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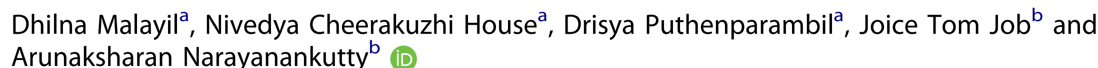
RESEARCH ARTICLE

Borassus flabellifer haustorium extract prevents pro-oxidant mediated cell death and LPS-induced inflammation



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ABSTRACT

Borassus flabellifer L., belonging to the Arecaceae family, is less explored for its nutritional and biological properties. The plant parts such as the tender kernel, tender water, *Borassus flabellifer* sap, and haustorium are consumed by various communities in India. The present study first time analyzed the cytoprotective and anti-inflammatory properties of *Borassus flabellifer* haustorium extract (BHE). The protective effect of BHE was estimated in the normal intestinal epithelial cell (IEC-6) against the pro-oxidant insults induced by hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Further, the change in redox status was estimated in terms of antioxidant enzyme activities or the oxidative stress markers. The anti-inflammatory potential of BHE was analyzed in macrophage cells (Raw 264.7) stimulated by lipopolysaccharide (LPS). BHE had a total polyphenols and flavonoids content of 37.92 ± 4.03 mg GAE and 7.02 ± 0.61 mg QE. The pretreatment with BHE has significantly reduced the cytotoxic effect of H_2O_2 and MDA ($p < 0.01$) in IEC-6 cells; restoration of cellular glutathione levels and catalase activity ($p < 0.05$), with a concomitant reduction in the thiobarbituric acid-reactive substances and conjugated dienes ($p < 0.05$) maybe the plausible mode of action. Further, H_2O_2 and MDA induced expression of apoptotic genes caspase 3/7 and apaf-1 was significantly reduced by BHE pretreatment ($p < 0.05$). Similarly, BHE dose-dependently reduced the LPS (1 μg/mL) induced release of pro-inflammatory cytokines including IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) in Raw 264.7 cells. The study concludes that *Borassus flabellifer* haustorium can be an efficient antioxidant and anti-inflammatory functional food.

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Borassus flabellifer L.; cytoprotective effect; anti-inflammatory activity; antioxidant; polyphenol content

1. Introduction

Plant products are the predominant dietary components for humans and other animals. Plants belonging to the family Arecaceae are predominantly cultivated for various food items as well as value-added products. *Borassus* palm (*Borassus flabellifer*) is a common variety found in India. Different parts of the plants including their fruit, tender water, kernel, and milk, or inflorescence sap derived from them are being consumed. Besides, during the germination time, the liquid endosperm inside the *Borassus flabellifer* kernel becomes a spongy tissue, which is also used as food and is thought to have nutritional, biological, or pharmacological properties. A study by Yoshikawa *et al.* (2007) has indicated the anti-diabetic properties of the methanolic extracts of the male flower of *Borassus flabellifer*; similarly, the root extracts (Debnath *et al.* 2013) and fruit extracts (Duraipandiyan *et al.* 2020) have hypoglycemic and lipid metabolizing properties. A dammarane triterpenoid isolated from the seed coat of *Borassus flabellifer* has been shown to inhibit the inflammatory enzymes in prostate cancer cells and thereby accelerating apoptotic events (Yarla *et al.* 2015). Antimicrobial properties are also attributed to the seed coat extract (Alamelumangai *et al.* 2014) and 2,3,4-trihydroxy-5-methyl acetophenone from the *Borassus flabellifer* syrup (Reshma *et al.* 2017). On contrary to other parts of the *Borassus flabellifer*, there is no available literature on the *Borassus flabellifer* haustorium (BH).

Cultured cells are suitable models for studying the pharmacological and toxicological aspects of various compounds, which helps to reduce animal usage for these studies. Multiple cells are used as the model of various organs, typical examples are intestinal cell lines (Sambruy *et al.* 2001). Various oxidative radicals are chosen as examples for inducing oxidative damage; the commonly used are hydrogen peroxide (H_2O_2) (Li *et al.* 2019) and malondialdehyde (MDA) (Cheng *et al.* 2011). These two molecules are physiologically relevant toxicity inducers and they are known to be involved in various pathological conditions (Lisanti *et al.* 2011, Cui *et al.* 2018). Apart from the oxidative damage models, inflammation can also be induced in monocyte/macrophage cells by using lipopolysaccharide (LPS) or phorbol esters (Narayanankutty *et al.* 2020a).

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Hence, the present study was aimed to evaluate the cytoprotective effect of *Borassus flabellifer* haustorium extract (BHE) against H_2O_2 and MDA induced cell death and its role on the cellular antioxidant status. Further, the anti-inflammatory activity of BHE was also studied in Raw 264.7 stimulated with LPS.

2. Materials and methods

2.1. Chemicals and cell lines

Diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), and Folin–Ciocalteu reagent were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Hydrogen peroxide, RPMI-1640, HEPES buffer, fetal bovine serum, MDA, penicillin and streptomycin antibiotics, L-glutamine, and sodium pyruvate were procured from Sigma Aldrich (St. Louis, MO).

Rat colon epithelial cells (IEC-6) and murine macrophages (Raw 264.7) were obtained from National Centre for Cell Science (Pune, India). The cells were maintained under standard conditions using complete growth media (RPMI-1640) with 10% FBS, 5% CO_2 at 37 °C.

2.2. Collection and extraction of Borassus flabellifer *haustorium*

The BH was collected from the Palakkad District, Kerala, India. The haustorium was collected by de-husking the fruit, which was then dried at 50 °C and powdered. The powder was extracted with 100% methanol in Soxhlet apparatus (Borosil, Mumbai, India) and under low temperature. Extracts were dissolved to a concentration of 100 mg/mL in dimethyl sulfoxide as stock for further use.

2.3. Estimation of total phenols and flavonoid content

Total polyphenol content was determined as per the Folin–Ciocalteu method (Ahmed and Tavaszi-Sarosi 2019), using gallic acid as the external standard. The quantity of polyphenols present was expressed as mg gallic acid equivalent. Total flavonoid content was estimated by the aluminum chloride method and results are expressed in terms of mg quercetin equivalent (Wang *et al.* 2020). The polyphenol composition was analyzed using LC/MS analysis as previously described (Illam *et al.* 2017).

2.4. Analysis of biologically safe concentration and cytoprotective effect

The nontoxic concentration of BHE and cytotoxicity of H_2O_2 and MDA were determined using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Mosmann 1983). Briefly, the IEC-6 cells (2.5×10^5/250 μL volume) were plated in a 48-well plate and were allowed to attach overnight. Cells were then exposed to different concentrations of H_2O_2, MDA, or BHE (10–100 μg/mL) and incubated further for 24 h. At the end of incubation, MTT (5 mg/mL) was added to each well and allowed to form formazan crystals, which were then dissolved in dimethyl sulfoxide. The absorbance was measured in a spectrophotometer (Shimadzu, Kyoto, Japan) percentage of cell death was calculated by comparing it with a control cell.

To study the protective effect, IEC-6 cells (2.5×10^5/250 μL volume) were plated in 48-well culture plates and allowed to grow to sub confluency. Cells were then pretreated for 24 h with biologically safe concentrations of BHE (10, 25, and 50 μg/mL) following which either H_2O_2 (350 μM) and MDA (450 μM) was exposed for another 24 h. The percentage cell viability of each was determined thereafter using MTT assay (Mosmann 1983).

2.5. Analysis of antioxidant and oxidative stress 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). Lipid peroxidation was estimated in terms of TBA reactive substances (Chiu *et al.* 2013) and conjugated dienes were quantified as per standard protocols (Narayanankutty *et al.* 2016). The cellular protein content was estimated by the Lowry method (Azimzadeh *et al.* 2016) and all the antioxidant and oxidative stress parameters were expressed per mg of protein.

2.6. qPCR mediated detection of apoptosis

The IEC-6 cells were treated according to the protocol mentioned in Section 2.4 and the cells were extracted using scrapers. Synthesis of cDNA was done using a direct cell to cDNA kit (CellAmp™, Takara Bio, New Delhi, India). The qPCR analysis was done using Applied Biosystem 7500 real-time PCR (Applied Biosystems, Foster City, CA) using SYBR™ Select Master Mix as per the manufacturer's instructions (Thermo Scientific, Waltham, MA). The genes and primers used were caspase 3 (forward 5′-GTG GAA CTG ACG ATG ATA TGG C-3′; reverse 5′-CGCAAAGTGACTGGATGAACC-3′), caspase 7 (forward 5′-GGACCGAGTGCCCACTTATC-3′; reverse 5′-TCGCTT TGTCGAAGTTCTTGTT-3′), apaf-1 (forward 5′-CTGGCAACGGG AGATGACAATGG-3′; reverse 5′-AGC GGA GCA CAC AAA TGA AGA AGC-3′), and the internal standard β-actin (forward 5′-AAG ATC CTG ACC GAG CGT GG-3′; reverse 5′-CAG CAC TGT GTT GGC ATA GAG G-3′). The CT value was obtained from the PCR software package and $\Delta\Delta$Ct values were calculated to determine the fold change in expression (Livak and Schmittgen 2001).

2.7. Anti-inflammatory activity of BHE on LPS-activated Raw 264.7 cells

The anti-inflammatory effect of BHE was analyzed against the pro-inflammatory cytokine production in macrophage cells activated by LPS (1 mg/mL) (Kwon *et al.* 2018, Nguyen *et al.* 2020b). Raw 264.7 cells were cultured in complete RPMI-1640 media for 24 hours with the different concentrations of BHE

Table 1. Composition of the polyphenols in *Borassus flabellifer* analyzed by LC–MS.

RT	*m/z* ratio	Compound
1.28	139	4-Hydroxybenzoic acid
1.98	165	p-Coumaric acid
2.19	155	Protocatechuic acid
2.91	193	Ferulic acid
3.38	181	Caffeic acid
6.15	291	Catechin
9.90	303	Quercetin

(10, 25, and 50 μg/mL). At the end of incubation, the cells were activated by adding LPS (1 μg/mL) concentration and incubated for another 24 h. The inflammation status was estimated in terms of cytokine profile and biochemical parameters.

Inflammatory markers such as IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) were estimated by ELISA kits of PeproTech (Rocky Hill, NJ) according to the manufacturer's instructions. Biochemical estimation of nitric oxide was done according to the procedures described previously (House *et al.* 2020).

2.8. Statistical analysis

All the results were expressed as mean ± SD for each concentration in triplicate. Statistical analysis was carried out by analysis of variance followed by the Tukey–Kramer *post hoc* test using GraphPad Prism software 7.0 version (La Jolla, CA). Values with variation $p < 0.05$ are considered statistically significant.

3. Results

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

Total polyphenol content in the BHE was 38.94 ± 3.75 mg GAE and the flavonoids content was estimated to be 7.02 ± 0.61 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 (Table 1).

3.2. Determination of cytotoxicity and biologically safe concentrations

The BHE was found to exert a very less cytotoxic effect on IEC-6 cells as well as raw 264.7 cells; event at a concentration of 100 μg/mL, cytotoxicity was less than 5%. Hence, the biological safe concentrations determined for the cytoprotective and anti-inflammatory study were 10, 25, and 50 μg/mL of BHE. The cytotoxic effect of H_2O_2 and MDA was determined to be 345.78 ± 18.5 and 457.46 ± 10.2 μM by MTT assay; therefore, the concentrations used to induce toxicity were determined as 350 and 450 μM (Figure 1).

3.3. Cytoprotective effect

The cytoprotective effect of BHE against H_2O_2-induced cell death is shown in Figure 2(a). Treatment with H_2O_2 alone reduced the cell viability to 50.8 ± 2.8%. However, pretreatment with BHE at

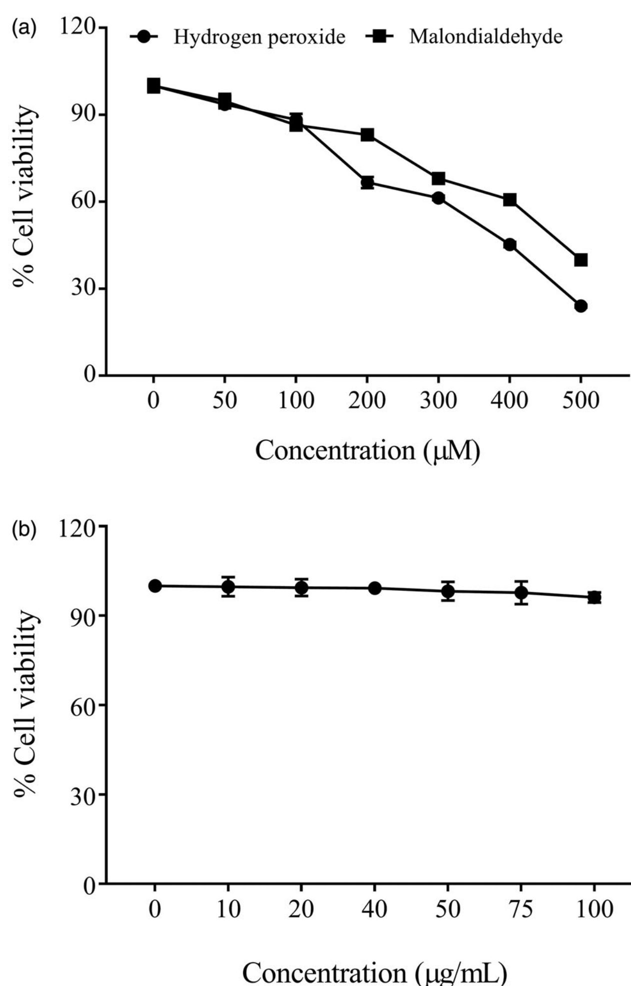

Figure 1. The cytotoxic effect of hydrogen peroxide and malondialdehyde (a) as well as *Borassus flabellifer* haustorium extract (b).

their respective doses 10, 25, and 50 μg/mL increased the cell viability by 13.8, 26.8, and 45.1%, respectively (Figure 2(a)).

Likewise, the treatment with MDA reduced the cell viability in IEC-6 cells to 48.2 ± 1.8%. Pretreatment with the BHE increased the cell viability by 12.0, 25.0, and 41.7%, at the respective doses 10, 25, and 50 μg/mL (Figure 2(b)). Compared to the H_2O_2 mediated toxicity, the protective effect of BHE was marginally reduced against MDA.

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

3.4.1. Redox status in H_2O_2 treated cells

The cytoprotective effect of BHE was found to be mediated through the establishment of redox balance in terms of elevated GSH levels, catalase activity and subsequently reduced lipid peroxidation markers. As indicated in Table 2, the GSH levels were significantly reduced by the treatment with H_2O_2 (350 μM); however, pretreatment with the BHE significantly increased the GSH levels at the doses 25 ($p < 0.05$) and 50 μg/mL ($p < 0.01$). Likewise, the increase in the catalase activity by H_2O_2 treatment was also improved by the pretreatment with 25 ($p < 0.01$) and 50 μg/mL ($p < 0.001$). Corroborating with the improved levels of antioxidants, there observed a significant reduction in the lipid peroxidation products. The TBARS and

conjugated diene levels were increased by the treatment with H_2O_2; however, pretreatment with 25 ($p < 0.01$) and 50 μg/mL ($p < 0.001$) reduced the levels (Table 2).

3.4.2. Redox status in MDA treated cells

In MDA treatment, the redox imbalance in terms of GSH depletion and elevated catalase activity was observed; however, the intensity was lesser than that of H_2O_2. On contrary, the lipid peroxidation induced by MDA was far higher than that of H_2O_2. The GSH depletion induced by the MDA was well reversed by BHE treatment with 25 ($p < 0.05$) and 50 μg/mL ($p < 0.01$). Likewise, the elevation in catalase activity was also reverted significantly by the BHE treatment in IEC-6 cells; the efficacy was dose-dependent (Table 2). The restoration of the cellular antioxidant system was also reflected in terms of reduction in the intracellular TBARS and conjugated diene levels, which was significantly reduced upon pretreatment with BHE 25 ($p < 0.05$) and 50 μg/mL ($p < 0.01$).

3.5. Gene expression analysis

The changes in the expression of the apoptotic genes are shown in Figure 3. The caspase 3 expression was significantly

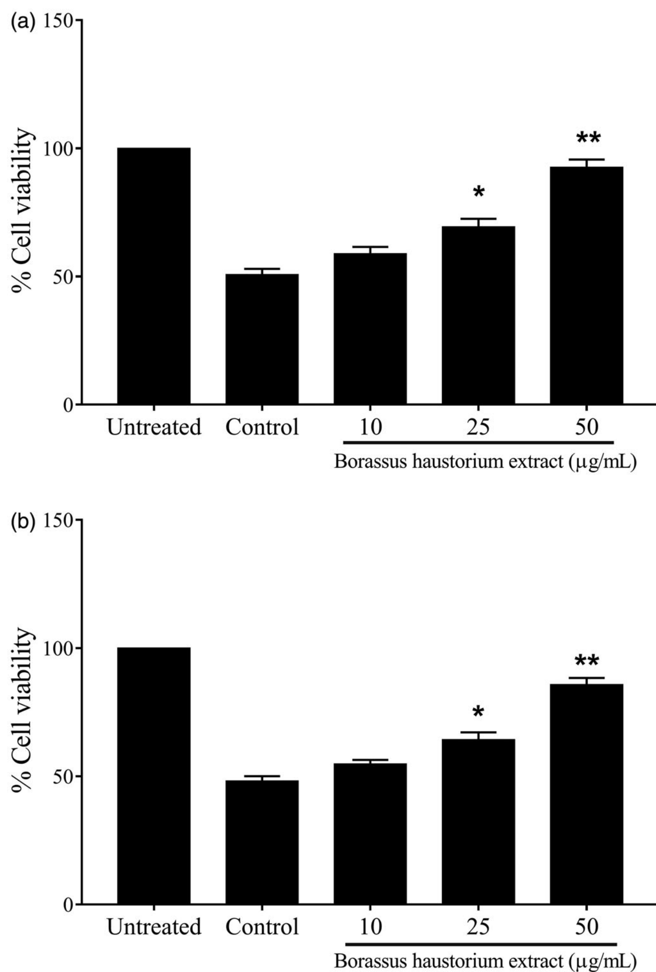

Figure 2. Cytoprotective effect of *Borassus flabellifer* haustorium extract against hydrogen peroxide (a) and malondialdehyde (b) induced toxicity in IEC-6 cells.

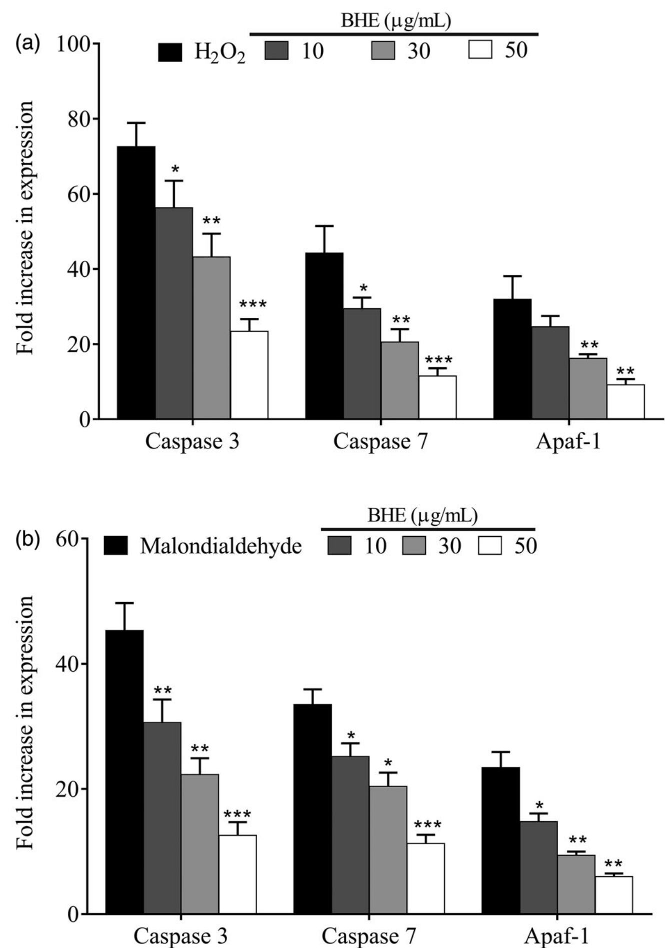

Figure 3. Expression of genes caspase 3, caspase 7, and apaf-1 in cells treated with pro-oxidants hydrogen peroxide (a) or malondialdehyde (b) and the protective effect of *Borassus flabellifer* haustorium extract.

Table 2. Antioxidant parameters in the IEC-6 cells exposed to MDA/hydrogen peroxide and the protective effect of *Borassus haustorium* extract.

Treatment	Catalase (U/mg protein)	GSH (μmol/mg protein)	TBARS (nmol/mg protein)	Conjugated dienes (nmol/mg protein)
Normal	40.91 ± 7.4	4.18 ± 0.41	2.29 ± 0.62	32.14 ± 5.11
H_2O_2 (350 μM)	79.33 ± 8.4	2.07 ± 0.25	6.97 ± 0.53	96.33 ± 7.27
BHE (25 μg/mL)	58.88 ± 4.1**	2.88 ± 0.31*	5.04 ± 0.98*	65.12 ± 7.04**
BHE (50 μg/mL)	47.95 ± 6.9***	3.39 ± 0.42**	3.88 ± 0.59**	54.17 ± 6.02***
MDA (450 μM)	69.22 ± 4.9	2.30 ± 0.32	8.16 ± 1.17	83.72 ± 6.93
BHE (25 μg/mL)	64.10 ± 5.2*	3.04 ± 0.40*	6.02 ± 0.73*	67.20 ± 7.19*
BHE (50 μg/mL)	56.26 ± 8.0**	3.56 ± 0.35**	4.36 ± 0.66**	50.22 ± 6.51**

The values are represented as mean ± SD of three independent experiments, each carried in triplicate.
*Significant difference $p < 0.05$.
**Significant difference $p < 0.01$.
***Significant difference $p < 0.001$.

Table 3. Anti-inflammatory activity of *Borassus haustorium* extract against lipopolysaccharide induced activation of Raw 264.7 cells.

	IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	TNF-α (pg/mg protein)	NO (μM/mg protein)
Untreated	72.8 ± 8.3	125.7 ± 22.5	169.3 ± 8.6	10.7 ± 1.67
LPS	458.1 ± 40.7	1452.4 ± 104.1	1828.7 ± 122.3	103.1 ± 10.5
BHE (10 μg/mL)	371.0 ± 46.4ns	1237.6 ± 117.8ns	1514.0 ± 143.2*	89.7 ± 7.2ns
BHE (25 μg/mL)	284.1 ± 51.5**	1063.2 ± 112.9**	1374.5 ± 138.6**	76.3 ± 9.5*
BHE (50 μg/mL)	219.5 ± 44.6***	956.8 ± 107.0***	1218.6 ± 106.5**	61.0 ± 7.2**

The values are represented as mean ± SD of three independent experiments, each carried in triplicate.
*Significant difference $p < 0.05$.
**Significant difference $p < 0.01$.
***Significant difference $p < 0.001$.

increased in the H_2O_2 (Figure 3(a)) and MDA (Figure 3(b)) alone treated IEC-6 cells, with a higher extent in the former. Pretreatment with the different concentrations of BHE dose-dependently reduced the expression ($p < 0.05$) of caspase 3. Likewise, the treatment with MDA and H2O2 induced the expression of pro-apoptotic genes caspase-7 and apaf-1. Pretreatment with BHE was capable of preventing the pro-oxidant mediated upregulation of these apoptotic genes, thereby improving cell viability.

3.6. Anti-inflammatory activity

Raw 264.7 cells exposed to LPSs were shown to secret higher amounts of pro-inflammatory cytokines and nitric oxide radicals. Table 3 summarizes the results of the anti-inflammatory activities of BHE in the LPS primed macrophage cells. Levels of pro-inflammatory cytokines were significantly elevated in LPS stimulated macrophages ($p < 0.001$); however, pretreatment with the BHE at the doses 25 ($p < 0.01$) and 50 μg/mL ($p < 0.001$) significantly reduced the elevation in IL-1β and IL-6 secretion. However, this reduction was marginal in the lowest dose of BHE (10 μg/mL). Further, the LPS-mediated elevation in TNF-α levels was efficiently brought down by all the three doses of BHE ($p < 0.05$). Together with the reduction in cytokine levels, the increase in nitric oxide radicals was also abrogated by the pretreatment with BHE 25 ($p < 0.05$) and 50 μg/mL ($p < 0.01$).

4. Discussion

Palm plants are important sources of edible items and other nutritious value-added compounds; various parts of these plants including fruit, kernel juice, tender water, and haustorium. Several products of the plants belonging to this family, especially coconut products, have been explored widely for their nutritional and pharmacological properties (Narayanankutty *et al.* 2018a, 2018b, 2020b). Among these, *Borassus flabellifer* is a less explored plant which is widely consumed in various parts of India. However, limited reports have indicated the biological efficacy of the plant (Alamelumangai *et al.* 2014, Yarla *et al.* 2015, Reshma *et al.* 2017). In this study, the cytoprotective efficacy and anti-inflammatory potential of BHE have been evaluated in cultured intestinal epithelial cells (IEC-6) and murine macrophages (Raw 264.7). During the phytochemical screening, the presence of higher amounts of phenolic and flavonoid compounds has been identified. Further, the LC/MS analysis suggested the presence of phenolic acids such as ferulic acid, coumaric acid, and protocatechuic acid; apart from that, flavonoids such as catechin and quercetin have also been detected in BHE.

In cultured intestinal epithelial cells, treatment with MDA and H_2O_2-induced significant toxicity was mediated through oxidative insults. Previous studies have also indicated a similar cytotoxic effect of MDA (Cheng *et al.* 2011) and H_2O_2 (Liu *et al.* 2005) compounds in multiple cell types. In this study, pretreatment with BHE reduced the cytotoxic effect of both these compounds in the cultured intestinal cells. Exposure to H_2O_2 and MDA has been shown to reduce the intracellular GSH levels (Narayanankutty *et al.* 2019), concomitantly elevating lipid peroxidation products such as TBARS and conjugated dienes (Al-Sheddi *et al.* 2016). Similarly, due to the exposure of cells to peroxidative damages, the activity of intracellular catalase is also elevated (Arnold *et al.* 2001, Konyalioglu *et al.* 2013, Martins and English 2014). Upon pretreatment with BHE, the cellular GSH levels were restored to near-normal and subsequently reducing the TBARS and conjugated diene levels. Corroborating with this, significant restoration of catalase activity has also been observed in cell pretreated with BHE. Expression of the antioxidant enzymes and GSH system are under the control of a transcription factor, nuclear factor erythroid 2-related factor 2 (nrf2), which binds to the antioxidant response element (ARE) (Liu *et al.* 2018, Tonelli *et al.* 2018). The compounds identified in BHE, such as caffeic acid, ferulic acid, and quercetin have been previously reported to activate the nrf2 signaling (Zhou *et al.* 2019). It is thus possible that these molecules may be responsible for the antioxidant mediated cytoprotective efficacy of BHE. Apart from this, H_2O_2 and MDA alone treatment have been shown to upregulate the expression of caspase 3/7 and apaf-1 genes, which are pro-apoptotic (Jan and Chaudhry 2019). However, pretreatment with the different concentrations of BHE has efficiently reduced the pro-oxidant mediated expression of these apoptotic genes. It is thus possible that BHE protects the cells from peroxide and MDA mediated cell death, which is partially mediated through the restoration of redox balance and inhibition of apoptotic gene expression. Since oxidative stress and associated chronic inflammation triggers neoplastic signaling in colon epithelial tissues (Carini *et al.* 2017), the BHE may help to prevent colon carcinogenesis.

Together with this, BHE pretreatment has been shown to inhibit inflammation in the LPS-stimulated macrophages. LPS treatment has been shown to induce cytokine secretion in macrophages, mediated through NF-kB, and toll like receptor signaling (Narayanankutty 2019, Sanjeewa *et al.* 2020). Inflammatory insults of LPS in macrophages are also mediated through nitric oxide production (Joo *et al.* 2014). Therefore, the reduced production of nitric oxide in BHE pretreated cells may indicate the possible inhibition of nitric oxide synthase by the polyphenols of BHE. Previously, various studies have indicated the possible role of natural products, as well as plant extracts in the prevention of the LPS, stimulated production of pro-inflammatory cytokines from Raw 264.7 cells (Torres-Rodríguez *et al.* 2016, Nguyen *et al.* 2020a). Hence, the BHE may develop as an anti-inflammatory drug candidate in the future.

The study thus concludes that the BHE exhibits significant cytoprotective effects mediated through the establishment of cellular redox balance. Besides, the BHE interfere with inflammatory cascades by blocking cytokine production and nitric oxide synthesis. Therefore, it is expected that by the antioxidant and anti-inflammatory potentials, BHE may be effective in preventing various chronic diseases, which needs to be ascertained in future studies. Together, BH may be considered as a functional food and regular intake of which may help in preventing various chronic diseases.

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Disclosure statement

The authors express no conflict of interest in the current study.

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