keratinocytes (A431) were seeded in 96-well plates at a density of 6000 cells/well. Cells were grown to near confluence. To investigate the protective or scavenging activities of the extracts, different protocols were used: cells were washed with phosphate-buffered saline (PBS), and the different concentrations of the extracts (TAE and TPE) and H_2O_2 or HX−XO in Hank's buffered salt solution (HBSS) were added simultaneously to cells. The controls were optimal and normal cell culture without the addition of extracts or oxidant agents and cells exposed to oxidative damage without the presence of extracts. In the pretreatment experiments, fibroblasts or keratinocytes were incubated with the extracts overnight. After the removal of media from the plates, cells were exposed to H_2O_2 (1×10^{-4} or 2×10^{-4} mol/L) or HX−XO (1×10^{-2}, 2×10^{-2}, or 4×10^{-2} units/mL) in HBSS with or without the presence of extracts. Cells were incubated for 3 h and washed twice with PBS, and fresh media were added to the wells. The 3-[4,5-dimethyltriazol-2-yl]-2,5-diphenyltretrazolium (MTT) assay was used for the assessment of both cell damage and the effects of the extracts on the extent of oxidative damage (*22*).

Statistical Analysis. Each experiment was performed at least 3 times. The sets of five wells for the MTT assay were used for each concentration of extracts, H_2O_2, and HX−XO or combinations of these compounds. Results are expressed as mean ± standard error of the mean (SEM) of the absorbance. Data were analyzed by one-way analysis of variance (ANOVA) and Scheffe's test using the SPSS for Windows, version 7.5.1. Differences at the 95% level were considered to be significant.

RESULTS AND DISCUSSION

Several methods have been used to determine the antioxidant activity of plants. The purpose of this present study was to investigate the antioxidant activity of phenolic compounds of bitter gourd fruits using cell-based assays (e.g., NIH 3T3 fibroblasts, Rat cardiac fibroblasts, and A431 keratinocytes) and evaluate the same using different *in vitro* chemical methods (e.g., DPPH radical-scavenging activity and hydroxyl radical-scavenging activity).

Plant phenols present in fruits and vegetables have received considerable attention because of their potential antioxidant activity (*23*). The TPC in comparison to standard gallic acid was found to be 324 ± 1.63 mg of GAE/g of dry sample. The HPLC chromatogram of TPE showed eight peaks along with the chromatogram of standards (viz. gallic acid, tannic acid, and catechin), showing the presence of these components with some unidentified components in the sample (**Figure 1**). Respectively, gallic and tannic acids contributed as second and fourth major constituents of TPE, while cetechin was one of the minor constituents.

The hydroxyl radical is the most reactive among ROSs, and it bears the shortest half-life compared to other ROSs and induces

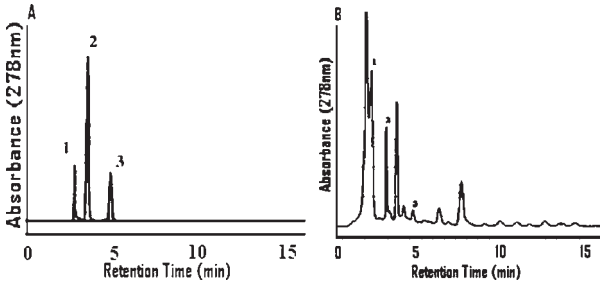

Figure 1. HPLC chromatogram of (A) standards and (B) phenolic compounds in bitter gourd fractions of green fruit. Detection was at 278 nm. Peak 1, gallic acid; peak 2, tannic acid; and peak 3, (+)-catechin.

Table 1. Antioxidant Activity of TAE on DPPH and H_2O_2 (HO•)

concentration of TAE (μg/mL)	percent inhibition of DPPH (M ± SD)	percent inhibition of HO• (M ± SD)
100	28.32 ± 1.3	26.5 ± 0.17
200	56.4 ± 0.52	51.4 ± 0.17
500	89.8 ± 0.41	83.5 ± 0.31
1000	92.7 ± 0.33	89.7 ± 0.23
2000	94.9 ± 0.38	91.4 ± 0.43

severe damage to adjacent biomolecules (*24*). The scavenging abilities of bitter gourd fruit extract (TAE) on hydroxyl radical inhibition by the 2-deoxyribose oxidation method are shown in **Table 1**. 2-Deoxyribose is oxidized by the Fenton reaction and degraded to malondialdehyde (*17*). The results are indicated as the inhibition rate increased with an increasing concentration, and more than 90% inhibition was found at 2000 μg/mL TAE.

The hydrogen atom or electron-donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of a purple-colored methanol solution of DPPH (*25*). DPPH is a free radical compound with a characteristic absorption at 517 nm, and as antioxidants donate protons, they neutralize DPPH, resulting in decreased absorption. Inhibition of DPPH by TAE was dose-dependent (Table**1**). There was almost 95% inhibition at 2000 μg/mL TAE.

In various *in vitro* studies of antioxidants on the cell models, fibroblasts have been used successfully (*26*, *27*). Although, cells were always incubated until near confluence (about 90%) before the assay were carried out, the number of cells were not exactly reproducible because values caused by each experimental condition varied. Different protocols were used to assess the effects of the extracts on H_2O_2- and HX−XO-induced damage.

In a first set of experiments, both fibroblasts, namely, NIH 3T3 and RCFs, were exposed to H_2O_2 at concentrations of 1×10^{-4} or 2×10^{-4} mol/L and to HX−XO at concentrations of 1×10^{-2}, 2×10^{-2}, or 4×10^{-2} units/mL with the simultaneous addition of the extracts. The concentrations of the TPE at 50, 100, 200, and 300 μg/mL and catalase at 250 units/mL showed almost complete protection of fibroblasts against H_2O_2 damage in two different doses. **Figures 2** and **3** show the protective effect of TPE against 2×10^{-2} units/mL H_2O_2.

In a second set of experiments, fibroblasts were preincubated with the extracts at a concentration of 300 μg/mL overnight before exposure to the same concentration of the extracts and H_2O_2 (1×10^{-4} mol/L). Fibroblasts were completely protected by the simultaneous addition of extracts from damage, while there was about 50% damage in cells with no extract or those only pretreated (**Figures 2** and **3**).

Xanthine oxidase catalyzes the conversion of oxygen to the superoxide anion, and this happens mostly in the case of chronic wound, ischemia, and tissue hypoxia (*28*). Three doses of xanthine

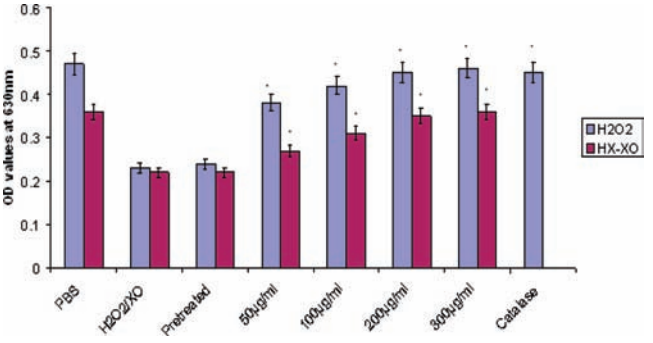

Figure 2. Protective effect of the TPE on H_2O_2 (2×10^{-4} mol/L) and HX−XO (2×10^{-2} units/mL xanthine oxidase) induced damage to RCFs. Bars represent mean ± SEM of five wells.

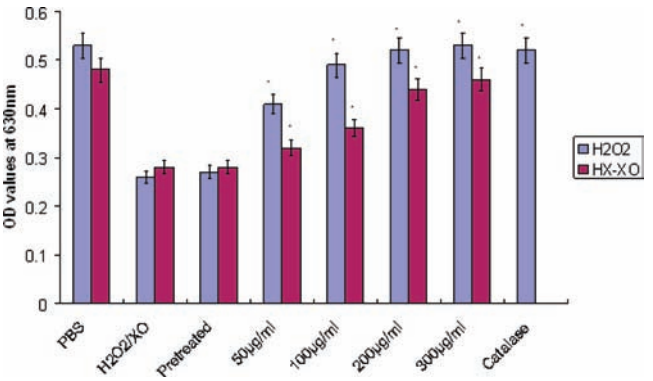

Figure 3. Protective effect of the TPE on H_2O_2 (2×10^{-4} mol/L) and HX−XO (2×10^{-2} units/mL xanthine oxidase) induced damage to NIH 3T3 fibroblasts. Bars represent mean ± SEM of five wells.

oxidase at 1×10^{-2}, 2×10^{-2}, or 4×10^{-2} units/mL were incubated with fibroblasts. The concentration at 1×10^{-2} units/mL did not cause statistically significant damage to cells (data not shown). Almost 60% fibroblasts were killed by xanthine oxidase at 2×10^{-2} units/mL (**Figures 2** and **3**), while 70% damage was observed with 4×10^{-2} units/mL (data not shown). TPE at 300 μg/mL was shown complete protection to fibroblasts against damage caused by the HX−XO reaction at 2×10^{-2} units/mL (**Figures 2** and **3**). Microscopically, 50 μg/mL TPE also showed slight protection but did not reach significant difference (data not shown).

The cardiac fibroblasts were more tolerant toward the xanthin oxidase in comparison to NIH 3T3 fibroblasts (**Figures 2** and **3**). This may be due to the difference in the source of origin, because the RCF has been isolated from a neonatal rat that may have been exposed to oxidants or antioxidants that could have upregulated the gene responsible for antioxidative enzymes (e.g., superoxide dismutase). In previous reported work (*29*), the extracts were found to restore antioxidant enzyme superoxide dismutase (SOD). This may be hypothesized that, because of pre-accumulation or upregulation of antioxidative enzymes, RCF could become more tolerant than NIH 3T3.

Keratinocytes are one of the constituents of the skin and have popularly been used for *in vitro* evaluation of antioxidants (*26*, *27*). Topical application of compounds with free-radical-scavenging properties on patients or animals has been used to significantly improve wound healing and protect tissues from oxidative damage (*30*). Unlike fibroblasts, the response of keratinocytes to oxidants or extracts was more complicated. The concentration of TPE at 50 μg/mL had basically no effect on keratinocytes against HX−XO-induced damage. Keratinocytes damaged by 1×10^{-4} mol/L H_2O_2 were completely protected by the TPE at 100, 200, and

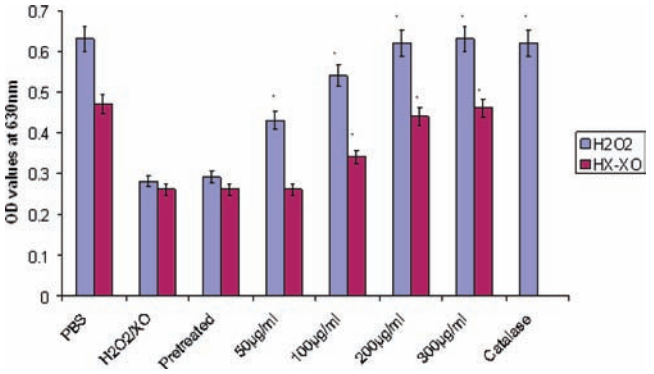

Figure 4. Protective effect of the TPE on H_2O_2 (2×10^{-4} mol/L) and HX−XO (1×10^{-2} units/mL xanthine oxidase) induced damage to keratinocytes (A431). One set of cells was incubated with catalase for comparison. Bars represent mean ± SEM of five wells.

300 μg/mL (data not shown). When the concentration of H_2O_2 was doubled to 2×10^{-4} mol/L, only about 60 and 70% keratinocytes survived under the 50 and 100 μg/mL treatment of the TPE but 100% of cells were protected under 200 and 300 μg/mL of TPE and 200 units/mL of catalase treatment (**Figure 4**).

All of the concentrations of xanthine oxidase at 1×10^{-2}, 2×10^{-2}, or 4×10^{-2} units/mL caused severe damage to keratinocytes. In the concentrations of 2×10^{-2} and 4×10^{-2} units/mL, only 20 or 15% of cells were alive in the group without either extract added (data not shown), whereas 60−70% of cells in 100 μg/mL and almost 95−100% of cells in 200 and 300 μg/mL survived under the protection of the TPE after 50% damage by 1×10^{-2} units/mL HX−XO (**Figure 4**). When the dose of xanthine oxidase was increased to 4×10^{-2} units/mL, the extract at 200 μg/mL failed to protect cells and there were only about 60% of living keratinocytes in the 300 μg/mL treatment group (data not shown).

Although we found that TPE was more cytoprotective than that of TAE, the differences were not statistically significant. Moreover, at higher concentrations (500 and 600 μg/mL), TPE was toxic to the cells, while TAE did not show any toxic effect at the same dosage level (data not shown).

In conclusion, the present study has demonstrated that bitter gourd (*M. charantia*) fractions are rich in phenolics, having a strong antioxidant activity and a radical-scavenging action in all of the tested methods. This also suggests that bitter gourd is a good source of natural antioxidants and has the potential beneficial activity of extracts using *in vitro* models to study the activity of cells involved in wound healing and cardioprotection. It also provides a rationale for the wide use of *M. charantia* preparations as antioxidant formulations.

ABBREVIATIONS USED

TAE, total aqueous extract; TPE, total phenolic extract; H_2O_2, hydrogen peroxide; HX−XO, hypoxanthine−xanthine oxidase; RCF, rat cardiac fibroblast; PBS, phosphate-buffered saline; HBSS, Hank's buffered salt solution; ROS, reactive oxygen species; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; NCCS, National Center for Cells Science; MTT, 3-[4,5-dimethyltriazol-2-yl]-2,5-diphenyltretrazolium; SOD, superoxide dismutase.

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