

Phytochemical Composition of Extracts Prepared from Dietary Cucurbits and their Cytoprotective Efficacies against Hydrogen Peroxide Induced Oxidative Stress in GI Cell-INT407

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Abstract

Compositions of phytochemicals in aqueous extracts prepared from *Lagenaria siceraria* (L_s), *Luffa cylindrica* (L_c) and *Cucurbita maxima* (C_m) fruits were identified by gas chromatography and mass spectroscopy. GC-MS analysis showed the presence of 83, 72 and 74 number of phytochemicals in L_s , L_c and C_m respectively. Extracts were evaluated for their role in protection of INT407 cells against injurious effect of H_2O_2 . MTT, LDH and colony survival assays (CSA) were employed for evaluating H_2O_2 -induced cytotoxicity. Antioxidant properties of extracts were evaluated by enzymatic assays in H_2O_2 induced oxidative stress in INT407 cells. LD50 and LD25 of H_2O_2 at 24 h on INT407 cells were evaluated and found 114.25 ± 0.24 and 64.01 ± 0.11 μ M respectively. Macromolecules were found to protect INT407 in dose dependent manner ($p \leq 0.05$). CSA showed enhanced growth in extracts of *Lagenaria siceraria* and *Luffa cylindrica* in comparison to *Cucurbita maxima* treated cells ($p=0.042$). H_2O_2 treated cells showed depleted superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities by 71.60, 68.81, 45.79 and 22.95% respectively. *Lagenaria siceraria* and *Luffa cylindrica* treated cells showed modulation of SOD by 8.88 and 8.95%, CAT by 22.34 and 14.20%, GPx by 5.56 and 1.42%, and GR by 2.20 and 3.22% respectively. *Cucurbita maxima* modulated CAT by 1.78%, and GR by 0.70%. Synergistic effects of phytochemicals present in these extracts were found non-toxic on INT407.

Keywords: Anti-oxidant; Gut; Cytotoxicity; DNA damage; ROS

Introduction

Plants of dietary nature contain rich sources of nutrients and secondary metabolites of therapeutic importance. Cucurbits belong to Cucurbitaceae family and are rich sources of nutritional and medicinal compounds [1-4]. Cucurbitaceae consists of nearly 100 genera and over 750 species [5-8]. Dietary intervention of these groups of plants can ameliorate various types of diseases through their prebiotics, modulation of proton pumping ATPase activity, antioxidant and immunomodulatory actions [1,2,4,9]. Despite of having immense potential to augment human health through their nutritional and medicinal effects [1-7], these group of plants have yet to be considered as potential therapeutic modalities by pharmaceutical industries and biomedical research funding agencies.

Gastrointestinal tract is constantly under threat of oxidative damage. Oxidative stress generated exogenously and endogenously in luminal as well as in the cellular environment of the gut is linked with genesis of various diseases including cancer. Reactive oxygen species (ROS) are frequently produced in the GI tract and is linked with the induction of inflammatory conditions [10,11]. Consequences are the manifestation of inflammatory bowel syndrome (IBS), diabetes, cancer and several other pathological conditions in the gut and associated organs [12-14]. Influx of immunoactive cells such as leukocytes, neutrophils and monocytes (associated with inflammation) may produce further ROS via respiratory burst enzymes as well as those involved in prostaglandin and leukotriene metabolism [15]. Therefore, protection of gut from ROS mediated induction and progression of pathogenesis is mandatory for better health. The living system has been evolved with endogenous antioxidant defense mechanism to quench leaked and free oxidant species [16]. Dietary intervention of antioxidant rich diet is the most important strategy to modulate antioxidant defense system.

Dietary cucurbits have been reported to contain adequate amount of secondary metabolites of excellent antioxidant activities [3-5].

Cucurbitacins, alkaloids, flavonoids, steroids, ribosome inactivating proteins and several other bioactive constituents of nutritional and pharmacological importance of cucurbits have been reported and reviewed [1,17,18]. For example, *Lagenaria siceraria* exhibits immune modulation, antioxidants and anticancer properties [19]. *Luffa cylindrica* acts as antimicrobial, antioxidants and anti-inflammatory agents [20]. *Cucurbita maxima* is known for its hepatoprotective and antioxidants activities [21]. These dietary cucurbits (Figure 1) modulate gut functions directly and through enteric microbial symbionts indirectly. Recently, we have reported the modulation of proton pumping ATPase activity of probiotic Lactobacilli by dietary Cucurbits [2]. In its continuation, the present study is designed to investigate the presence of phytochemicals and their mechanistic role in antioxidant defense system against hydrogen peroxide (H_2O_2) induced oxidative stress using INT407 as a model cell line of human gastrointestinal tract. There are numerous studies which support INT407 as normal intestinal cells [22-24]. Further it is important to mention that INT407 cells show the characteristic properties of normal cell as optimized during the present study. This cell line maintains their actual morphology only for some passages (about 20-30) which is characteristic properties of normal cells whereas cancer cell line like Hela, HepG2 and HT29 show continued growth without losing their morphology for uncountable

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Received November 09, 2016; Accepted January 27, 2017; Published February 03, 2017

Citation: Ahmad I, Irshad M, Zafaryab M, Asad Khan M, Syed HM, et al. (2017) RPhytochemical Composition of Extracts Prepared from Dietary Cucurbits and their Cytoprotective Efficacies against Hydrogen Peroxide Induced Oxidative Stress in GI Cell-INT407. J Plant Biochem Physiol 5: 181. doi: 10.4172/2329-9029.1000181

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Figure 1: Dietary cucurbits used in the present study. Ls: *Lagenaria siceraria*; Lc: *Luffa cylindrica* and Cm: *Cucurbita maxima*

passages. Therefore, INT407 was screened as the most suitable normal intestinal cell line for the present as well as several other studies underway in our laboratory.

Materials and Methods

Chemicals and reagents

Growth medium and other constituents for cell culture were purchased from HiMedia (Mumbai, India). Tissue culture flasks and microtiter plates were procured from Nunc (USA). LDH kit was purchased from G-Biosciences (USA). All other chemical constituents were purchased from Sigma (Germany) and Merck (Mumbai, India).

Extract preparation

Lagenaria siceraria (Ls), *Luffa cylindrica* (Lc) and *Cucurbita maxima* (Cm) were purchased from local market and their generic identification was authenticated by Prof. Santosh Kumar (Department of Botany, BRA Bihar University, Muzaffarpur, India). Fruits were washed, homogenized in triple distilled water and filtered by muslin cloth. Centrifuged at 6000 rpm to remove particulate matters, lyophilized and finally got aqueous extract (AqE) of cucurbits. Samples were kept in vacuum desiccators for further studies.

Gas Chromatography and Mass Spectroscopy (GC-MS)

Identification of phytochemicals present in the extracts (Ls, Lc and Cm) was done by GC-MS analysis. Analytical gas chromatography was performed on Shimadzu-2010 system equipped with AB-WAX column (30 m × 0.25 mm thick with 0.25 μm internal diameter). Helium gas was used as the carrier (1 mL/min). The column temperature was programmed at 6000°C for 1 min and then raised to 1800°C for the total analysis time of 60 min. Sample (0.1 mL) was injected in the splitless mode. The chemical components were identified by comparing the retention time of the chromatographic peaks with that of authentic compound using the WILEY8.LIB and NIST05s.LIB. A list of 25 compounds was provided for each spectral peak of GC-MS Chromatogram. Only one compound from this list was carefully selected based on possible presence of this compound in similar plant group already reported in the published phytochemical research publications.

Cell line and culture conditions

Human intestinal cell (INT407) was procured from National Centre for Cell Sciences, Pune, India (NCCS, Pune, India). Cells were grown in Dulbecco's Modified Eagle's Medium containing 10% fetal

bovine serum and antibiotics (100 U/mL Penicillin and 100 mg/L Streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C in T-75 flasks. Cells were sub cultured twice a week to optimize and retain their actual morphology.

MTT assay

Cytoprotective activity of cucurbit extracts against H₂O₂ induced oxidative stress was assessed by MTT assay [5,22]. Briefly, cells (~2 × 10⁴ cells/well) were seeded into 96 well plate and culture overnight next day, cells were treated with H₂O₂ (LD₂₅=64.1 μM) alone and along with various concentrations of AqE of cucurbits (0-300 μg/mL) and incubated for next 24 h. AqE (0-300 μg/mL) in INT407 without H₂O₂ treatment was also performed for cytotoxic/cytoproliferative profiling of the extracts. Cells were then incubated in 20 μl of MTT (5 mg/mL in PBS) in fresh medium for 4 h at 37°C. Formazan crystals formed by the live cells were solubilized in DMSO (200 μL/well) and the plate were read at 570 nm using iMark Microplate Reader (Bio-Rad, USA). Positive control was used with extract treated cells and negative control was only H₂O₂ treated cells.

Percentage cell viability was calculated:

$$\% \text{ Cell Viability} = \frac{\text{Control} - \text{Experimental (OD}_{570})}{\text{Control (OD}_{570})} \times 100$$

Lactase Dehydrogenase (LDH) assay

Cytoprotective activity of extracts against H₂O₂ induced oxidative damage was further assessed by the lactate dehydrogenase (LDH) enzyme leakage in the culture medium. Similar to MTT assay, cells were seeded in 96 well plate (~2 × 10⁴ cells/well) overnight and treated with H₂O₂ (LD₂₅=64.1 μM), thereafter, treated with extracts (0-300 μg/mL) for 24 h. The LDH enzyme activity in culture medium was determined by the Cytoscan[™] LDH Cytotoxicity Assay Kit (G-Biosciences, USA) according to the manufacture instruction.

Colony survival assay

Colony survival assay reconfirmed cell viability [5]. Cells (3 × 10⁴ cells) were seeded in 12 well flat bottomed microtiter plates for overnight. Cells were treated with H₂O₂ (LD₂₅=64.1 μM), thereafter, treated with various concentration of extracts (0, 50, 100, 150, 200 μg/mL) for 24 h time point. After 24 hours, cells were washed with PBS and incubated with fresh growth medium. The medium was changed at the intervals of every 24 h for 3 days. Positive control was used without extract or H₂O₂ treated cells and negative control was H₂O₂

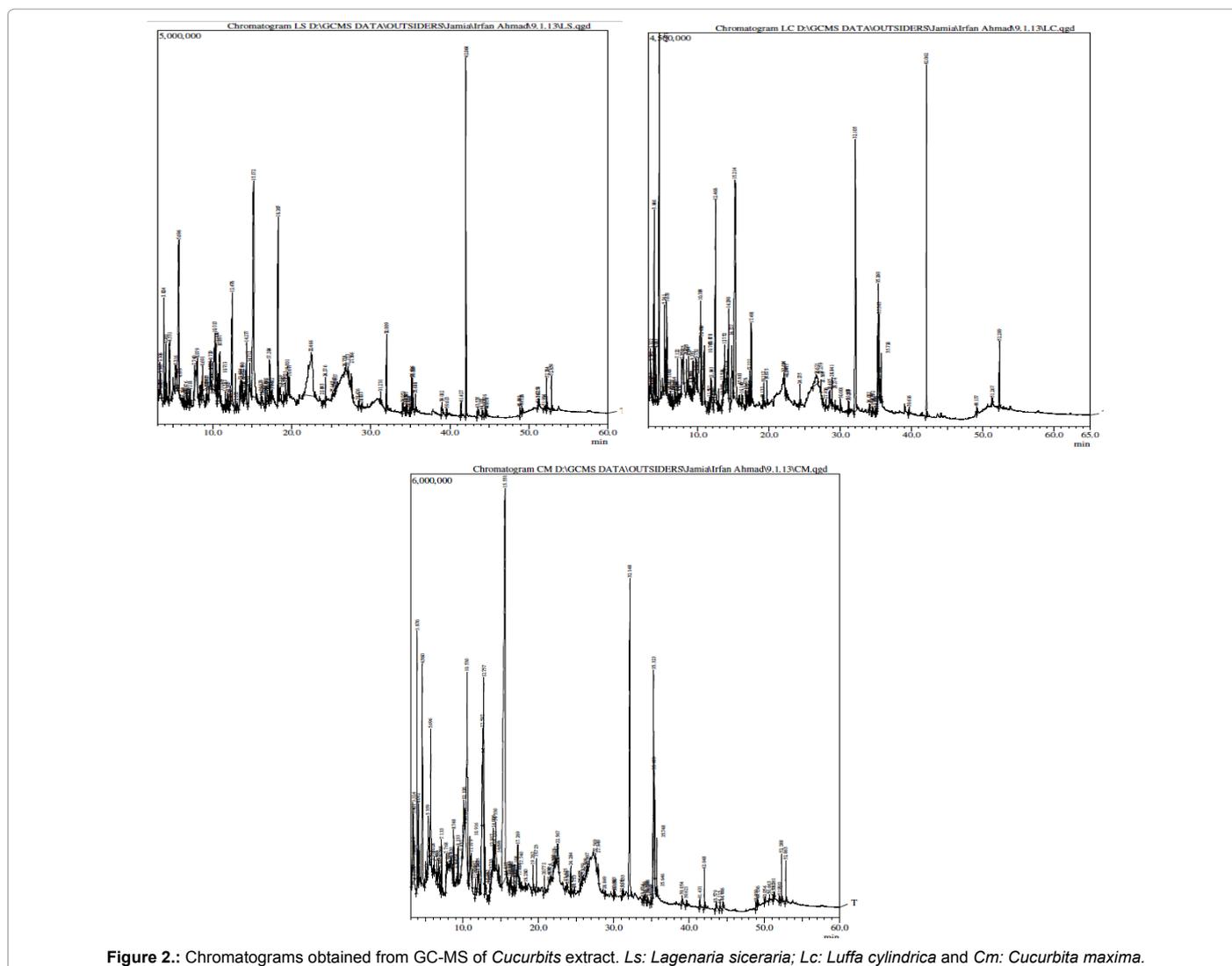


Figure 2.: Chromatograms obtained from GC-MS of Cucurbits extract. Ls: *Lagenaria siceraria*; Lc: *Luffa cylindrica* and Cm: *Cucurbita maxima*.

treated cells. At the end, cell colonies were counted under the inverted microscope (Motic, China). Colonies (>30 cells/colony) were counted and compared with control (untreated) colonies. Percentage survival colonies were calculated.

Antioxidant Assays

Preparation of cell free extract (CFE)

Overnight seeded INT407 cells in 25 mm culture plate were treated with H_2O_2 ($LD_{25}=64.1 \mu M$) for 2 h and thereafter various concentration of extracts (0-200 $\mu g/mL$) was added and incubated for further 24 h separately for each extract. At the end, cells were scraped and washed with PBS at 4°C. Cell free extract (CFE) was prepared by mechanical disruption at 4°C using homogenizer buffer (1 mmol/L phenyl methyl sulphonyl fluoride, 250 mmol/L sucrose, 10 mmol/L Tris-HCl, pH 7.5) and 0.45-0.50 μm diameter glass beads in a homogenizer equipped with CO_2 cylinder for cooling. Homogenate was centrifuged at $1000 \times g$ for 5 min at 4°C to remove glass beads. The pellet was re-centrifuged at $10000 \times g$ for 45 min in suspension buffer (10 mmol/L Tris-HCl, pH 7.5) to separate CFE in the supernatant. Total protein content in the CFE was estimated by Bradford assay (HiMedia, Mumbai, India). BSA (Bovine Serum Albumin) was used as standard. CFE isolated from

non-treated H_2O_2 cells was used as positive and H_2O_2 treated CFE as negative controls.

Catalase activity

Catalase (CAT) was measured by the method of Claiborn [23]. Briefly, reaction mixture containing 1.99 ml phosphate buffer (0.05 mol/L, pH 7.0), 1 ml H_2O_2 (0.019 mol/L), and 10 μl CFE (final volume 3 mL) was taken in a Quartz cuvette and absorbance was read at 230 nm using spectrophotometer. At intervals of 30 secs, depletion of absorbance due to disappearing H_2O_2 was monitored for 3 min. At the end activity of catalase enzyme was calculated and expressed as nmole H_2O_2 consumed per minute per mg protein.

Super oxide dismutase activity

Super oxide dismutase (SOD) was determined following the method adopted by Marklund and marklund [24]. SOD activity was measured with the help of spectrophotometer at 420 nm. Reaction mixture was prepared with 2.9 ml Tris buffer (50 mmol/l Tris and 1 mmol/l EDTA, pH, 8.5), 0.1 ml pyrogallol solution (20 mmol/l pyrogallol) and 0.1 ml CFE and a control without CFE. After an induction period of 90 secs, absorbance of control and treated samples were read respectively in

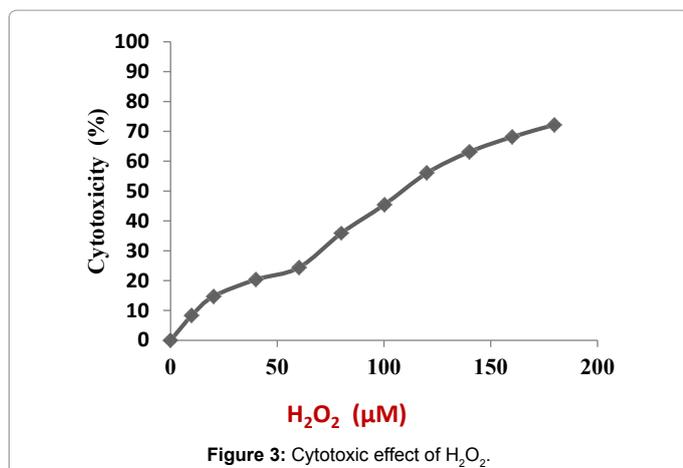


Figure 3: Cytotoxic effect of H₂O₂.

Compounds	L _s	L _c	C _m
Number of compounds	83	72	74
Major (>5%)	6	6	4
Minor (<5% but not >1%)	11	14	10
Compounds in trace amount (CTA) (1 or 1%)	66	52	60

Table 1: Compounds identified from extracts of cucurbit fruits.

Compound with (M. Formula)	L _s	L _c	C _m
2-Furancarboxaldehyde, 5-(hydroxymethyl)	16.4	13.01	-
2-Deoxy-D-galactose	10.5	-	0.12(T)
2, 5-Dimethyl-4-hydroxy-3(2H)-furanone	5.11	6.08	7.52
α-Angelic lactone	6.89	0.14(T)	-
4-Nitro-3-oxobutyric acid, methyl ester	5.84	9.07	3.61(M)
Phthalic acid dioctyl ester	6.66	-	0.49(T)
Palmitic acid	1.79 (M)	8.44	9.21
Furanmethanol	-	3.47(M)	5.42
2-Pyrimidinol	2.66(M)	-	6.76
Hydroxymethylfurfural	-	2.94(M)	28.3
2, 4-Dimethylimidazole	-	13.48	-

'M and T' denote minor compounds and compounds in trace amount respectively.

Table 2: Major compounds identified in cucurbit fruits (Numerical value expressed in percentage).

every 30 secs for 3 min at 420 nm. The induction period could achieve a steady state of authorization of pyrogallol. The rate of change in absorbance per minute was noted to calculate SOD activity.

$$\text{SOD activity} = \left[\frac{(A-B)/A}{100} \right] \times 10$$

Where A denotes: The difference of absorbance/min in the control sample,

B is the difference in absorbance/min in the treated sample, and 10 is the dilution factor.

Finally, results were expressed in units per milligram of protein.

Glutathione peroxidase

Glutathione peroxidase (GPx) was measured according to the procedure adopted by Mohandas et al. [25]. The reaction mixture containing 1.53 ml phosphate buffer (0.05 ml/l, pH 7.0), 0.1 ml glutathione (1 mmol/l), 0.1 ml of 0.2 mmol/l NADPH, 0.01 ml of 0.25 mmol/l H₂O₂, and 100 µl CFE in a final volume of 2.0 ml were measured in terms of the decrease in absorbance at 340 nm at an interval of 30 secs for 3 min at room temperature. At the end, enzyme activity was calculated and expressed as nmoles of NADPH oxidized per minute per milligram protein [25].

Glutathion reductase

The activity of glutathion reductase (GR) was monitored following the method adopted by Carlberg and Mannervik [26]. The assay mixture was placed in a 3 ml cuvette and consisted of 1.68 ml phosphate buffer (0.1 mol/l, pH 7.4), 0.1 ml 0.1 mmol/l NADPH (freshly prepared by dissolving 0.883 mg in 10 ml 0.1 mol/l phosphate buffer, pH 7.4), 0.1 ml 0.5 mmol/l EDTA (1.86 mg in 10 ml distilled water), 0.05 ml/l oxidized GSH disulphide (freshly prepared by dissolving 6.126 mg in 10 ml 0.1 mol/l phosphate buffer, pH 7.4), and 70 µl CFE in a total volume of 2 ml. Decrease in absorbance was measured at 340 nm at an interval of 30 sec for 3 min at room temperature. Enzymatic activity was calculated by measuring the disappearance of NADPH and results were expressed as nmoles of NADPH oxidized per minute per milligram of protein.

Statistical analysis

Results were expressed as mean ± SEM (n=3). Statistical significance was examined with Student's t test and p<0.05 was significant.

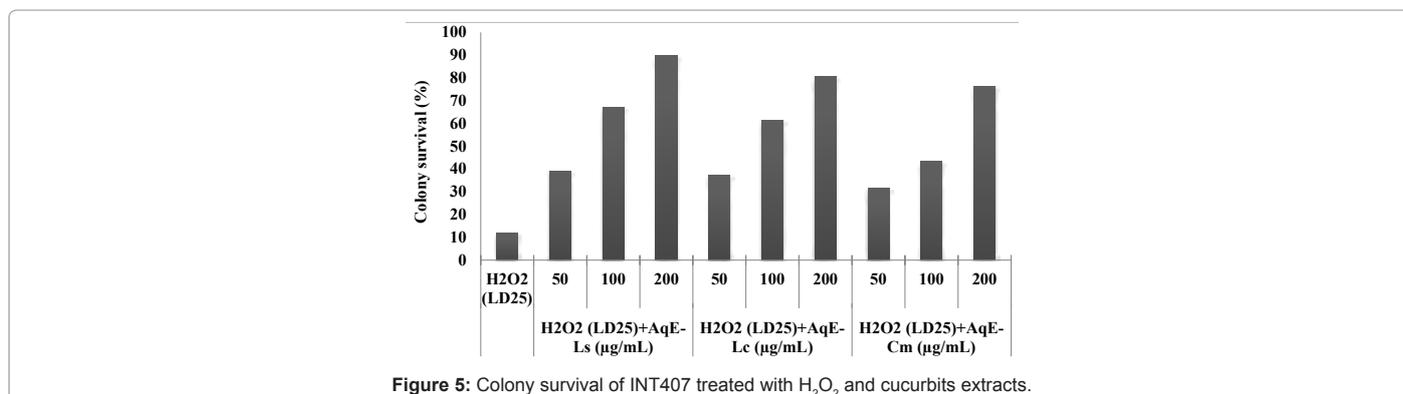
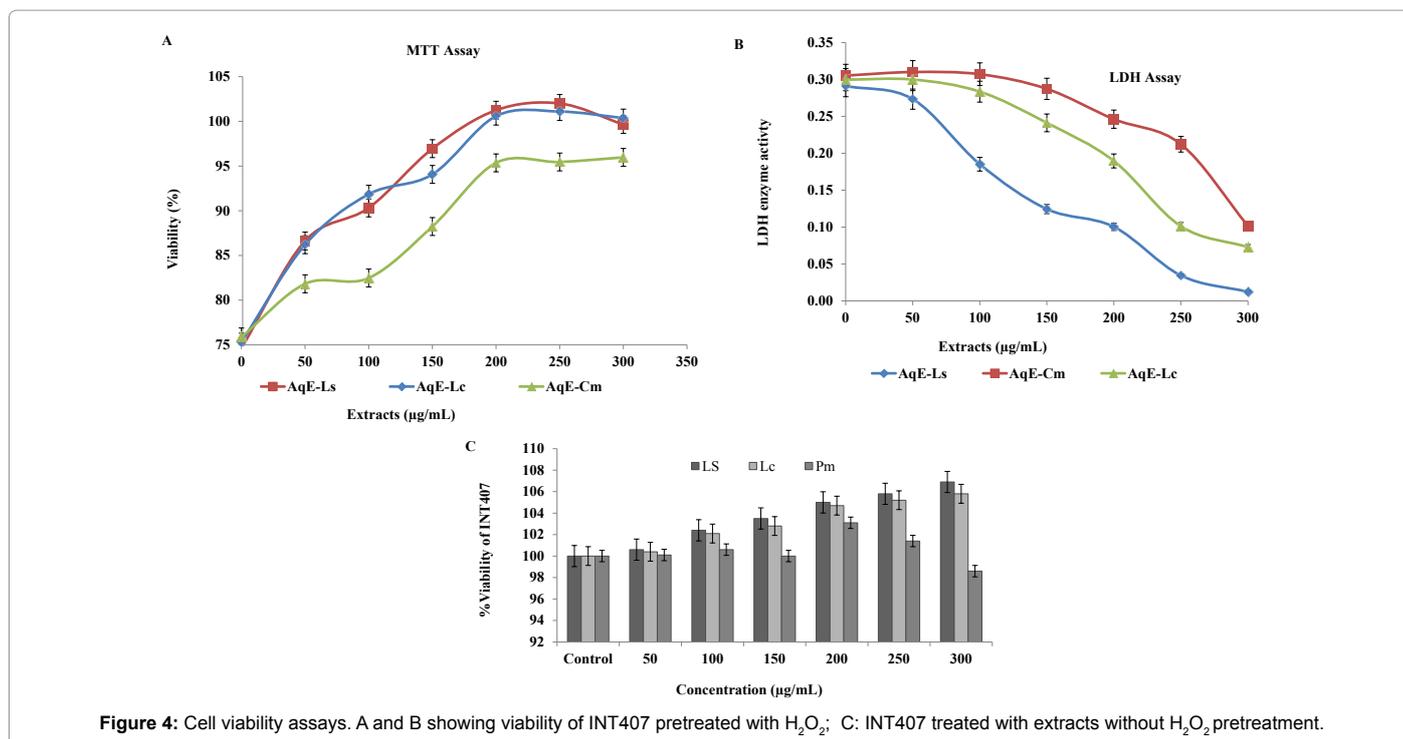
Results and Discussion

Comparative analysis of phytochemicals identified from three dietary cucurbits

Compounds in extracts were identified by the inbuilt library software WILEY08.LIB and NIST05.LIB in GC-MS apparatus. Our results showed that L_s, L_c and C_m contained 83, 72 and 74 compounds respectively. Figure 2 shows the GC-MS chromatogram L_s, L_c and C_m where the characteristic peaks represent the presence of a specific compound in the extracts. Tables 1 and 2 show the major compounds and essential fatty acids found to be present in L_s, L_c and C_m respectively. Complete list of compounds is available with us and may be provided if asked.

Cytoprotective efficacies of cucurbits

Lethal (LD50) and sub-lethal doses (LD25) of hydrogen peroxide (H₂O₂) against INT407 cells were found to be 114.12 ± 1.25 µM and 64.01 ± 0.91 µM respectively as determined by MTT and LDH assays (Figure 3). Exposure of H₂O₂ at the dose LD25 was found to inhibit cell growth by 25% after 24 h time point in comparison to control. This concentration (LD25) was considered as reference point for further evaluation of cyto-protective and anti-oxidant defense enzyme activities. Extracts in pre-H₂O₂ treated INT407 cells showed significantly cytoprotective or anti-cytotoxic effect as determined after 24 h (p<0.05) (Figure 4). There was no cytotoxic effect of the extracts itself as shown in Figure 4c. The trends of cytoprotection were found to be enhanced with increasing concentration of extracts. Cells showed exponential growth at the dose 200 µg/mL of extracts as compared to the control (untreated cells). It was observed that AqE-L_s and AqE-L_c showed more cytoprotective effect against H₂O₂ than the AqE-C_m. At the dose 100 µg/mL of AqE-C_m showed 82.48% viability, whereas AqE-L_s and AqE-L_c showed 93.26 and 91.85% in comparison to untreated control respectively. However, further increase of concentration (200 µg/mL) of AqE-L_s and AqE-L_c extracts showed plateau of cell growth, whereas AqE-C_m extract treatment showed marginally similar cell growth as compared to the control. At the concentration of 350 µg/mL of AqE-L_s, AqE-L_c and AqE-C_m showed cytotoxicity activity 6.74, 7.62 and 10.74 respectively. LDH enzyme activity was also decreased after the treatment of AqE-C_m at concentration of 50 to 300 µg/mL (Figure 4). The IC₅₀ of AqE-L_s, AqE-L_c, and AqE-C_m against INT407 cells were 718.54 ± 0.56, 704.43 ± 1.01, and 697.16 ± 0.93 µg/mL respectively.



Colony survival

Colony survival is one of the most important parameter to evaluate cell survivals *in vitro*. Results of this assay showed that H₂O₂ treated cells did not show growth however, simultaneous treatment of H₂O₂ and extracts showed cell survivals with formation of colonies (Figure 5). At 200 µg/mL of AqE-Ls, AqE-Lc and AqE-Cm treated INT407 cells showed growth of 89.7, 80.7 and 76.2% colonies respectively.

Aq-Ec-induced modulation of anti-oxidant enzymes activity

Antioxidant defense system involves scavenging of ROS generated within cells. In present study, exposure of H₂O₂ to INT407 cells showed depletion of endogenous anti-oxidant enzyme activities (Figures 6 and 7). As compared to untreated control, CAT, SOD, GPx and GR activities was found to be decreased by 68.81, 71.60, 77.12 and 25.89% respectively. Interestingly it was found that treatment of H₂O₂ with extract protect cells from depletion of antioxidant enzymes and helped in its modulation in concentration dependent manner (Figure 3).

Aqueous extracts of *Lagenaria siceraria* (AqE-Ls)

Treatment of AqE-Ls not only protect from the toxic effect of H₂O₂ on INT407 cells but also helped in modulation of antioxidant enzymes defense system (Figures 6 and 7). Treatment of AqE-Ls helped to inhibit antioxidant enzymes depletion in concentration dependent manner. As compared to untreated control, CAT enzyme activity was shown to be enhanced by 9.38 and 22.34% at the concentration of 150 and 200 µg/ml respectively. SOD enzyme activity was shown to increase by 7.93 and 8.88% at concentration of 150 and 200 µg/ml respectively. Similarly, GPx enzyme activity was found to increase by 1.62 and 5.56%, whereas, GR enzyme activity was found to increase by 1.53 and 2.20% at concentration of 150 and 200 µg/ml respectively.

Aqueous extracts of *Luffa cylindrica* (AqE-Lc)

Treatment of AqE-Lc with H₂O₂ showed protection from the depletion of antioxidant enzyme as compared to H₂O₂ negative control at concentration of 50 and 100 µg/mL whereas increase of antioxidant enzymes activities as compared to untreated control at concentration of 150 and 200 µg/mL (Figures 6 and 7). Treatment of AqE-Lc extracts

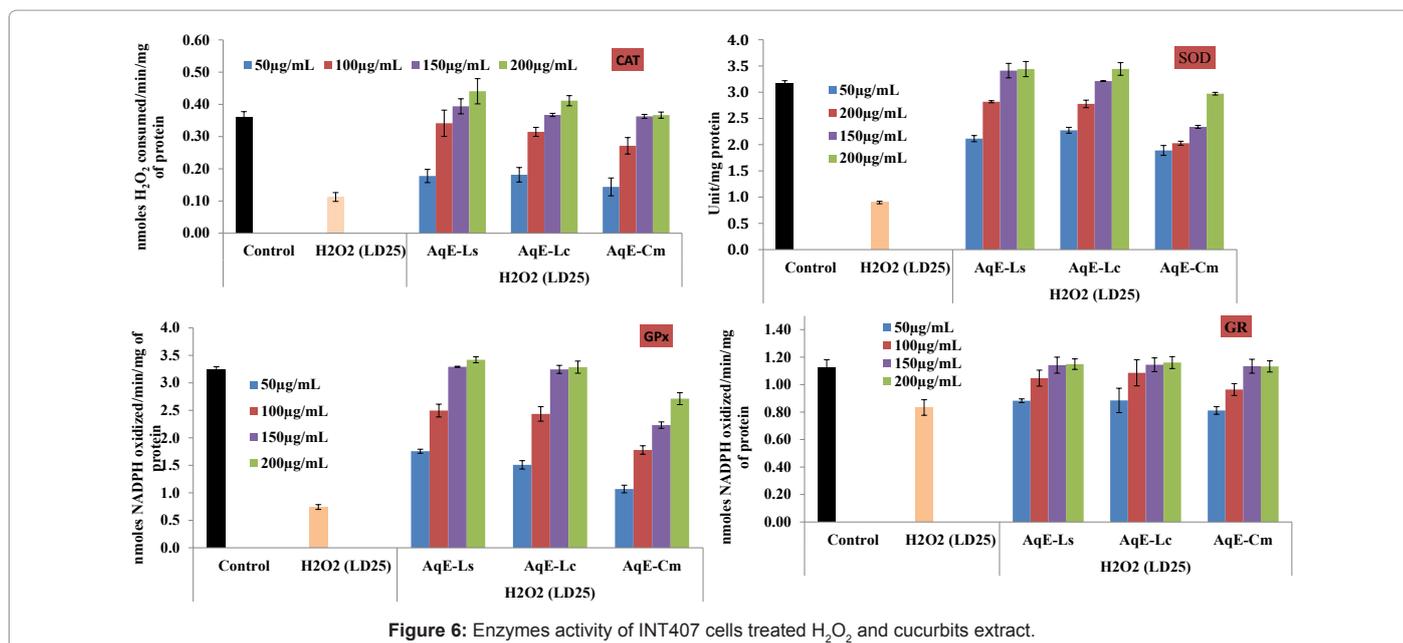


Figure 6: Enzymes activity of INT407 cells treated H₂O₂ and cucurbits extract.

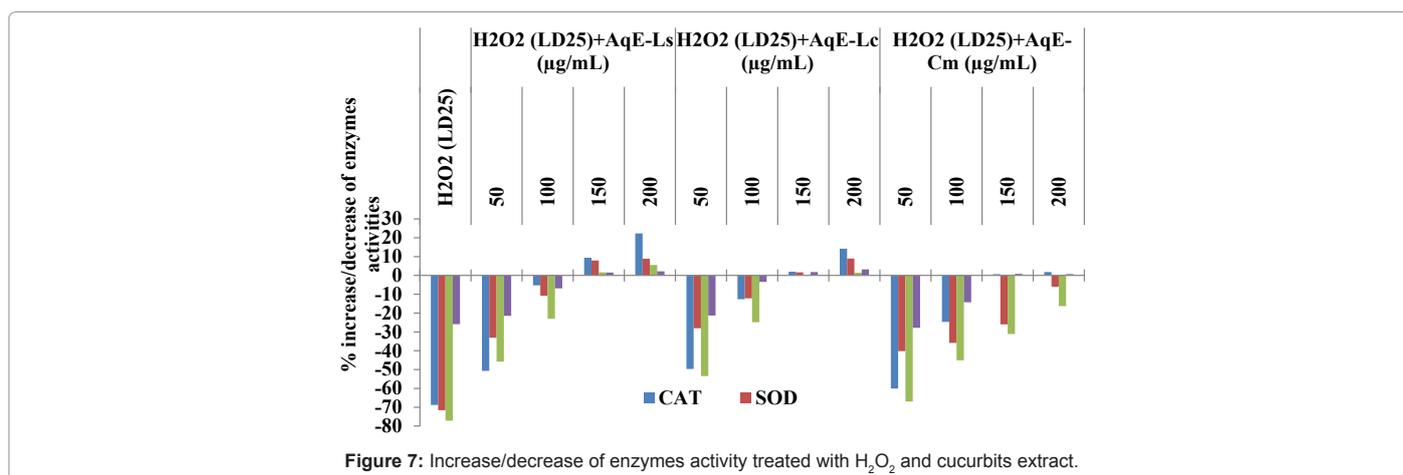


Figure 7: Increase/decrease of enzymes activity treated with H₂O₂ and cucurbits extract.

helped in modulation of CAT enzyme activity by 1.94% at concentration of 150 µg/mL and by 14.20% at concentration of 200 µg/mL. Similarly, increased activity of SOD enzyme was observed at concentration of 150 and 200 µg/ml AqE-Lc by 1.63 and 8.95% respectively. GPx enzyme activity was also found to increase by 0.16% at concentration of 150 µg/ml and by 1.42% at concentration of 200 µg/ml. GR enzyme activity was also found to increase by 1.79 and 3.22% at concentration of 150 and 200 µg/ml respectively.

Aqueous extracts of *Cucurbita maxima* (AqE-Cm)

Treatment of AqE-Cm did not show similar effect to AqE-Ls and AqE-Lc. Treatment of AqE-Cm with H₂O₂ inhibit/protect depletion antioxidant enzyme activity in concentration dependent manner (Figures 6 and 7). As compared to untreated control CAT enzyme activity was enhanced by 0.67 and 1.78% and GR enzyme activity by 0.85 and 0.70% at concentration of 150 and 200 µg/mL respectively. However, SOD and GPx enzymes activity was found to low by 25.95 and 31.12% at concentration of 150 µg/ml whereas 5.96 and 16.28% at the concentration of 200 µg/ml respectively.

Discussion

Phytochemical composition of cucurbits

Gas chromatography and mass spectroscopy (GC-MS) is the most important technique employed in natural product research to analyze the presence of individual compounds present in the sample. Compounds were identified by inbuilt spectral library equipped with the GC-MS instrument [5]. GC-MS revealed the presence of 83, 72 and 74 compounds in L_s, L_c and C_m respectively in varying concentrations. Compounds were categorized in three different groups based on their percent concentration. Major (>5%); minor (>1%, but <5%) and compounds in trace amount (<1%) (Table 1). Some compounds were found common in all the three cucurbits investigated. A number of compounds resembling with saccharides were identified in all three types of cucurbit fruits. Among major compounds, 2-Furancarboxaldehyde, 5-(hydroxymethyl)-, C₆H₆O₃ (16.40%), 2-Deoxy-D-galactose, C₁₂H₂₂O₁₁ (10.50%), and 2, 5-Dimethyl-4-hydroxy-3(2H)-furanone, C₆H₈O₃ (5.11%) were found in L_s and this compound were of carbohydrate groups. Among these compounds, some were also found to be present in L_c and C_m with varying concentration (Tables 1 and 2).

Compound with (M. Formula)	L _s	L _c	C _m
Palmitic acid (C ₁₆ H ₃₂ O ₂)	1.79%	8.44%	9.21%
Myristic acid (C ₁₄ H ₂₈ O ₂)	0.23%	0.17%	0.26%
Stearic acid (C ₁₈ H ₃₆ O ₂)	0.88%	0.68%	0.71%
Linoleic acid (C ₁₈ H ₃₂ O ₂)	0.59%	2.51%	1.19%
Linolenic acid (C ₁₈ H ₃₀ O ₂)	0.66%	1.33%	NF

Table 3: Essential fatty acids identified in cucurbit fruits (NF: Not found).

Various compounds containing benzene ring with or without phenyl group were identified in all three cucurbits. These were 4-Pyrimidinol, C₄H₄N₂O (2.66%), benzene acetaldehyde, C₈H₈O (0.15%), 2-Ethoxy-4-methylphenol, C₉H₁₂O₂ (0.20%), 3-Hydroxybenzene methanol C₇H₈O₂ (0.80%), and Diethyl phthalate C₁₂H₁₄O₄ (0.52%). Several compounds of ester and carboxylic acid were identified in extracts. Eight fatty acids have been reported in *Lagenaria siceraria* [27]. Our extract of Ls was found to contain essential fatty acids such as palmitic acid, stearic acid, linolenic acid, linoleic acid, and myristic acid (Table 3). Palmitic acid and its ester, stearic acid, eicosanoic acid and its ester, linolenic acid and its ester, glycolic acid, acetic acid and its ester were identified commonly in all samples of cucurbits used in this study in varying concentration. Compounds of sterols such as β-sitosterol, stigmasterol and ergosterol were found in all three cucurbits in different concentration. Compound containing lactone group such as α-angelic lactone (C₅H₆O₂) was also found in all samples. Extracts were found to contain compounds of anhydrides. Carbinol, maltol sorbitol was found in extracts in varying concentration. Compounds of azole groups such as 2-Methyl-4, 5-dihydro-1H-imidazole (0.73%), N-Methyltetrazole (2.85%), and methyltetrazole (1.76%) were identified in all the three fruits extract respectively.

Cytoprotection and antioxidant activity

Hydrogen peroxide (H₂O₂) is capable of generating intracellular ROS *in vitro*. In the present study, H₂O₂ was used for generation of ROS which leads to increase intracellular oxidative pressure. H₂O₂ is an oxidizing agent, capable of crossing plasma membrane, triggers inactivation of certain enzymes leading to inappropriate cellular antioxidant defense and hence produce cells injury or cells death [28-30]. Our study shows that H₂O₂ treated cells have less viable cells as evaluated by MTT and LDH assays. Cytotoxicity result of H₂O₂ was further confirmed by the inhibition of colony formation which readily discriminate prolonged growth arrest of cells or cell death. Our result also showed that the H₂O₂ depleted CAT, SOD and GPx enzymes activities found more than GR enzymes activity in INT407 cells. The result is consistent with the previous studies in which H₂O₂ treatment depletes GSH, reduced glutaredoxin and decreased SOD and GPx enzymes activities [31,32]. Similarly, high levels of intracellular H₂O₂ (>1 μM) depletes cellular thioredoxin pool and increases oxidation of glutathione (GSH) and oxidative inactivation of general cellular thiolate-dependent enzymes [33]. Also, H₂O₂ inhibits thiolate dependent enzymes by oxidation of their active-site thiolate moieties [34]. Similarly, SOD activity was inhibited by H₂O₂ associated with the reduction of the active site of SOD (Cu²⁺ to Cu⁺), and/or the structural alteration of SOD that restricts access to Cu²⁺ [35,36].

High level of intracellular ROS induces extracellular signal-regulated protein kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and p38-mitogen-activated protein kinases (p38-MAPK) leading to apoptosis through activation of various downstream signal [26,28-30,37]. Also, intracellular ROS accelerate DNA damage which induces apoptotic cell death [28]. Hence, the protection of GI epithelial layer against lethal effect of free radicals is necessary to augment enteric and

systemic functions to improve human health. Our results indicate that extracts treated INT407 cells were found to elicit enhanced activities of antioxidant enzymes in H₂O₂ pre-treated cells (p<0.05) in the trends AqE-Lc>AqE-Ls>AqE-Cm. Recent studies indicates that polyphenols; a group of typical antioxidant compounds are capable of not only scavenging the ROS but also they lead to activate the defense system of an individual [38,39]. Cucurbit fruits contain adequate amount of secondary metabolites including phenolic compounds showing antioxidant activities as evaluated in our previous study using *in vitro* chemical methods [3,4]. We found that the radical scavenging activities of cucurbit extracts were proportional to the polyphenolic and flavonoid contents. The present result is also consistent with the previous one in which AqE of *Lagenaria siceraria* and *Luffa cylindrica* have high concentration of polyphenolic compound as compared to *Cucurbita maxima* [4,5]. *Lagenaria siceraria* and *Luffa cylindrica* contained significant amount of polyphenolic compounds and found to be a potent scavenger of ferric and hydroxyl radicals than *Cucurbita maxima* extract [4,5]. It was also reported that oxidative damage of cells or tissues triggered by generation of ROS through a cascade of reactions is efficiently blocked by various antioxidants to overcome their potentially injurious actions [40-42].

Overall, our result indicates that ROS generated in H₂O₂ exposed intestinal cell line *in vitro* were found to be scavenged either by antioxidant compounds present in cucurbit extracts or enhanced the defensive activities of cell's endogenous antioxidant enzymes system. Our present and previous studies indicate that *Lagenaria siceraria* and *Luffa cylindrica* extracts have more antioxidant defence activities than *Cucurbita maxima*. Present studies provide considerable line of evidences concerning nutraceutical implications and antioxidant properties of Cucurbit extracts to augment enteric organ systems. Hence, dietary intervention of cucurbits for regular basis might be potential site therapeutic strategies to cure and prevent gastrointestinal ailments generated by oxidative stress. Extracts may also be encapsulated as herbal medicine following preclinical and clinical evaluation towards emerging new therapeutic product in therapy and prophylaxis of various diseases including cancer [43-45].

Conclusion

In the present study three selected dietary cucurbits mentioned elsewhere in this article were investigated for the presence of phytochemicals. Aqueous extracts containing these phytochemicals were evaluated for protecting INT407 cells of intestinal origin against intracellular ROS generated by hydrogen peroxide. Extracts were found to protect gastrointestinal cells from injurious effect of H₂O₂ by modulating the activities of cellular anti-oxidant defense system. Based on this and our previous studies on dietary cucurbits [1-4,46] it can be said that the consumption of adequate amount of these vegetables might be essentially helpful to ameliorate ROS induced gut associated pathogenesis. Enhanced nutrient uptake was previously evaluated on probiotic Lactobacilli through modulation of proton pumping ATPase activities [2] expose light towards uptake of antioxidant phytomedicines present in the extracts. Therefore, it is finally concluded that these extracts seem to have potential of therapeutic implications and can be used as nutraceutical and non-toxic medicine for better health.

Acknowledgements

The authors report no conflict of interest. This study was financially supported by Defense Research and Development Organization (DRDO) and University Grant Commission (UGC), Government of India.

Conflict of Interest

Author discloses that there is no conflict of interest.

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