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RESEARCH ARTICLE

Borassus flabellifer Linn haustorium methanol extract mitigates fluoride-induced apoptosis by enhancing Nrf2/Haeme oxygenase 1 –dependent glutathione metabolism in intestinal epithelial cells

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ABSTRACT

Fluoride is the most common cause of drinking water-associated toxicity and is known to induce various metabolic imbalances and dental/skeletal fluorosis. The present study analyzed the protective effect of *Borassus flabellifer* Linn. haustorium extract (BHE) against fluoride-induced intestinal redox metabolism and apoptosis. The total polyphenols and total flavonoids present in BHE were estimated to be 39.67 ± 5.14 mg gallic acid equivalent/g extract and 8.59 ± 0.74 mg quercetin equivalent. In cultured intestinal epithelial cells (IEC-6), sodium fluoride exposure-induced apoptosis mediated through antioxidant enzyme inhibition and subsequent oxidative damages. Further, there observed an increased expression of caspase-3, caspase-7, and apoptotic protease activating factor-1 (apaf-1) genes, increased cytochrome C release, and caspase 3/7 activity indicating the apoptosis- mediated cell death ($p < 0.05$). Upon pretreatment with BHE, the cytotoxic effect of fluoride was reduced by decreasing the expression of apoptotic genes and increased the cytochrome release as well as caspase 3/7 activity ($p < 0.01$). Providing the mechanistic basis, the expression of nuclear factor erythroid 2-related factor-2 (Nrf2)/haeme oxygenase-1 (HO1) gene was increased in the BHE pretreated cells; corroborating to these, there observed increased activity of glutathione biosynthetic enzymes ($p < 0.05$) and glutathione reductase. Hence, the protective effect of BHE may be mediated through Nrf2-mediated glutathione biosynthesis, the subsequent establishment of redox balance, and inhibition of apoptosis in intestinal epithelial cells.

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Borassus flabellifer Linn.; Fluoride toxicity; antioxidant activity; apoptosis; redox balance

1. Introduction

Fluoride is a trace element that is commonly known for its toxic impacts, especially on teeth, bone, and soft tissues. Drinking water is the primary source of fluoride ion and several countries in the world are having a fluoride level above 1.5 ppm (Tkachenko *et al.* 2021). Fluoride ions are absorbed partly in the stomach and prominent portion in the small intestine (Villa *et al.* 1993). As the intestine is the major site of fluoride absorption, high intake of fluoride has been associated with alterations in the gut microbiota, redox imbalance in intestinal cells, and thereby resulting in rectal barrier damages (Fu *et al.* 2020, Wang *et al.* 2020b). It has also been proposed that increased fluoride intake may be associated with the risk of inflammatory bowel diseases (IBD), however, no specific dose-limit was reported for the same (Follin-Arbelet and Moum 2016). Besides, the risk of various cancers including that of the colon has been associated with increased water fluoride (Yang *et al.* 2000, Takahashi *et al.* 2001). Fluoride intake has been shown to inhibit the activity of Na/K + dependent ATPase activity in multiple cell types (A and G 2015, Waugh 2019). Subsequently, the absorption of glucose in intestinal cells also gets reduced upon exposure to fluoride (Chen *et al.* 2016). The health impact associated with fluoride toxicity is usually observed as dental and skeletal fluorosis (Shankar *et al.* 2021). Together with this, the fluoride ions are also known to induce enzyme inactivation, redox imbalance, and DNA damage (Zhong *et al.* 2021). It has been proposed that dietary intake of essential nutrients and antioxidants are possible ways to limit the fluoride-induced toxic health impacts (Susheela and Bhatnagar 2002).

Plants belonging to the Arecaceae family are predominantly consumed in southern India and other south Asian countries. *Borassus flabellifer* Linn. is one among these; the fruit, tender water, kernel, inflorescence, and haustorium are the commonly consumed parts of the plant. The cytoprotective and anti-inflammatory effects of methanolic extract of Borassus flabellifer haustorium have been reported in our earlier studies against hydrogen peroxide, malondialdehyde, and lipopolysaccharide-induced models (Malayil *et al.* 2020). A similar report has also been made by Tusskorn *et al.* (2021), where the protective effect of male flower extract of *Borassus flabellifer* is observed against cisplatin-induced

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toxicity in kidney cells. A report by Yoshikawa *et al.* (2007) showed the anti-diabetic activity of male flower extract of *B. flabellifer Linn.* Likewise, hypoglycemic activity has also been observed in the root extract of the plant (Debnath *et al.* 2013). Fruit extract of the plant has been found to improve lipid metabolism in high fat/streptozotocin-induced diabetic rats (Duraipandiyan *et al.* 2020). A terpenoid compound from the seed coat has been shown to inhibit inflammation in prostate cancer cells and thereby inducing apoptosis in these cells (Yarla *et al.* 2015). Apart from these, antimicrobial properties are also observed for the seed coat extract of the plant (Alamelumangai *et al.* 2014) and 2,3,4-trihydroxy-5-methyl acetophenone isolated from the *B. flabellifer Linn.* sirup (Reshma *et al.* 2017).

Thus, the present study analyzed the protective effect of *B. flabellifer Linn.* haustorium extract (BHE) against fluoride oxidative stress and subsequent apoptosis induction in cultured intestinal cells. The intestinal cells were chosen as models because the fluoride ions are absorbed prominently across the small intestinal epithelium (Villa *et al.* 1993). Further, it has been reported that excessive intake of fluoride has been associated with altered barrier functioning, glycoprotein transport, and immune function. Further, the study was limited to the protective effect alone, as the BHE is being emerged as a functional food instead of a drug candidate.

2. Materials and methods

2.1. Chemicals and cell lines

Sodium fluoride (NaF), RPMI-1640, fetal bovine serum, HEPES buffer, penicillin and streptomycin antibiotics, L-glutamine, and sodium pyruvate were procured from Sigma Aldrich (St. Louis, MO, United States). A two-step Cell to cDNA kit (CellAmp^{TM}) was purchased from Takara Bio (Bengaluru, India) and qPCR master mix (SYBR^{TM} Select Master Mix) was obtained as a sample from Thermo Fisher Scientific (Massachusetts, United States).

Rat intestinal epithelial cell line (IEC-6) was procured from National Center for Cell Science, Pune. The cells were cultured in complete RPMI-1640 with 10% FBS, 5% CO_2 at 37 °C.

2.2. Collection and extraction of B. flabellifer *Linn.* haustorium

The germinated *B. flabellifer* Linn., fruit was collected from the Palakkad district, Kerala, India; the haustorium (BH) was collected by mechanical de-husking. The collected haustorium was dried at 50 °C in an incubator, powdered, and extracted with 100% methanol using Soxhlet apparatus. The extract was concentrated to dryness and subsequently diluted using dimethyl sulfoxide (DMSO) for cell culture applications.

2.3. Estimation of total phenols and flavonoid content

The quantitative estimation of the total polyphenol content was determined according to the methods described by Ahmed and Tavaszi-Sarosi (2019). Briefly, 0.1 mL of BHE (1 mg/mL) was mixed with 2 mL of Folin-Ciocalteu reagent (1 N) and mixed well. Further, to this mixture 3 mL of 7.5% sodium carbonate solution was added and incubated at room temperature for 30 min. The optical density was estimated in a UV-Vis double beam spectrophotometer (Model 2205, Systronics, India) at 765 nm. A standard curve was plotted similarly using gallic acid as the external standard; the final concentration was expressed as mg Gallic acid equivalent. The total flavonoid content of BHE was estimated by the Aluminum chloride method (Wang *et al.* 2020a); the final quantity is expressed in terms of mg Quercetin equivalent. Briefly, the BHE or different concentrations of quercetin (0.5 mL) was mixed with an equal volume of 2% aluminum chloride. The solution was incubated at room temperature for 1 h and absorbance was measured at 420 nm in a spectrophotometer. Liquid chromatography-Mass spectrometry was adopted to determine the polyphenol composition and the protocols were the same as described previously (Illam *et al.* 2017, Malayil *et al.* 2020).

2.4. Analysis of biologically safe concentration and cytoprotective effect

The cytotoxic effect of *B. flabellifer* haustorium extract (BHE) and sodium fluoride were estimated by employing MTT assay according to the standard procedures (Mosmann 1983). The protective effect of BHE against NaF-induced cytotoxicity was determined as follows. Briefly, IEC-6 cells (1×10^6/mL) were plated in a 24-well culture plate; after complete adherence for 24 h, the cells were pretreated with biologically safe concentrations of BHE (10, 25, and 50 μg/mL). After 24 h of incubation, sodium fluoride was added to each well to make the final concentration of 3.5 mM. The percentage cell viability in each concentration of BHE pretreatment was determined and the percentage of protection was calculated by comparing it with the control (NaF alone treated well).

2.5. Analysis of antioxidant and oxidative stress parameters

The cells were treated as described in section 2.4 in T-75 cm2 culture flasks and were collected by cell scrapers. The collected cells were then lysed using repeated freeze-thaw cycles. The lysate was then centrifuged at 10,000 rpm at 4 °C for 15 min; the clarified supernatant was used for the analysis of various biochemical parameters. Cellular antioxidant level such as reduced glutathione (GSH) was determined using standard protocols as described by Nair *et al.* (2016) and catalase activity was determined according to the protocols of Al-Oqail *et al.* (2020). Glutathione biosynthetic enzymes including γ-glutamyl cysteine synthetase (GCS) and glutathione synthetase (GS) were estimated as per standard methods described by Volohonsky *et al.* (2002). Enzymes involved in glutathione regeneration such as glutathione reductase (GR) (Mannervik 2001), glutathione-dependent detoxification enzymes such as glutathione-s-transferase (Moatamedi Pour *et al.* 2014), and glutathione peroxidase (De Vega *et al.* 2002)

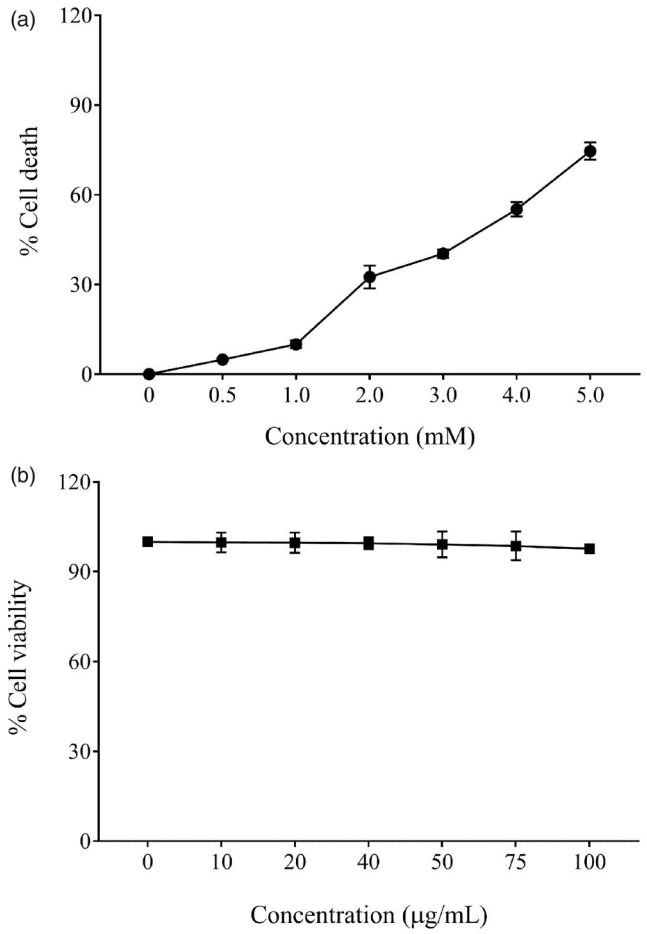

Figure 1. The cytotoxic effect of sodium fluoride on IEC-6 cell by MTT assay (a) and cytotoxicity of *B. flabellifer haustorium* extract (b).

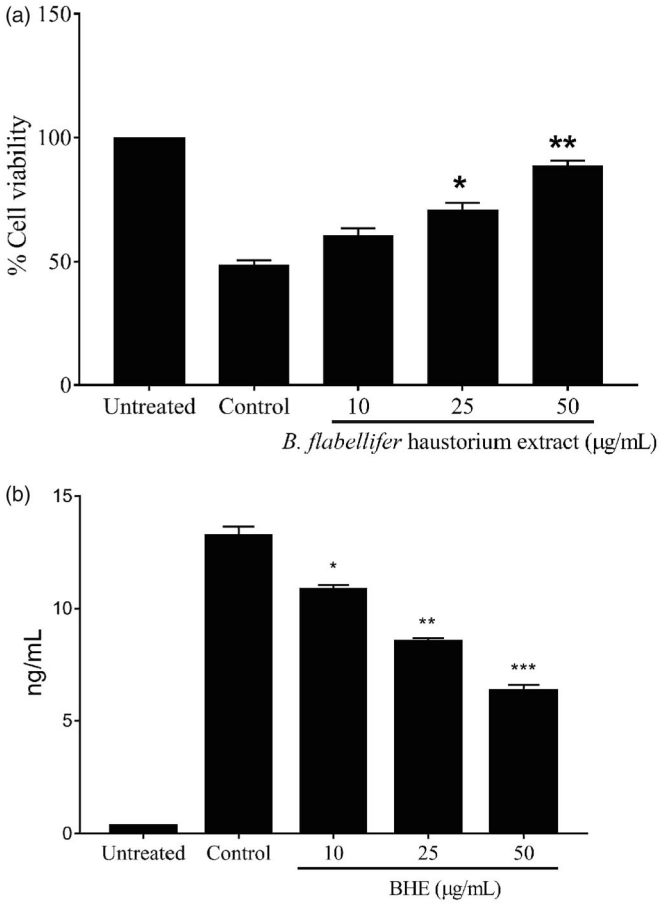

Figure 2. Cytoprotective effect of *Borassus flabellifer* Linn., haustorium extract against sodium fluoride-induced toxicity in IEC-6 cells (a). Release of cytochrome c from the cells which are exposed to fluoride, and those pretreated with different doses of BHE (b).

were also determined spectrophotometrically in terms of NADPH usage. Oxidative stress markers including lipid peroxidation in terms of thiobarbituric acid reactive substances (Chiu *et al.* 2013) and conjugated dienes as per standard protocols (Narayanankutty *et al.* 2016).

2.6. qPCR and apoptosis detection

The cells were treated as described in section 2.4 and collected using mechanical scrapers. The cDNA was synthesized using CellAmp™ (Takara Bio, India) and qPCR analysis was carried out in Applied Biosystem 7500 real-time PCR using SYBR™ Select Master Mix according to the manufacturer's instructions (Thermo Scientific, Massachusetts, United States). The expression of genes associated with apoptosis such as caspase 3, caspase 9, and apaf-1 was determined using specific primers with beta-actin as the internal standard (Supplementary material 1). The ΔΔCT value was calculated using the CT value and fold change in expression with respect to the normal untreated cells were estimated according to the $2^{-\Delta\Delta CT}$ method. Apart from these, the extent of cytochrome C release was estimated in the cells using ELISA kits (Sigma, St. Louis, USA). The Caspase 3/7 activity was estimated using Caspase-Glo® 3/7 Assay System (Promega, Wisconsin, USA) according to the manufacturer's instructions.

2.7. Statistical analysis

The results are expressed as mean ± SD and each *in vitro* experiment is carried out in triplicate. Statistical analysis was done using one-way analysis of variance followed by Tukey Kramer test using Graph pad Prism software 7.0 version A variation in the p values <0.05 is considered as statistically significant.

3. Results

3.1. Total phenolic and flavonoid content as well as phenolic composition

Total polyphenol content in the BHE was 39.67 ± 5.14 mg GAE and the flavonoids content was estimated to be 8.59 ± 0.74 mg QE. The predominant phenolic acids identified in the BHE by LC/MS analysis were ferulic acid, *p*- coumaric acid, protocatechuic acid, catechin, and quercetin (Supplementary Material 2).

3.2. Determination of cytotoxicity and biologically safe concentrations

The cytotoxicity of sodium fluoride was estimated from 0 to 5 mM range and there observed as dose-dependent

cytotoxicity in the IEC-6 cells. The IC50 value of sodium fluoride has been estimated using regression analysis as 3.49 ± 0.16 mM and hence, a dose of 3.5 mM was chosen for inducing cytotoxicity in these cells (Figure 1(a)). Further, the cytotoxic effect of BHE for 48 h was very less even at a concentration of 100 μg/mL. Hence, doses 10, 25, and 50 μg/mL of BHE were estimated to be biological safe concentrations for further studies.

3.3. Cytoprotective effect

The cytoprotective effect of BHE against sodium fluoride-induced cell death is shown in Figure 2(a). Treatment with sodium fluoride was capable of reducing the cell viability to 48.7 ± 1.9%. However, pretreatment with BHE at their respective doses 10, 25, and 50 μg/mL increased the cell viability to 60.4 ± 3.1, 70.9 ± 2.8, and 88.6 ± 2.2%, respectively (Figure 2(a)).

3.4. Effect on cellular (IEC-6) antioxidant defense

The levels of intracellular reduced glutathione as well as catalase activity were significantly reduced by fluoride treatment ($p < 0.01$). Further, the fluoride exposure significantly inhibited the glutathione biosynthetic enzymes such as GCS and GS ($p < 0.01$) in the cells and subsequently reduced the intracellular reduced glutathione content. Besides, the glutathione regenerating enzyme- GR was also found to be inhibited by fluoride exposure, which indicates the reduced regeneration of GSH from its oxidized form ($p < 0.05$). Apart from these, the activity of the glutathione peroxidase enzyme was elevated in fluoride exposed intestinal cells, which might have also contributed to the observed reduction in cellular GSH content (Table 1).

On contrary, the pretreatment with the BHE significantly reduced the fluoride-induced GSH depletion at doses 10 ($p < 0.05$), 25 ($p < 0.01$), and 50 μg/mL ($p < 0.001$). Supporting the above observation, there observed an increase in the activities of glutathione biosynthetic enzymes- GCS and GS; the activity of GR was also elevated in BHE-treated cells, indicating an improved glutathione regeneration ($p < 0.05$). Likewise, there observed an increase in the catalase activity in cells pretreated with 10 ($p < 0.05$), 25 ($p < 0.01$), and 50 μg/mL ($p < 0.001$). Corroborating with the improvement in cellular antioxidant defense, significant reduction was observed in the lipid peroxidation products such as TBARS and conjugated diene levels, in cells pretreated with BHE ($p < 0.05$) (Table 2).

3.5. Cytochrome-c release and caspase 3/7 activity

The cells exposed to fluoride were also shown to have increased cyt. c leakage from the mitochondrial membrane (Figure 2(b)). Further, during the pretreatment with the different doses of BHE, the levels of cytochrome c was significantly reduced ($p < 0.01$), possibly indicating the reduced mitochondrial damage. Apart from these, the activity of caspase 3/7 was also measured using bioluminescence; the activity of these executioner caspases was significantly elevated in the fluoride exposure, whereas pretreatment with the BHE doses prevented the fluoride-induced caspase 3/7 activity estimated in terms of luminescence ($p < 0.001$) in the intestinal epithelial cells (Figure 3(a)).

3.6. Changes in the gene expression pattern by fluoride and protection by BHE

The expression of caspase-3 and caspase-7 were elevated with the NaF treatment compared to untreated cells (Figure 3(b)); however, pretreatment with BHE has shown a considerable reduction in the caspase expression induced by NaF treatment ($p < 0.001$). Likewise, the expression of apoptotic protease activating factor 1 (Apaf-1) was also found to be elevated by fluoride treatment. Significant inhibition of apaf-1 expression was observed in BHE treatment, with the highest in 25 and 50 μg/mL ($p < 0.01$).

Further, the expression of the Nrf-2 gene and associated haeme oxygenase-1 was analyzed for the mechanism of antioxidant activity. The fluoride exposure didn't elevate the Nrf-2/HO1 expression; however, there observed a dose-dependent increase in the gene expression of both Nrf-2 and HO1 in the IEC-6 cell pretreated with BHE ($p < 0.05$).

4. Discussion

Fluoride is a naturally occurring element, which usually presents in our drinking water at low levels. However, exposure to higher concentrations is known to cause deleterious health effects including dental and skeletal fluorosis in humans. The mechanistic basis of action includes the induction of enzyme inhibition and oxidative damages. Results indicated the cytotoxic effect of fluoride in cultured intestinal epithelial cells; the cytotoxicity has been mediated through the inhibition of catalase activity and intracellular glutathione depletion. Providing further insights, the glutathione biosynthetic enzymes such as GCS and GS are inhibited by the fluoride ions. Besides, the activity of glutathione reductase, which is an enzyme involved in the regeneration of oxidized glutathione, is also found to be reduced with fluoride exposure. The reduced activity of GCS, GS, and GR may be the possible reason for the reduced level of intracellular glutathione. Concomitantly, a significantly higher level of lipid peroxidation products has been observed in fluoride-treated cells. The results are in corroboration with previous reports, where oxidative stress and apoptosis induction has been evident (Lu *et al.* 2017).

Pretreatment with BHE at low, medium, and high doses protected the intestinal epithelial cells from fluoride-induced cell death. The expression of Nrf-2/haeme oxygenase 1 is increased in BHE supplementation; the Nrf2/HO1 system is key in regulating the glutathione biosynthesis (Loboda *et al.* 2016). Corroborating with the increased Nrf2/HO1 expression, the increased activity of GCS and GS enzymes has been noted in BHE pretreated cells; which may be responsible for the increased level of GSH in these cells. In addition, the increase in the activity of GR, which regulates the salvage

Table 1. Changes in the redox status of IEC-6 cells exposed to sodium fluoride and the reversal potential of *Borassus flabellifer* Linn., haustorium methanolic extract.

Treatment	Catalase (U/mg protein)	GSH (μmoles/ mg protein)	TBARS (nmoles/mg protein)	Conjugated Dienes (nmoles/mg protein)
Normal	52.55 ± 7.3	5.23 ± 0.60	1.82 ± 0.36	29.55 ± 4.73
NaF (3.5 mM)	28.12 ± 3.6	2.61 ± 0.34	6.03 ± 0.38	78.10 ± 8.02
BHE 25 μg/mL	37.60 ± 5.0*	2.99 ± 0.22*	4.87 ± 0.43*	60.78 ± 6.93*
BHE 50 μg/mL	44.14 ± 3.7***	3.88 ± 0.45**	3.93 ± 0.61**	45.06 ± 6.67***

The values are represented as mean ± SD of three independent experiments, each carried in triplicate. (*Indicate significant difference $p < 0.05$; ** indicate significant difference $p < 0.01$; and *** indicate significant difference $p < 0.001$).

Table 2. Changes in the glutathione metabolizing enzyme activities in intestinal epithelial cells (IEC-6 cells) exposed to sodium fluoride (NaF) and the protective effect of *Borassus flabellifer* Linn haustorium methanol extract. The values are represented as U/mg protein.

Treatment	γ-GCS (U/mg protein)	GS (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	GR (U/mg protein)
Normal	5.18 ± 0.11	7.86 ± 0.33	3.56 ± 0.30	8.29 ± 0.15	6.22 ± 0.42
NaF (3.5 mM)	2.57 ± 0.09	3.91 ± 0.27	5.88 ± 0.28	4.90 ± 0.23	3.67 ± 0.32
BHE 10 μg/mL	2.94 ± 0.13*	4.67 ± 0.15*	5.01 ± 0.24*	5.81 ± 0.41*	4.18 ± 0.22
BHE 25 μg/mL	3.39 ± 0.17**	5.23 ± 0.22**	4.18 ± 0.24**	6.42 ± 0.20**	4.80 ± 0.34**
BHE 50 μg/mL	3.98 ± 0.12***	5.91 ± 0.20***	3.55 ± 0.37***	7.09 ± 0.36***	5.48 ± 0.17***

The values are represented as mean ± SD of three independent experiments, each carried in triplicate. (*indicate significant difference $p < 0.05$; ** indicate significant difference $p < 0.01$ and *** indicate significant difference $p < 0.001$).

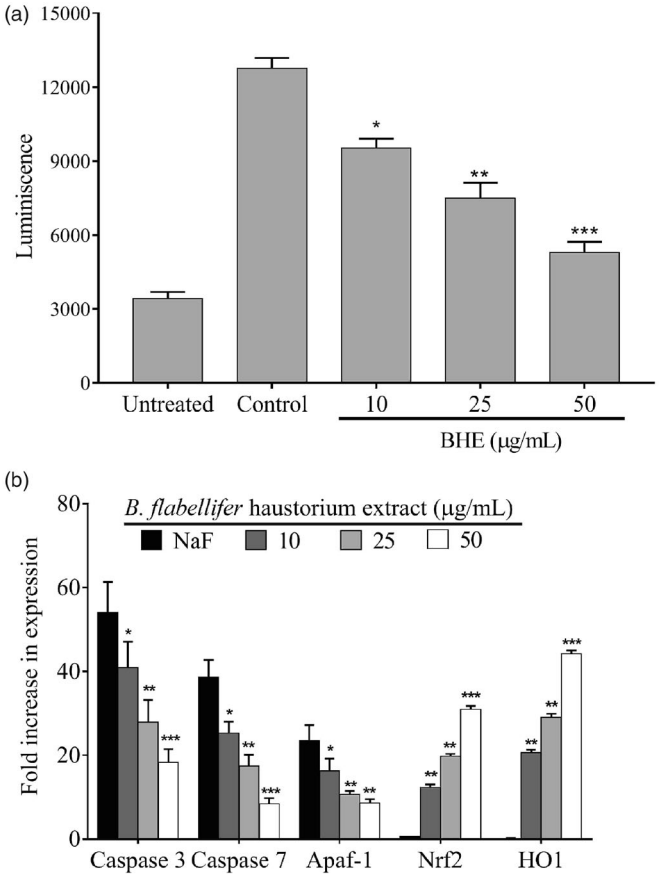

Figure 3. Detection of the caspase 3/7 activity by bioluminescence method in untreated cells, fluoride along exposed cells as well as BHE pretreated cells (a). Expression of Caspase-3, capsase-7 and Apaf-1 genes in NaF alone treated cells and those pretreated with BHE. The fold change in expression was estimated by qPCR with respect to the normal cells (b). (*Indicate significant difference $p < 0.05$; ** indicate significant difference $p < 0.01$; and *** indicate significant difference $p < 0.001$).

pathway of glutathione, may also have contributed to the increased GSH levels. Further, a reduction in catalase activity induced by fluoride treatment has also ameliorated with the BHE pretreatment. The improved antioxidant status with the BHE thus may be responsible for the observed reduction in TBARS as well as conjugated diene levels. It is thus evident that the BHE efficiently reduces the redox imbalance induced by fluoride ions.

Oxidative stress is often associated with tissue damages and cell death; in colon epithelial tissues the oxidative stress has also been shown to induce pro-carcinogenic signaling (Carini *et al.* 2017). Fluoride exposure is known to induce reactive radical formation and thereby apoptosis in a variety of cell lines (Deng *et al.* 2016, RIBEIRO *et al.* 2017). Supporting their reports, the present study also observed increased expression of caspase 3/7 and Apaf-1 genes, which are actively involved in apoptotic signaling (Parrish *et al.* 2013). Supporting the results, we observed increased caspase 3/7 expression and cytochrome c release in the fluoride-treated cells. The release of cytochrome c release is an important marker of the apoptotic cell death mediated through mitochondrial damage (Zhou *et al.* 2018, Lee *et al.* 2015). However, pretreatment with BHE successfully prevented the apoptotic signaling and thereby fluoride-induced apoptosis in the intestinal epithelial cells. Hence, our study demonstrates the protective effect of BHE against fluoride-induced toxicity in cells; these results are in concordance with the previous studies by Susheela and Bhatnagar (2002), where they proposed the possible roles of dietary antioxidants in ameliorating the fluoride-induced damages.

Hence, the present study concludes that the haustorium extract of *B. flabellifer* Linn. exhibits protective effects against fluoride-induced intestinal cell death. Reestablishing the fluoride-induced redox imbalance has been pivotal in the beneficial effects of BHE. Further, protection against free radical-induced apoptosis in intestinal cells may also indicative of the possible role of BHE in preventing ailments including ulcerative colitis and colorectal cancers. The antioxidant and cytoprotective properties thus indicate the possible value of *B. flabellifer* Linn., haustorium as a functional food.

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Ethics approval

The study involved no human or animal models; hence, no ethical issues persist.

Informed Consent

No third party material is used in the manuscript, for which permission is required.

Authors contributions

JTJ conducted the cytoprotective analysis and data analysis under the supervision of AN. Rajagopal R., Alfarhan A., and Job J.T. contributed toward the phytochemical analysis as well as initial cytotoxicity analysis. The study was designed, qPCR experiments, and the manuscript was prepared by Dr. Narayanankutty, and the manuscript was approved by all authors.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data may be made available upon valid request.

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