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Toxic effects of fluoride in intestinal epithelial cells and the mitigating effect of methanol extract of coconut haustorium by enhancing *de novo* glutathione biosynthesis

Joice Tom Job [a], Rajakrishnan Rajagopal [b], Ahmed Alfarhan [b], Varsha Ramesh [c], Arunaksharan Narayanankutty [a,*]

[a] Division of Cell and Molecular Biology, Post Graduate & Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut, Kerala, India
[b] Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box-2455, Riyadh, 11451, Saudi Arabia
[c] Department of Biotechnology, Deakin University, Melbourne, Australia

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ABSTRACT

Fluoride ions are an important environmental contaminant and pollutant found in a wide variety of environmental conditions. The fluoride in drinking water is evident to induce toxic effects including neurodegeneration, skeletal and dental fluorosis as well as organ damage. Nutraceuticals and functional foods are emerging as possible preventive agents against fluoride toxicity. Hence, the possible use of an emerging functional food-the coconut haustorium is being evaluated against sodium fluoride-induced toxicity in intestinal cells (IEC-6). The cells exposed to fluoride showed significant cell death mediated through the increased lipid peroxidation and glutathione depletion. The glutathione biosynthetic enzymes were inhibited by the exposure to fluoride and the apoptotic genes (caspases 3/7 and apaf-1) were upregulated. The CHE pre-treatment improved the activity of enzymes involved in the *de novo* biosynthesis of glutathione and subsequently improved the intracellular GSH pool. The improved antioxidant defense was also evident from the reduced expression of apoptotic genes ($p < 0.05$). Overall, the study concludes that fluoride ions induce oxidative stress-mediated apoptosis in intestinal epithelial cells, via inhibiting glutathione biosynthesis. Methanol extract of coconut haustorium increased glutathione biosynthesis and subsequently prevented fluoride toxicity in IEC-6 cells by virtue of its antioxidant potentials.

1. Introduction

Fluoride is a common pollutant in various environmental systems including terrestrial, aquatic, and even groundwater sources (Su et al., 2021). It is well-known for its ill effects on animals and humans; these effects include skeletal and dental fluorosis, oxidative stress, and organ toxicities (Farmus et al., 2021; Shankar et al., 2020). The mode of toxicity is through the dietary intake through drinking water, which is very common in tropical countries (Shashi and Meenakshi, 2015; Tkachenko et al., 2020; Waugh, 2019). Based on the recommendations of various studies, World Health Organization has fixed 1.5 ppm of fluoride as the maximum permitted level in drinking water, whereas, there are several countries with a drinking water fluoride content that is above this limit (Tkachenko et al., 2020). In humans and animals, the fluoride ions are directly absorbed from the gastrointestinal tract, especially from the stomach and across the intestinal epithelial tissues (Villa et al., 1993). This in turn reported to cause alterations in the composition of gastrointestinal microflora and thereby causing changes in the colorectal barrier functioning (Wang et al., 2020). Besides, there is evidence that high dietary fluoride intake often tends to increase the risk for the overall development of inflammatory bowel diseases (Follin-Arbelet and Moum, 2016). In addition, studies have reported that the incidence of colorectal cancers is also positively associated with the fluoride intake through drinking water (Takahashi et al., 2001; Yang et al., 2000). The possible role of nutraceuticals and functional foods in prevention of fluoride toxicity has been recently proposed (Susheela and Bhatnagar, 2002).

Among the functional foods, the products from *Cocos nucifera* L or Coconut are important. Various parts and products of coconut are widely used for edible purposes in various South Asian countries; the

* Corresponding author. .
E-mail address: arunaksharan1990@gmail.com (A. Narayanankutty).

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predominant products from the coconut include its edible oils, milky extract of fresh coconut kernel, and water from tender coconuts. The edible oils from fresh, grated coconut kernel or copra and the liquid endosperm been emerged as functional foods and have been evaluated for their biopharmaceutical potentials. Virgin coconut oil is an important functional food and has been known for its antioxidant, anti-inflammatory (Narayanankutty et al., 2018), cytoprotective and anti-diabetic (Illam et al., 2017), cancer preventive (Narayanankutty et al., 2020), and chemoprotective properties (Famurewa et al., 2020a, 2020b). Apart from these, Coconut water (Rao and Ikram, 2019), coconut inflorescence sap (Mammen et al., 2020), and coconut milk (Ratheesh et al., 2017) are also known for their health benefits. Preliminary studies on the coconut haustorium as reported by Manivannan et al. (2018) and independent studies from Arivalagan et al. (2018) have reported the presence of nutritionally active products in it. Despite the presence of these nutritional factors in coconut haustorium, the product has not been explored for its biological or pharmaceutical effects.

Due to the significant impact of fluoride on intestinal tissues, the present study evaluated the mechanism of fluoride toxicity and the protective effect of CHE on fluoride toxicity and emphasizing its roles in redox balance and apoptosis.

2. Materials and methods

2.1. Chemicals and cell lines

The fluoride source, sodium fluoride of extra pure grade (NaF), fetal bovine serum, RPMI-1640 (without L-glutamine and Sodium pyruvate), HEPES buffer (1 M), L-glutamine (200 mM), and sodium pyruvate (100 mM) were purchased from Sigma Aldrich (St. Louis, MO, United States). The two-step Cell to cDNA kit (CellAmp™) was purchased from Takara Bio (Bengaluru, India) and qPCR master mix (SYBR™ Select Master Mix) was obtained as a generous sample from Thermo Scientific (Massachusetts, United States). The remaining chemicals used in the experiment were of reagent grade that was procured from SRL Ltd. (Mumbai, India).

Rat immortalized (normal) intestinal cell line (IEC-6) was obtained from the cell repository at NCCS, Pune, India. The IEC-6 cells were maintained in complete RPMI-1640 supplemented with 10% FBS, 5% CO_2 at 37 °C and passaged every third day to a fresh T25 cm^2 flask (Tarson, India).

2.2. Collection and extraction coconut haustorium

Mature coconuts were collected and they were maintained in shady and moist areas to germinate. The germinated coconut was then dehusked and haustorium was collected and dried at 50 °C in an incubator. The dried haustorium (50 g) was then powdered and extracted with methanol (250 mL) using the Soxhlet apparatus (Borosil, India). The quantity of dried coconut haustorium and yield of extract was calculated and expressed s percentage. The methanol extract was then dried and re-dissolved in dimethyl sulfoxide (DMSO) for further assays; a portion of which was dissolved in HPLC grade methanol for LCMS analysis.

2.3. Characterization of polyphenols by LCMS

The total phenolic content of the methanol extract of coconut haustorium (CHE) was estimated according to the standard protocols described by Folin–Ciocalteau method (Fahmi et al., 2021). About, 100 μL of the coconut haustorium extract was mixed with 800 μL of water and 2 mL of 1 N Folins reagent and mixed well. After 5 min, 7 mL of 10% (w/v) of Na2CO3 was added and the optical density was measured at 700 nm after a 30-min incubation. A standard curve was plotted using gallic acid as standard and the final quantity was indicated as mg gallic acid equivalent (GAE) per gram of tissue. The phenolic compounds present in the sample were analyzed using liquid chromatography-mass spectrometry by gradient elution method as per the protocols described in the studies of Illam et al. (2017).

2.4. Analysis of the cytoprotective effect

The cytotoxicity of both CHE and sodium fluoride was assessed by a thiazolyl blue tetrazolium bromide assay for 48 h (Mosmann, 1983). The doses of CHE, which is causing less than 1% of cell death are considered to be biologically safer for cytoprotective studies. The dose corresponding to the IC50 value of sodium fluoride was used to induce toxicity in further cytoprotective studies.

The cells were cultured in T25 cm^2 flasks, and actively dividing cells at 60–70% confluency were used for the experiment. The collected cells were added to 24-well tissue culture-treated plates and allowed to attach and grow for 12 h. The cells were then pre-treated with 10, 20, and 40 μg/mL doses of CHE and kept for 24-h incubation, and the media containing the CHE was replaced with fresh RPMI-1640 media containing 3.5 mM of NaF. The cell viability in NaF alone treated cells and that of CHE pre-treated ones were determined by MTT assay at the end of 24-h incubation.

2.5. Analysis of redox parameters including glutathione metabolizing enzymes

Rat intestinal epithelial cells (IEC-6) were plated in T75 cm^2 flasks at a density of 1×10^7 cells/mL of complete RPMI-1640 media. The attached cells were then pre-treated with different concentrations of CHE for 24 h and then exposed to NaF (3.5 mM). A normal flask without any treatment and a NaF alone flask was maintained as a negative control. At the end of 24 h, the media was removed and washed with phosphate-buffered saline, and cells were harvested using mechanical cell scrapers. A portion of the cells was used to synthesize cDNA and the remaining portion was lysed in phosphate buffer (pH7.0) containing protease inhibitor and the lysate was centrifuged at 2000g for 15 min in a cold centrifuge (Remi, India) at 4 °C to collect the supernatant and which was used for biochemical analysis.

The intracellular reduced glutathione content was quantitatively determined using Ellman's reagent (Nair et al., 2016). Briefly, the reaction mixture contained 0.1 mL of 20% cell lysate along with 7.5 mM DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)), 2.5 mM EDTA, and 0.33 mM sodium dihydrogen orthophosphate; the optical density of the samples was measured at 412 nm using a spectrophotometer. The activities of intracellular *de novo* glutathione biosynthetic enzymes such as γ-glutamyl cysteine synthetase (GCS)- that is used for the synthesis of γ-glutamyl cysteine, and glutathione synthetase (GS)- that is involved in the formation of glutathione, were determined as per the methods described in an article published by Volohonsky et al. (2002). The activity of detoxification enzymes including glutathione-s-transferase was conducted by kinetic spectrophotometric scanning method (Moatamedi Pour et al., 2014). Briefly, the reaction mixture contained 200 mM reduced glutathione, 100 mM 2,4-Dinitrochlorobenzene in 0.1 M phosphate buffer (pH 7.4). The enzymatic activity of glutathione reductase was initiated by the addition of 0.05 mL of cell lysate and continuous spectrophotometric determination for 5 min. The glutathione peroxidase activity (De Vega et al., 2002) was determined indirectly in terms of the NADPH utilized for the conversion of oxidized glutathione (that is formed by GPx activity) to reduced form by glutathione reductase (GR). The intracellular GR activity was estimated as per standard protocols available (Mannervik, 2001); briefly, 2 mM oxidized glutathione was mixed with 2 mM NADPH in phosphate buffer (0.05 M, pH 7.4). The rate of NADPH utilization during enzyme activity was estimated as the change in optical density at 340 nm was used to calculate.

The activity of catalase, an enzyme associated with detoxification of peroxide radicals, was determined as the rate of cleavage of hydrogen peroxide as reported by Al-Oqail et al. (2020). The reaction system was

composed of 50 mM phosphate buffer (pH 7.0), 0.036% of hydrogen peroxide, and 0.1 mL of cell lysate. The change in absorbance at 240 nm was recorded and the rate was calculated. Cellular lipid peroxidation status was measured in terms of thiobarbituric acid reactive substances (TBARS) and conjugated dienes according to the standard protocols available (Chiu et al., 2013; Narayanankutty et al., 2016). The TBARS was estimated as malondialdehyde content using a reaction mixture contained 8% thiobarbituric acid, 0.8 sodium dodecyl sulfate, and 20% acetic acid; the optical density was measured at 532 nm. The conjugated diene levels were measured in terms of absorbance at 234 nm.

2.6. qPCR analysis and detection of apoptosis

The untreated cells, NaF alone treated cells, and those treated with different doses of CHE were collected as described in section 2.5. The collected cells were then used for cDNA synthesis using standard protocols described in the instruction manual of CellAmp™ (Takara Bio, India). The cDNA quantity and purity were analyzed and the qPCR analysis for the gene expression changes was conducted in Applied Biosystem 7500 real-time quantitative PCR using SYBR™ Select Master Mix as per the instruction manual (Thermo Scientific, Massachusetts, United States). The expression of target genes in different treatment groups was conducted against beta-actin as the internal standard. The details of primers used have been appended in Table 1.

The PCR conditions were as follows; the sample was initially denaturation at 95 °C for 2 min, further 40 cycles composed of denaturation at 95 °C for 30 s, primer annealing was conducted at 58 °C for 45 s, and polymerase-mediated extension at 72 °C for 30 s. Apart from these, a final annealing and extension step was added with 58 °C and 72 °C for 2 min. The CT value was calculated by the system software and the fold change in expression of target genes was estimated concerning the normal cells using the $2^{-\Delta\Delta CT}$ method as per the protocols described by (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The in vitro studies were carried out in 24 well plates with 4 replicas of each concentration analyzed; the experiment was repeated three times for concordant values. The data in the manuscript has been given as mean ± SD for each experiment. Gene expression was conducted in triplicate for each dose. The Statistical operations were conducted with one-way ANOVA followed by the Tukey Kramer test (Graph pad Prism 7.0, La Jolla, USA).

3. Results

3.1. Yield, polyphenol content, and composition analysis by LCMS

The fresh coconut haustorium yielded 10.97 ± 0.87% as dry coconut kernel upon drying; the yield of extract was estimated to be 2.17 ± 0.11% from the dried coconut haustorium. The total polyphenol content was estimated to be 57.26 ± 3.65 mg GAE/g of dried coconut haustorium. The LC/MS analysis revealed the presence of both simple phenolic acids and complex polyphenols and flavonoids in the CHE. The phenolic acids reported in the CHE include ferulic acid, protocatechuic acid, and *p*-coumaric acid; further, complex flavonoids such as catechin, quercetin, Myricetin-3-glucoside was also present in the sample (Table 2).

3.2. Cytoprotective effect of CHE against NaF-induced cell death

Methanol extract of Coconut haustorium showed very low cytotoxicity towards IEC-6 cells with no-observable toxicity till 100 μg/mL over 48 h. Therefore the study selected doses 10, 20, and 40 μg/mL for further cytoprotective studies. Exposure to sodium fluoride has been found to reduce the cell viability significantly with an IC50 value of 3.37 mM and therefore a dose of 3.5 mM was selected for cytoprotective studies (Supplementary material 1).

The IEC-6 cells were growing normally in the RPMI-1640 media; however, exposure to a dose of 3.5 mM NaF reduced the cell viability significantly to 48.19 ± 2.1% (Fig. 1a) compared to the untreated cells. Pre-treatment with 10, 20, and 40 μg/mL alleviated the toxic effect of NaF and improved the cell viability respectively to 56.62 ± 2.8, 70.91 ± 3.1, and 85.11 ± 2.2% (Fig. 1a). As the lower dose CHE was less effective in alleviating the cytotoxic effect of NaF, the doses 20 and 40 μg/mL were only used for evaluating the antioxidant defense system.

3.3. Effect on CHE on glutathione metabolism and antioxidant status

Normal cellular activities of glutathione metabolizing enzymes are listed in Table 3. Exposure to NaF (3.5 mM) significantly reduced ($p < 0.001$) the activities of glutathione biosynthetic enzymes (GCS and GS) as well as glutathione-dependent detoxification enzymes (GST and GR) compared to the untreated cells. On contrary, the activity of GPx was heightened in NaF alone treated cells ($p < 0.05$) subsequently resulting in a reduced level of GSH in cells. Further, the catalase activity was also found to be significantly reduced in IEC-6 cells exposed to NaF, concomitantly resulting in an elevation in the TBARS and conjugated diene levels (Table 3).

In connection with the cytoprotective properties, the antioxidant status in terms of glutathione biosynthetic enzymes was increased upon treatment with CHE. Concomitant with an increased GSH level, the GPx activity of these cells was restored to normal (Table 4). Further, the improvement in cellular antioxidant defense was also reflected in the levels of intracellular TBARS as MDA and conjugated dienes in these cells ($p < 0.05$).

3.4. Apoptosis induction by NaF and protective effect by CHE

In comparison with the normal cells, the intracellular expression of pro-apoptotic genes including caspase-3, caspase-7, and apaf-1 had been significantly elevated in the NaF treated IEC-6 cells (Fig. 1b). The increased expression of executioner caspase indicates the pro-apoptotic role of NaF. However, pre-treatment with the different concentrations of CHE, being the lowest level of expression in the highest dose, protected the cells from fluoride-induced oxidative insults and subsequent cell death ($p < 0.05$).

Table 1
The sequence of primers used in apoptosis detection in IEC-6 cells against the internal control gene β-actin.

Gene	Direction	Sequence
Caspase-3	Forward	5′-GTGGAACTGACGATGATATGGC-3′
	Reverse	5′-CGCAAAGTGACTGGATGAACC-3′
Caspase-7	Forward	5′- GGACCGAGTGCCCACTTATC-3′
	Reverse	5′-TCGCTTTGTCGAAGTTCTTGTT-3′
Apaf-1	Forward	5′-CTGGCAACGGGAGATGACAATGG-3′
	Reverse	5′-AGCGGAGCACACAAATGAAGAAGC-3′
β-actin	Forward	5′-AAGATCCTGACCGAGCGTGG-3′
	Reverse	5′-CAGCACTGTGTTGGCATAGAGG-3′

Table 2
LC-MS analysis result of the phenolic composition of Coconut haustorium methanol extract.

RT	m/z ratio	Compound
1.28	139	4-hydroxy benzoic 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

(RT- Retention Time; m/z ratio-mass to charge ratio).

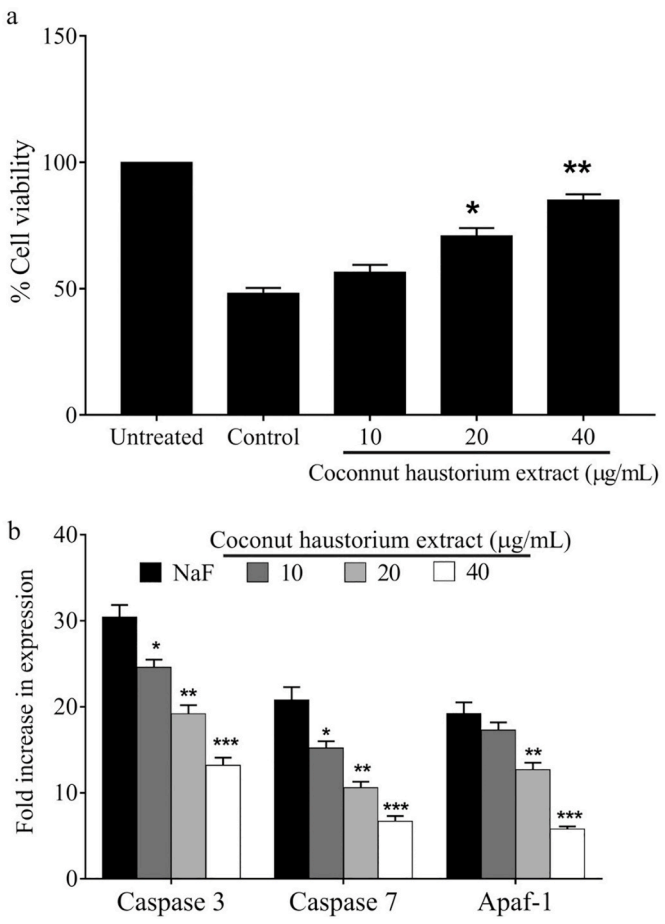

Fig. 1. Cytoprotective effect of the methanol extract of Coconut haustorium against sodium fluoride-induced toxicity in immortalized intestinal cell (IEC-6) (a). Change in the expression of genes such as Caspase-3, capsase-7 and Apaf-1 cells exposed to NaF alone and those pre-treated with CHE (b). The fold change in expression is estimated with respect to the untreated normal cells (*indicate significant difference with NaF $p < 0.05$; ** indicate significant difference with NaF $p < 0.01$; and *** indicate significant difference with NaF $p < 0.001$).

Table 3
Alterations in the antioxidant and lipid peroxidation status in IEC-6 cells exposed to fluoride (3.5 mM) and the alleviating ability of coconut haustorium methanolic extract.

Treatment	Catalase (U/mg protein)	GSH (µmoles/mg protein)	TBARS (nmoles/mg protein)	Conjugated Dienes (nmoles/mg protein)
Untreated	84.37 ± 2.8***	5.81 ± 0.23***	1.42 ± 0.15***	19.43 ± 2.10***
NaF (3.5 mM)	36.02 ± 4.2	3.04 ± 0.17	5.78 ± 0.26	80.43 ± 3.11
CHE 20 µg/mL	52.25 ± 4.8*	3.68 ± 0.22*	4.67 ± 0.31*	55.92 ± 3.40*
CHE 40 µg/mL	67.01 ± 5.1***	4.34 ± 0.30**	4.05 ± 0.27**	41.29 ± 5.16***

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

4. Discussion

Fluoride toxicity is the prevailing concern in the health aspects as well as environmental issues associated with that. The toxic effects of fluoride ions are numerous and largely affect the ecosystem, plants, animals, and humans. Health effects of fluoride include skeletal and dental fluorosis as well as multiple organ toxicities. Recent studies suggested the application of dietary agents like nutraceuticals and functional foods in the prevention of fluoride toxicity significantly. Therefore the present study evaluated the preventive efficacy of coconut haustorium methanol extract, a fewer studies antioxidant functional food in alleviating the fluoride toxicity in intestinal cells.

Table 4
Changes in the activities of glutathione-dependent detoxification enzymes in IEC-6 exposed to fluoride and the mitigating efficacy of the Coconut haustorium methanol extract.

Treatment	γ-GCS (U/mg protein)	GS (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	GR (U/mg protein)
Untreated	4.88 ± 0.22***	6.83 ± 0.16***	3.44 ± 0.24***	8.79 ± 0.41***	7.83 ± 0.24***
NaF (3.5 mM)	2.56 ± 0.15	4.19 ± 0.24	5.39 ± 0.17	4.57 ± 0.32	4.87 ± 0.19
CHE 20 µg/mL	3.17 ± 0.34*	4.90 ± 0.18	4.76 ± 0.14	5.04 ± 0.28*	5.54 ± 0.32*
CHE 40 µg/mL	3.94 ± 0.19***	5.87 ± 0.27**	4.02 ± 0.20**	5.99 ± 0.31***	6.38 ± 0.31***

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

The study went on to analyze the mitigating potential of CHE in the toxicities induced by sodium fluoride. The intestine is one of the predominant sites of fluoride absorption and therefore more direct damages are expected in intestinal epithelial cells (Nopakun et al., 1989). In different in vitro models, fluoride exposure has been shown to induce the formation of reactive radical formation subsequently oxidative damages and apoptosis (Deng et al., 2016; Ribeiro et al., 2017). Excessive doses of fluoride exposure are also known to induce damages to the intestinal mucosa and changes in gut microbiota (Fu et al., 2020; Wang et al., 2020). Supporting their reports, the present study also observed a significant reduction in glutathione levels and a subsequent increase in lipid peroxidation in intestinal cells. In addition, increased expression of caspase genes and apaf-1 are observed in fluoride exposed cells; as these genes are actively involved in the apoptosis, it is clear that the cell death induced by fluoride in IEC-6 cells is also through apoptosis (Parrish et al., 2013). Reports have indicated that fluoride induces apoptosis in cells mediated through mitochondrial membrane disruption and subsequent activation of intrinsic and extrinsic pathways (Agalakova and Gusev, 2012). Interaction of Fas/FasL interaction and P38/MAP kinases pathways are also proven to be involved in fluoride-induced apoptosis (Thrane et al., 2001). It is therefore possible that the cytoprotective effect of the CHE may be mediated through the modulation of mitochondrial biogenesis or inhibition of apoptotic pathways.

However, CHE pre-treatment dose-dependently inhibited the cytotoxicity induced by fluoride. Providing a mechanistic basis of the cytoprotection, the treatment with CHE has been shown to enhance the enzymatic activity of γGCS and GS; these enzymes are responsible for the *de novo* biosynthesis of glutathione in cells (Janowiak et al., 2006). Further, the activity of glutathione reductase (GR), which is responsible for the reduction of oxidized glutathione thereby contributing to the intracellular reduced glutathione pool (Couto et al., 2016). Therefore, it is possible that increased activity of these enzymes may have resulted in the observed increase in reduced glutathione levels. In corroboration with these, the apoptotic genes are also downregulated with the treatment of CHE as its different doses. It is therefore clear that the CHE pre-treatment inhibits the apoptotic cell death in intestinal epithelial cells. The anti-apoptotic effect has been accorded to the individual polyphenol compounds including ferulic acid and coumaric acid in multiple models (Daroi et al., 2021; Ren et al., 2017). It is therefore possible that the individual polyphenols identified in CHE by LCMS may

have offered the protective effect against fluoride-induced toxicity by inhibiting apoptotic cell death. Oxidative stress and subsequent tissue damage are often associated with various degenerative diseases of intestinal tissue, especially in the colon (Carini et al., 2017); it is, therefore, possible that the use of coconut haustorium may be a useful functional food in alleviating these diseases.

Overall, the present study concludes that the methanol extract of coconut alleviates the cell death induced by fluoride. The mechanistic basis of action is mediated through the restoration of redox balance and inhibition of apoptosis. Hence, the study indicates the possible use of coconut haustorium as a functional food in preventing fluoride toxicity and its complications.

Credit author statement

JTJ- Data collection, Initial draft preparation; VR- Analysis, Initial draft preparation; RR- Data collection, Analysis, Review of Draft; AA-Design, Review of Final draft; AN- Study design, Methodology approval, Data analysis, Review of the final draft.

Availability of data and materials

The data may be made available upon valid request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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