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Protective Effect of *Trigonella foenum-graecum* and *Foeniculum vulgare* Mature Leaf Against t-BHP Induced Toxicity in Primary Rat Hepatocytes

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Abstract

Objective: *Trigonella foenum-graecum* (TFG), is used to treat diabetes, liver and spleen diseases, whereas *Foeniculum vulgare* (FV), is used to cure liver disorder, cardiovascular diseases, cancer, aging etc. in Ayurvedic system. Both plants have variety of pharmacological activities, including anticancer, antidiabetic, antihepatotoxic, antibacterial activities etc. The present study was carried out to explore and compare the hepatoprotective activities of ethanolic extracts of mature TFG and FV leaf against tert-butyl hydroperoxide (t-BHP) induced cell death in primary rat hepatocytes.

Methods: Hepatocytes were isolated from liver of overnight fasted rat after subjecting it to two stage collagenase perfusion with HEPES {4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid} buffer. Only preparations with cell viability greater than 95% were used for subsequent experiments. Cell viability was determined by MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay, whereas hepatoprotective activities of plant extracts were determined by estimation of superoxide dismutase and catalase activities, lipid peroxidation, total glutathione content and nitric oxide release against t-BHP induced oxidative stress.

Results: Treatment of TFG and FV leaf extracts (1.0 to 7.5 µg) significantly reduced the impact of t-BHP (250 µM) induced toxicity. TFG and FV leaf extracts showed 2.3 and 2.7 fold (P<0.001) increase in superoxide dismutase (SOD) activity, 3.4 fold and 2.2 fold (P<0.001) increase in catalase (CAT) activity at concentration of 5 µg whereas maximum glutathione (GSH) content was restored at concentration of 2.5 µg and 5.0 µg, respectively as compared to stressed cells. Both the leaf extracts were found to decrease the lipid peroxidation (P<0.001) and inhibit NO release (P<0.001) at concentration of 5.0 µg in cells as compared to stressed cells.

Conclusion: The results suggest cytoprotective, antioxidant and hepatoprotective properties of TFG and FV leaf extracts which may be used in the form of dietary component and also in formulations against liver diseases.

Introduction

Liver is the pivotal organ in the human body and has intense metabolic activities. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply and energy provision [1]. Thus, to maintain a healthy liver in human is of great importance for the normal metabolism of the body. Reactive oxygen species (ROS), the by-products of cellular metabolism, can damage the macromolecules like lipid, nucleic acid, protein which may leads to various diseases, like heart disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts and age related functional disorders [2-4]. Exogenous and endogenous harmful chemicals are processed by the liver to be eliminated from the body. There is less availability of such therapy in western medicines which can cure these diseases without side effects [5]. In recent year, there has been a global trend towards the use of natural phytochemicals present in fruits, vegetables, oil seed and herbs as antioxidant and functional food [6].

Trigonella foenum-graecum (Hindi: Methi, English: Fenugreek), an annual herb, belonging to the family *Fabaceae* is widely grown in India, Pakistan, Egypt, and Middle Eastern countries [7]. It is used as dietary antioxidant as well as to treat various diseases due to the presence of strong aroma and medicinal properties [8]. The leaves of *Trigonella foenum-graecum* (TFG) plant are commonly used as vegetables because they are rich source of calcium, iron, β-carotene and other phytochemicals while the seeds are used as a spice in food preparations [9]. The main chemical constituents of *T. foenum-graecum* are fibers, flavonoids, polysaccharides, saponins and some identified alkaloids viz., trigonelline, trigocoumarin and choline [10]. TFG plant possesses different activities such as anticancer, antidiabetic [11,12], antioxidant [9,13], antimicrobial [13], antiulcerogenic [14] and gastro protective activities [10]. *Foeniculum vulgare* (Hindi: Saunf, English: Fennel) is an annual herb, belonging to family *Umbelliferae*, widely grown in the Mediterranean area and in the Asia minor, but is commonly cultivated in the US and Europe. Fennel contains essential oil anethole (40-70%), fenchone (1-20%) and estragole (2-9%) [15,16]. *Foeniculum vulgare* (FV) plant has certain pharmacological activities like antioxidant [17]; antimicrobial [18]; anticancer [19]; anti-inflammatory [20]; antidementia [21] and antispasmodic activities [22].

Previously, we reported that mature leaf of TFG and FV plants have better antioxidant activities as compared to early leaf of these plants [23,24]. Hence, mature leaves of TFG and FV plants were selected to evaluate its hepatoprotective activities under *in vitro* condition on the basis of its utility profile in the traditional system of medicine. The isolated perfused liver is a one of the most important systems for studying the toxicity and metabolic activity of many compounds *in vitro*. Tert-butyl hydroperoxide (t-BHP) is an organic lipid hydroperoxide analogue, used as pro-oxidant to evaluate mechanisms involving oxidative stress in cells and tissues [25]. This study has been carried out to investigate the hepatoprotective effect of mature TFG and FV leaf extracts against t-BHP induced hepatotoxicity in male Wistar rats. Antioxidant enzymes (superoxide dismutases and catalase), glutathione content, lipid peroxidation and NO release were assessed.

Materials and Methods

Reagents

RPMI media, fetal bovine serum, reduced glutathione, reduced nicotinamide adenine dinucleotide (NADH), tertiary-butyl hydroperoxide (t-BHP), nitro blue tetrazolium (NBT), thio-barbituric acid (TBA), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) were purchased from Sigma–Aldrich Co., USA. Antibiotic and antimycotic solution (with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml) and all other chemicals were of analytical grade.

Collection of plant materials

Plants samples were collected from vegetable growing areas of eastern Uttar Pradesh at fruiting stage of plants. Collected plant samples were washed under running tap water, dried, powdered and stored in polythene bags at 4°C. The plant identification was confirmed by National Botanical Research Institute (NBRI), Lucknow, India. The voucher specimens were also deposited at the institute.

Extraction

Twenty grams of dried mature leaf was extracted with 70% ethanol until decoloration. The extracted solvent was evaporated at 40°C in a vacuum rotary evaporator and lyophilized till dryness. The powdered form of plant extract was stored at -4°C and used for the hepatoprotective activity determination [26].

Isolation of hepatocytes

Animal handling in all experimental procedures was approved by the Institutional Animal Ethics Committee, (ITRC/IAEC/20/2006). Male Wistar rats weighing 200 ± 20 g from Indian Institute of Toxicology Research (IITR) animal colony were used for the experiment. Rats were housed in an air conditioned room at 25 ± 2°C temperature with 60-70% humidity and a controlled 12 h light/dark cycle. Rats were fed on standard pellet diet (Ashirwad Pellet Diet, Mumbai, India) and water ad libitum. Chloroform was used for euthanasia.

Primary cell-culture

Hepatocytes were isolated from liver of overnight fasted rat after subjecting it to two stage collagenase perfusion with HEPES buffer [27]. Cell viability was determined by MTT test. Hepatocytes were maintained in RPMI-1640 media supplemented with heat- inactivated 10% fetal bovine serum and 1% of 10,000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B, 1 mM sodium pyruvate, 2 mM glutamine under an atmosphere of 5% CO_2-95% air in an incubator (Thermo-forma) with controlled humidity at 37°C. The cells were seeded at a density of 1.0×10^4 cells/well (counted on hemocytometer) in 0.1% collagen pre-coated 96 well plates, and used for drug exposure experiments after being cultured for 24 h.

Treatment of cells

t-BHP was dissolved in distilled water and filtered through 0.22 µM filter and used for subsequent treatment.

Treatment schedule

Co-treatment schedule was followed throughout the study with t-BHP. In the co-treatment hepatocytes were incubated with different concentration of extracts followed by addition of 250 µM of t-BHP, the oxidative stress agent, and were incubated in CO_2 incubator for 1 h. This schedule gives an insight of protective effect of the co-exposure of the extracts.

Quantitative analysis of viable cells

Cell viability was determined by a colorimetric MTT assay, as described by Mosmann [28]. After the treatment, culture medium was carefully aspirated and MTT was added to each well. After 4 h incubation, 0.2 ml dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal and incubated for 20 min. Absorbance was measured at 530 nm using Spectramax PLUS 384 microplate reader (Soft max pro version 5.1; Molecular Devices, USA). The data are expressed as percentage of control viability measurement in untreated cells.

Estimation of antioxidant enzymes

Superoxide dismutase (SOD): SOD assay was based on the spectrophotometric assay of the inhibition of nitro blue tetrazolium-NADH and phenazine methosulphate (PMS) mediated formazan formation. Briefly, to 10 µl of cell lysate, 90 µl of 50 mM sodium tetra pyrophosphate buffer (pH 8.3), 30 µl of 0.3 mM nitro blue tetrazolium, 10 µl of 0.96 mM PMS and 40 µl of double distilled water (DDW) was added. The reaction was initiated by the addition of 20 µl 0.72 mM NADH. After incubation at 30°C for 90 s, the reaction was terminated by the addition of 50 µl glacial acetic acid. Absorbance was measured at 560 nm. 50% inhibition of formazan formation under assay condition in 1 min is taken as one unit of enzyme activity/minute [29].

Lipid peroxidation (LPO): Lipid peroxidation was quantified by measuring malondialdehyde (MDA), a breakdown product formed from polyunsaturated fatty acids (PUFA) hydroperoxides. In this assay, the evaluation of end product malondialdehyde (MDA) formed due to membrane lipid peroxidation was measured. Briefly, to 10 µl lysate, 10 µl of DDW, 50 µl of 50 mM phosphate buffer, 10 µl of 1 mM butylated hydroxy toluene (BHT), 75 µl of 1.3% thiobarbituric acid (TBA) was added. The lipids were isolated by precipitating them with 50 µl of 50% trichloro acetic acid. The mixture was then incubated at 60°C for 40 min and then kept on ice for 15 min. Reaction was stopped by the addition of 10 µl of 20% sodium dodecyl sulphate. This assay measures the amount of pink colored MDA-TBA adduct at 530 nm and to

account for the interference of phytochemicals it is also read at 630 nm [30]. The concentration of thiobarbituric acid reactive substances (TBARS) was expressed as nmoles of MDA formation. Diluted l, l, 3,3-tetraethoxypropane was used for the standard calibration curve.

Nitric oxide (NO) production: Accumulation of nitrite, the end product of NO metabolism in culture medium was determined using Greiss reagent. In brief, 100 µl of the cell supernatant (1×10^4 cells/100 µl) was incubated with 100 µl Greiss reagent consisting of 1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% H_3PO_4 at 37°C for 30 min and the absorbance was recorded at 542 nm. A range of concentration of sodium nitrite was used to generate the standard curve [31].

Reduced glutathione (GSH): Total glutathione was measured using GSH reductase–DTNB recycling assay and the rate of color developed was measured at 412 nm. Total glutathione was measured by incubating 50 µl of lysate with 2.5 µl of 2-vinyl pyridine for 1 h at room temperature prior to recycling assay [32].

Catalase (CAT): CAT was assayed spectrophotometrically using the method of Aebi et al. [33]. Briefly, the assay mixture of 1.0 ml contained 970 µl of 50 mM sodium phosphate buffer pH 7.0 and 20 µl of homogenate. Reaction was started by addition of 10 µl hydrogen peroxide. The decrease in absorbance was then observed for 180s at every 15s interval at 240 nm. Catalase activity is expressed as Unit/mg of protein.

Total protein content: Protein estimation was done by using standard protocol of Bradford et al. [34]. Bovine serum albumin was used as standard, and the colour developed was read at 595 nm.

Statistical analysis: All analytical data were subjected to an analysis of variance (ANOVA). Each value is mean ± SD of three replications (n=3). Results were considered significant when $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Statistical analysis was done by using Prism software (Graph Pad Prism software version 3.0, USA).

Results

Quantitative analysis of viable cells

Varying concentrations of mature TFG and FV leaf extracts (1.0-7.5 µg/ml) were examined to determine the protective effect of plant extracts. In order to evaluate the cytotoxic potential of t-BHP, the hepatocytes were treated with 250 µM of t-BHP and cell viability was determined by MTT assay. t-BHP caused 36.38% decrease in cell viability as compared to control. To see the protective effect of mature TFG leaf at varying concentrations against t-BHP induced oxidative stress in cultured hepatocytes, cells were co-treated with 250 µM of t-BHP and TFG leaf extract (at concentrations of 1.0, 2.5, 5.0 and 7.5 µg) which showed increased cell viability by 1.22 (P<0.001), 1.24 (P<0.001), 1.91 (P<0.001) and 1.64 fold (P<0.001), respectively as compared to t-BHP alone treated cell (Figure 1a). In mature FV leaf, cells treated with t-BHP alone (at 250 µM), cell viability decreased by 31.27% (P<0.001) with respect to control, whereas cells co-treated with t-BHP and mature FV leaf at concentration of 1.0, 2.5, 5.0 and 7.5 µg showed increased cell viability by 1.84 (P<0.001), 1.04 (P<0.001), 1.78 (P<0.001), and 1.54 fold (P<0.001) as compared to t-BHP stressed cells (Figure 1b). Results also revealed that mature TFG leaf extracts showed better cell viability at concentration of 5.0 and 7.5 µg whereas mature FV leaf showed better cell viability at concentration of 1.0 and 5.0 µg among tested concentrations.
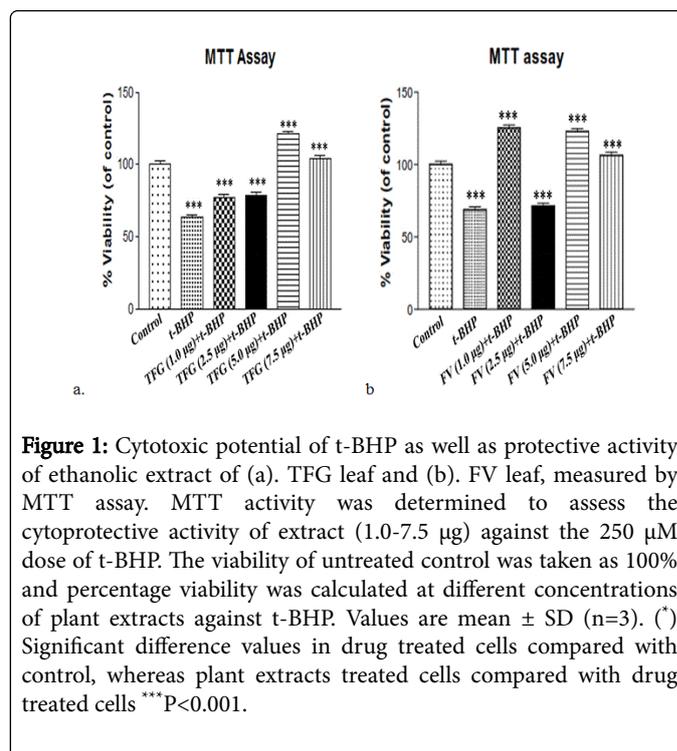

Figure 1: Cytotoxic potential of t-BHP as well as protective activity of ethanolic extract of (a). TFG leaf and (b). FV leaf, measured by MTT assay. MTT activity was determined to assess the cytoprotective activity of extract (1.0-7.5 µg) against the 250 µM dose of t-BHP. The viability of untreated control was taken as 100% and percentage viability was calculated at different concentrations of plant extracts against t-BHP. Values are mean ± SD (n=3). (*) Significant difference values in drug treated cells compared with control, whereas plant extracts treated cells compared with drug treated cells $^{***}P<0.001$.

Effect on superoxide dismutase activity

Cultured hepatocytes when treated with t-BHP (250 µM) showed decrease in SOD activity by 33.78 Unit/min/10^4 cells (P<0.01). When cells were co-treated with t-BHP (250 µM) and TFG leaf extract at concentration of 1.0, 2.5, 5.0 and 7.5 µg, SOD activity was shown to be 67.07 (P<0.001), 51.14 (P<0.01), 78.74 (P<0.001) and 36.26 Unit/min/10^4cells, respectively (Figure 2a).
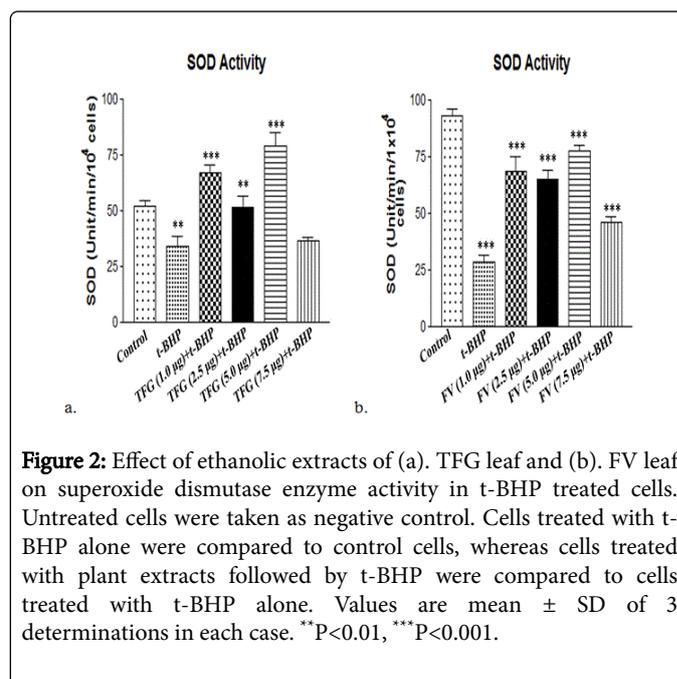

Figure 2: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on superoxide dismutase enzyme activity in t-BHP treated cells. Untreated cells were taken as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. $^{**}P<0.01$, $^{***}P<0.001$.

In FV leaf extract treated cells, the SOD activity was reported to be 68.19 (P<0.001), 64.73(P<0.001), 77.17 (P<0.001), 45.72 Unit/min/10^4cells (P<0.001), respectively at concentrations of 1.0, 2.5, 5.0, and 7.5 μg as compared to stressed cells which has SOD activity of 18.49 Unit/min/10^4 cells (P<0.001) (Figure 2b). Results revealed that mature TFG and FV leaf extracts showed best SOD activity at concentration of 5.0 μg.

Effect on lipid peroxidation

Cultured hepatocytes treated with the t-BHP at concentration of 250 μM, generated 11.78 nM MDA/10^4cells. When cells were co-treated with t-BHP (250 μM) and TFG leaf extract at concentrations of 1.0, 2.5, 5.0 and 7.5 μg, the extract reduced the formation of MDA by 7.31 nM (P<0.001), 7.44 nM (P<0.001), 5.04 nM (P<0.001) 6.15 nM MDA/10^4cells (P<0.001), respectively (Figure 3a). In FV leaf extract with same treatment, it were 7.74 nM (P<0.01) 7.55 nM (P<0.01), 5.07 nM (P<0.001), 5.50 nM MDA/10^4cells (P<0.001), respectively as compared to t-BHP stressed cell which has 10.78 nM MDA/10^4cells (Figure 3b). The results showed that mature TFG and FV leaf extract has best anti-lipid peroxidation activity at concentration of 5.0 μg.
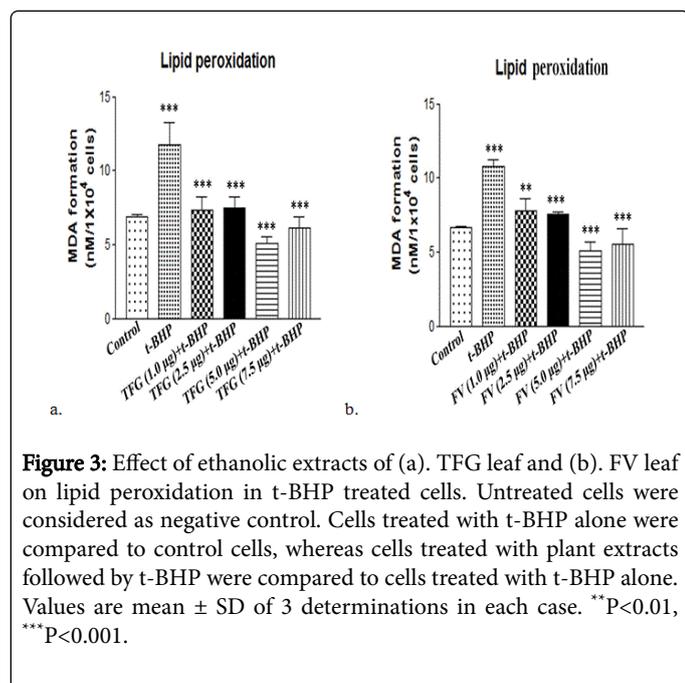

Figure 3: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on lipid peroxidation in t-BHP treated cells. Untreated cells were considered as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. **P<0.01, ***P<0.001.

Effect on NO release

In cultured primary hepatocytes, the NO concentration was found to be 2.68 pg/10^4cells. Cultured hepatocytes, when treated with t-BHP at concentration of 250 μM showed 3.74 pg NO released/10^4cells. When cells were co-treated with t-BHP (250 μM) and TFG leaf extract at concentrations of 1.0, 2.5, 5.0 and 7.5 μg, it released 2.31 pg (P< 0.001), 2.21 pg (P<0.001), 2.04 pg (P<0.001) and 2.67 pg (P<0.001) NO/1 x 10^4cells, respectively (Figure 4a). With the same treatment with FV leaf extract, it released 2.93 pg (P<0.01), 1.94 pg (P<0.001), 1.88 pg (P<0.001) and 2.22 pg (P<0.001) NO/1 x 10^4cells, respectively as compared to t-BHP stressed cell which produces 4.47 pg NO/1 x 10^4cells (Figure 4b). Result showed that TFG and FV leaf have best activity at concentration of 5.0 μg.
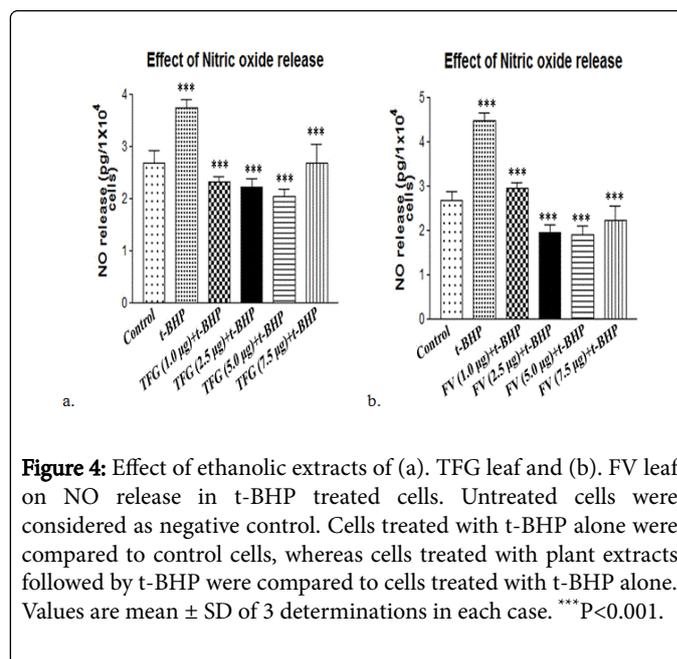

Figure 4: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on NO release in t-BHP treated cells. Untreated cells were considered as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. ***P<0.001.

Cellular GSH level

In our experiment, it was found that 1h treatment with t-BHP caused reactive oxygen species (ROS) production in cultured primary hepatocytes which decreased GSH content. When cultured hepatocytes were treated with t-BHP (250 μM), it showed 1.88 μg GSH/mg of protein (P<0.01) which is 1.36 folds less than the untreated cells. When cells were co-treated with t-BHP (250 μM) and extract of TFG leaf at concentration of 1.0, 2.5, 5.0 and 7.5 μg, protection of GSH depletion was observed by 1.36, 1.55, 1.23 and 1.54 fold, respectively as compared to t-BHP stressed cells (Figure 5a).
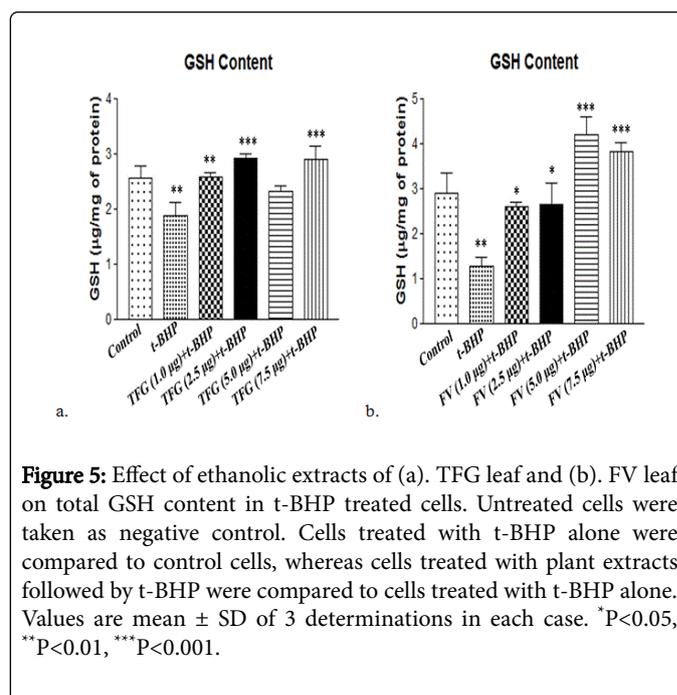

Figure 5: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on total GSH content in t-BHP treated cells. Untreated cells were taken as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. *P<0.05, **P<0.01, ***P<0.001.

The FV leaf extract showed protection of GSH depletion by 2.05, 2.10, 3.31 and 3.00 fold, respectively at the same concentrations as compared to stressed cell which had 1.27 µg GSH/mg of protein ($P<0.01$) (Figure 5b). The results reflect that treatment of hepatocytes cells with mature TFG and FV leaf extracts efficiently blocked t-BHP-induced GSH depletion at concentration of 2.5 and 5.0 µg, respectively.

Catalase activity

Cultured hepatocytes when treated with t-BHP (250 µM) showed depletion in CAT activities by 2.90 fold as compared to control, but when cells were co-treated with t-BHP (250 µM) and TFG leaf extract at concentration of 1.0, 2.5, 5.0 and 7.5 µg, cells showed 1.97, 2.53, 3.60 and 3.30 fold increase in CAT activity as compared to t-BHP stressed cell (Figure 6a). On FV leaf extract treatment with same concentrations, cells showed 1.13, 1.56, 2.22 and 1.92 fold higher enzymatic activities as compared to t-BHP alone (Figure 6b). Results showed that mature TFG and FV leaf extracts have high enzymatic protection activity at concentration of 5.0 µg.
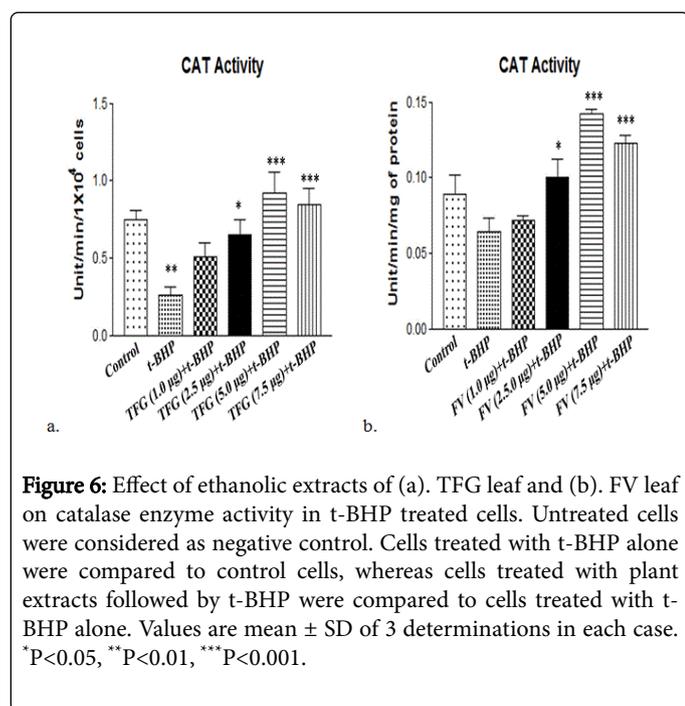

Figure 6: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on catalase enzyme activity in t-BHP treated cells. Untreated cells were considered as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. *P<0.05, **P<0.01, ***P<0.001.

Total protein content

Cultured hepatocytes treated with t-BHP alone at concentration of 250 µM, decreased the total protein content by 0.6 fold as compared to control, but when cells were treated with t-BHP (250 µM) and TFG leaf extract at concentrations of 1.0, 2.5, 5.0 and 7.5 µg, protection of protein content depletion was observed and it increased by 1.66, 1.63, 2.21 and 1.68 fold, respectively as compared to t-BHP treated cells (Figure 7a). In case of FV leaf, the extract protected protein content depletion and it increased to the extent of 1.37, 1.36, 1.43 and 1.36 fold, respectively as compared to t-BHP treatment alone (Figure 7b). The results indicate that treatment of hepatocytes with mature TFG and FV leaf extracts efficiently protected the protein content depletion at concentration of 5.0 µg. In the present study, we found that the total protein content of cell lysate also clearly indicate health status of cells.
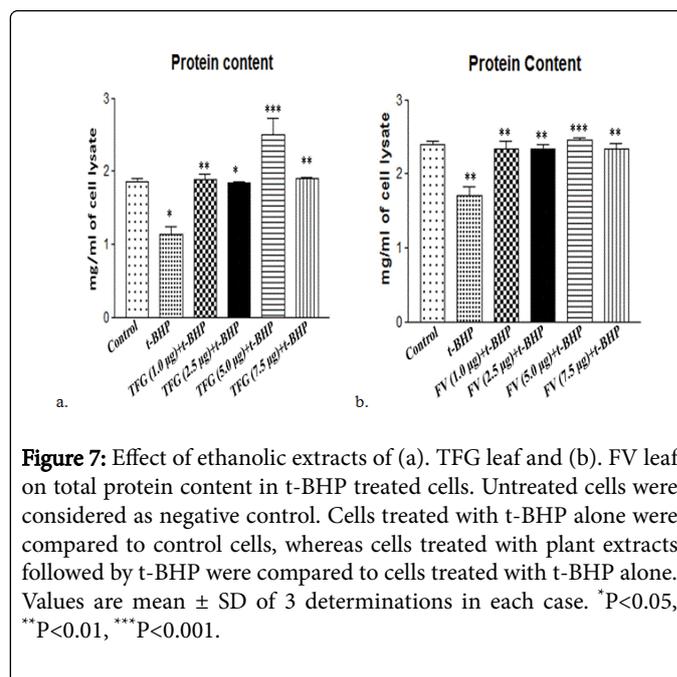

Figure 7: Effect of ethanolic extracts of (a). TFG leaf and (b). FV leaf on total protein content in t-BHP treated cells. Untreated cells were considered as negative control. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean ± SD of 3 determinations in each case. *P<0.05, **P<0.01, ***P<0.001.

Discussion

In animal system, liver is considered as most sensitive organ to toxic agents [35]. The study of antioxidant enzyme activity (SOD and CAT), lipid peroxidation, total glutathione and protein content have been found to be of great importance in the assessment of experimental liver cells damage. Decreased levels of antioxidant enzymes (SOD and CAT) and glutathione content and increased level of MDA and NO formation in hepatocytes are indicators of hepatotoxicity [36]. The levels of these biomarkers were significantly reverted toward normal values in the t-BHP pretreated rat hepatocytes administered with mature leaf of TFG and FV plant extracts when compared with the cells treated with t-BHP alone. Plant extracts are rich sources of antioxidant and can minimize the damage caused by oxidative stress [23]. Mammalian cells are equipped with antioxidant systems to combat oxidative stress-induced liver damage [37,38].

The purpose of this study was to observe the cytoprotective and antioxidative potential of mature TFG and FV leaf extracts, to overcome t-BHP induced toxicity in cultured hepatic cells. The toxic effect of t-BHP on cell membrane integrity was shown by MTT assay resulting in significant decrease in the percent viability of isolated rat hepatocytes. MTT is taken up into the cells and it gets reduced mainly by mitochondrial enzymes, to yield a purple formazan product [39]. This formazan product accumulates within the cell and cannot pass through the plasma membrane [40]. Formazan can be readily detected and quantified by spectrophotometer due to solubilisation of the cells in the medium. Higher color intensity provides an indication of mitochondrial integrity and activity which may be interpreted as a measure of cell viability [41]. Cell viability was reduced in t-BHP treated hepatocytes as compared to normal cells. Mature TFG and FV leaf extracts downplayed the toxic effects induced by t-BHP in term of loss in the cell viability in a dose dependent manner. The decreased cell viability caused by hepatic damage is believed to be due to breakage of the structure of the cellular membrane [42].

SOD provides defense against the toxicity of superoxide radical by scavenging it from the system [43]. With t-BHP treatment, a significant decrease in the SOD level was observed, which reverted back toward normal value with plant extract co-treatment. Superoxide radicals produced in various cellular metabolic processes can react with free iron or copper ions and form hydroxyl radical (Fenton's reaction) or react with NO to produce ONOO⁻. These radicals, if unchecked, cause damage to cellular macromolecules resulting in cellular dysfunctions [44]. Superoxide dismutase enzyme dismutates the superoxide anion, thus saving the cell from the deleterious effects of the superoxide radical and further generation of ROS. The plant extracts i.e. mature TFG and FV leaf extracts in our study showed protection of cells from the toxic effects of superoxide ions by enhancing the activity of superoxide dismutase enzyme as compared to t-BHP stressed cells.

Lipid peroxidation is the result of ROS attack on unsaturated fatty acids present in lipids of cell membrane. Peroxidation of membrane phospholipids results in increased peroxides which eventually lead to disruption of membrane structure, membrane fluidity and function when there is a lack of adequate antioxidant defense [45,46]. Thiobarbituric acid reactive substances (TBARs), which indicate the level of lipid peroxidation, have been extensively used as markers of oxidative stress in biological system. t-Butyl hydroperoxide produces alkoxy or peroxy radicals, which cause lipid peroxidation leading to the formation of various TBA reactive substances [47]. Higher levels of unscavenged free radicals result in oxidative deterioration of polyunsaturated lipids leading to malondialdehyde formation. These products may participate in the pathogenesis of vascular complications [48]. The results of present study suggest that mature TFG and FV leaf extracts are capable of managing lipid peroxidation at concentration of 5.0 µg. Therefore, the role of dietary antioxidants in the form of food ingredients can be an effective strategy to strengthen the immune system, healthy ageing and homeostatic balance during oxidative stress [49].

The production of NO by activated macrophages against pathogens may cause cellular injury to macrophages as well as to the neighbouring tissues. Excess NO can react with superoxide radicals leading to formation of harmful peroxynitrite radicals that are responsible for the damage to macromolecules in biological systems, resulting in protein nitration, DNA strand breakage and guanine nitration etc. [50]. Jeong et al. [51] revealed that excess NO generated in body system causes tissue damage and inflammation. The approach to reduce NO production is emerging as an important therapeutic strategy for treatment of inflammatory disorders. In our study, the treatment of cells with extracts reduced the NO concentration which indicates better antioxidant status of cells against t-BHP induced oxidative stress. Results showed that mature TFG and FV leaf extracts have best activity at concentration of 5.0 µg against NO formation.

GSH is an important non-enzymatic cellular antioxidant, which helps in preventing the oxidation of protein sulfhydryl groups by free radicals generated during oxidative stress. Thiol based antioxidant system plays second line of cellular defense against reactive free radical and other oxidant species mediated oxidative damage. GSH with its -SH group functions as catalyst in the disulfide exchange reaction. Its function is to reduce free radicals. So, the decrease in the concentration of GSH is related to neutralization of free radicals [52]. It is suggested that during oxidative stress, GSH depletion occurs frequently, which may also alter mitochondrial membrane fluidity [53]. In this study, we found that cells were sensitive to t-BHP stress, and GSH level of cells depleted during t-BHP treatment. The administration of TFG or FV leaf extracts along with t-BHP inhibited GSH depletion. The protective effect of FV leaf extract on GSH depletion was higher at concentration of 5.0 and 7.5 µg whereas protective effect of TFG leaf extract was higher at concentration of 2.5 and 7.5 µg.

CAT is a detoxification antioxidant enzyme that protects the cells against oxidative stress or highly reactive free radicals. Activities of these enzymes get induced when free radicals are generated in the cells. Catalase converts H_2O_2 to H_2O. The TFG and FV extracts showed enhanced activity of this enzyme ensuring better antioxidant and protective effect on the stimulated cells against the deleterious effects of the t-BHP and generation of other radicals.

Conclusions

TFG and FV leaf extracts showed significant cytoprotective and antioxidant property against t-BHP induced oxidative stress in primary rats hepatocytes. t-BHP at 250 µM concentration, causes cell death in primary cultured hepatocytes via mitochondria dependent pathways. Induction of ROS caused depletion in antioxidant enzyme activity and GSH level. The protective activity of TFG and FV leaf extracts against t-BHP induced cell death observed was confirmed by increased cell viability; increased antioxidant enzyme activity, reduced NO release and MDA formation. The reversal of altered antioxidant enzyme status and peroxidative activity suggests that mature TFG and FV leaf extract has antioxidant and anti-peroxidative properties. The present work suggests a beneficial effect of TFG and FV leaf extract for preventing t-BHP induced oxidative stress and hepatotoxicity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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