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Natural combination of phenolic glycosides from fruits resists pro-oxidant insults to colon cells and enhances intrinsic antioxidant status in mice

Soorya Parathodi Illam, Ashif Hussain, Anu Elizabeth, Arunaksharan Narayanankutty, Achuthan C. Raghavamenon*

Amala Cancer Research Centre, Amala Nagar, Thrissur, 680 555 Kerala, India

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ABSTRACT

A combination of fresh fruits adequately supplying required nutrients is likely to have better health benefits by virtue of the synergistic/additive effect of its natural constituents. With this view and aiming to obtain phenolic glycosides in combination, fresh apple, grape, orange, pomegranate, and sapota fruit juices were combined and lyophilized. An aqueous extract of this fruit combination (AEFC) had polyphenols as a major constituent (47.36 μg GAE/mL) and LC–MS analysis documented the presence of cyanidin and pallidol 3-O-glucosides, phloridzin, delphinidin-3-O-rutinoside, kaempferol-3-O-pentoside, quercetin-3-O-rutinoside, trans-caffeic acid. Corroborating this, AEFC exhibited significant DPPH and superoxide radical scavenging activities (IC_{50}values 43.63 and 49.01 μg/mL) and protected colon epithelial cells (HCT-15) against H_2O_2 and AAPH induced cell death by 40 and 72.62% and buthionine sulfoximine (BSO) induced GSH depletion by 52.43%. In normal Swiss albino mice, administration of AEFC for over 30 days improved hepatic and renal GPx, SOD, and catalase activities and GSH levels. The study thus suggests the combinatorial effects of natural phenolic glycosides from fruits in resisting oxidative insults and associated disease pathology.

1. Introduction

A volume of experimental evidence is available to support the view that oxidative modifications of biomolecules are fundamental in most degenerative diseases including cancer [1]. These oxidative modifications to biomolecules can be the result of direct interactions of exogenous or endogenous oxygen or lipid-derived free radicals [2]. Reactive oxygen species (ROS) cause tissue damage by a variety of different mechanisms: lipid peroxidation; DNA damage (base hydroxylation and strand breaks); modifications to proteins including gingival hyaluronic acid and proteoglycans; oxidation of important enzymes; and induction of release of pro-inflammatory cytokine by depleting intracellular thiol compounds and activating nuclear factor κB [3,4]. Direct interaction of ROS on membrane lipids initiates peroxidative modifications, generating peroxides and aldehyde species of lipids which exert the similar macromolecular damage. The result is cellular physiological alterations and death [5].

The human body has a well-defined antioxidant and detoxification system to defend these free radical-mediated damages. This system includes enzymatic antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase and non-enzymatic molecules such as reduced glutathione (GSH) which detoxify lipid-derived as well as other peroxides, vitamin E that impede free radical chain reactions and vitamin C that neutralize various free radicals [6]. However, at instances of severe oxidative stress, the supplies of exogenous antioxidants are necessary to combat various ailments.

Antioxidants of plant origin have gained much momentum in combating oxidative stress. Polyphenols and flavonoid class of compounds, iso-thiocyanates, diallyl sulfides, resveratrol etc. are important bioactive compounds that are reported to alleviate oxidative insults [7–10]. However, due to low bioavailability as well as species-specific efficacy of many of these individual molecules, very little clinical success has been achieved. Combination of antioxidants, however, has shown to offer improved bioavailability [11]. Additionally, a combination of different antioxidant can offer intervention at different oxidative and inflammatory signalling, the effect of which may prevent chronic degenerative diseases with multifactorial etiology [12]. Apart from antioxidants, several bioactive phytochemicals such as fibres, alkaloids, and terpenoids are also available in these dietary items. Nevertheless, it has been shown that health advantageous of plant derived food supplements may not be due to an individual compound, but a combinatorial or synergistic actions of mixtures of phytonutrients it

* Corresponding author at: Department of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala 680 555, India.
E-mail address: raghav@amalaims.org (A.C. Raghavamenon).

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contains [13]. Fruits are rich in vitamins, minerals, proteins carbohydrates, fibres, and fats as well as bioactive phytochemicals, such as resveratrol, zeaxanthin, and carotenoids that help to prevent human ailments [14,15]. There is convincing evidence that a diet rich in vegetables and fruits lessen the risk of CVDs and diabetes and also protect against cancer [8].

Thus, a dietary nutraceutical supplement could be effective in impeding of many degenerative ailments. In this study, we prepared a combination of five different fruits orange, grape, sapodilla, apple and pomegranate that provide all vitamins, minerals, proteins, carbohydrate, lipids, and fibres meeting the daily requirement for the body and also a different class of bioactive compounds. An aqueous fraction of this fruit combination is expected to provide phenolic glycosides as they are mostly polar and increase the bioavailability of individual glycosides providing synergistic/additive effects. This combination has been tested for its effectiveness on the redox system in cells challenged with various oxidant insults and also in normal young rodents.

2. Materials and methods

2.1. Cell lines and chemicals

AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), 3-(4, 5 dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT), H_2O_2, and RPMI-1640, were from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin and other reagents for cell culture were purchased from Invitrogen (Grand Island, NY, USA). Plastic wares for cell culture used in the study were procured from Tarsons (Bangalore, India). All other reagents used in the study were analytical graded.

HCT-15 cell line (Human colonic epithelial cells) was obtained from National Centre for Cell Sciences, Pune, India. The cells were grown in RPMI-1640 media with 10% FBS at 37 °C and 5% CO_2.

2.2. Animals

Male Swiss albino mice of 6 to 7 weeks age (25–26 g) were procured from the small animal breeding station, Kerala Veterinary and Animal Sciences University, Thrissur, Kerala. The animals were housed in polypropylene cages under standard conditions and allowed to acclimatize for a period of two weeks. Animals were allowed to access the food and water *ad libitum*. Use of animals was according to the regulations described [16], with prior approval from the Institutional animal ethics committee, Amala Cancer Research Centre, Thrissur, (149/PO/Rc/S/99/CPCSEA).

2.3. Preparation of 'AEFC'-a multi-fruit combination

The multi-fruit combination- AEFC was prepared by mixing the five different fruits in equal quantities. The combination was composed of apple (*Malus domestica*), orange (*Citrus sinensis*), sapodilla (*Manilkara zapota*), grapes (*Vitis vinifera*) and pomegranate (*Punica granatum*). The fruits were collected from a reputed and reliable shop of local market ensuring that the fruits obtained were harvested within one week. This is because the age of the plant, time of harvest, colour and temperature determine the polyphenol content of fruits [17].

Individual fruits were weighed (100 g) accurately; juices were prepared and mixed together. The combined juice was then dried in vacuum concentrator to remove water content and the residue was collected. The residue was dissolved in fresh distilled water (100 mg/mL) where 10% solubility was observed. The water extract was stored under refrigeration at −20 °C to avoid deteriorations.

2.4. Qualitative determination of polyphenols by UPLC-Q-TOF

Ultra-performance liquid chromatography coupled to Quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) was used to identify the individual polyphenolic components. UPLC equipped with a binary pump system (Waters, Milford, MA, USA) using an AcQuity UPLC TM BEH C18 column (1.7 µm, 100 mm × 2.1 mm). The solvent system and analysis settings were according to previously described protocols [18]. Individual phenolic compounds were qualitatively assessed based on retension time and the mass spectra obtained using software library.

2.5. Quantitative estimation of polyphenols

Briefly,0.5 mL of the AEFC extract (1 mg/mL) was mixed with 2 mL of the 10% Folin- Ciocalteu reagent and incubated at dark for six (6) minutes and neutralized the reaction system with 4 mL of 7.5% sodium carbonate solution. The reaction mixture was further incubated at room temperature for 30 min with intermittent shaking. The absorbance was taken at 765 nm, and total phenolic content of AEFC was documented as mg gallic acid equivalent (GAE) [19].

2.6. Determination of antioxidant activity by DPPH and Superoxide radical scavenging assays

DPPH and superoxide free radical scavenging efficacies of AEFC was assesed as per the standard protocols described by Kumar et al. [20]. The DPPH radical assay system contained 187 µL DPPH reagent (0.3 mM in methanol) and different concentrations of extract (10–100 µg/mL) in a final volume of 1 mL. The solution was immediately mixed and incubated at 37 °C for 20 min in dark condition. The reduction in absorbance of the test and experimental tubes was measured using UV/Vis spectrophotometer at 517 nm. DPPH solution (0.3 mM) was taken as blank. The percentage (%) radical scavenging was calculated by the formula.

$$\%\ \text{Free radical scavenging activity} = \frac{\text{Ac} - \text{As}}{As} * 100$$

Where Ac = Absorbance of control at 517 nm; As = Absorbance of the sample.

Superoxide scavenging activity of AEFC was determined by Chun et al. [21] with slight modifications. The superoxide radical scavenging assay system contained Nitro blue tetrazolium (0.18 mM), riboflavin (0.12µM), NaCN/EDTA (0.3 mM NaCN in 0.1 M EDTA), phosphate buffer (0.06 M, pH 7.8) and the drug at different concentrations (10–100 µg/mL) in a volume of 3 mL. The tubes were illuminated for 15 min. The absorbance at 560 nm was measured before and following illumination for 15 min. The % inhibition and IC_{50} was calculated according to the formula;

$$\%\ \text{Inhibition} = \frac{\text{Ac} - \text{As}}{As} * 100$$

Where Ac-Absorbance of control, As- Absorbance of the test. Unit activity of SOD is referred to as the amount of enzyme required to produce 50% inhibition of photo reduction of NBT.

2.7. Determination of cytotoxicity

HCT-15 cells (1×10^5/mL) were seeded in 48 well plates. At subconfluency, cells were exposed with various concentrations of aqueous extract of AEFC (10–200 µg/mL), Following 24 h period, the cell viability was determined by using 3-(4, 5- dimethyl thiazol-2-yl)- 2, 5-diphenyl tetrazolium bromide (MTT).

Similarly, H_2O_2, AAPH or BSO (200–1000µM), were added to the HCT-15 cells in culture (1×10^5cells/mL) over 24 h period to test their toxicity. The untreated cells were kept as control [22].

2.8. MTT assay

Following incubation with the additives as mentioned above, the

spent medium was removed and HCT-15 cells were washed thrice with PBS and then added fresh media containing MTT (500μg/mL) and further incubated for 4 h. During this incubation process, no additive is present in the media that can interact with MTT. Therefore very little chance of interaction of MTT with polyphenols is expected. After the incubation, 500 μL of solubilization reagent (5 mL Triton-X 100, 45 mL isopropanol and 1 drop HCl (12 M) was added to each well and mixed thoroughly. Absorbance was taken at 570 nm.

2.9. Cytoprotective assay

HCT-15 cells (1×10^5/mL) were plated in 48-well culture plates and allowed to adhere and grow for subconfluency. The cells were then pretreated for 24 h with biologically safe concentrations of AEFC (here 25, 50, 75 and 100 μg/mL) following which either H_2O_2 (200 μM) or AAPH (400 μM) or BSO (3 mM) were exposed for another 24 h. Cells exposed only to pro-oxidant served as control. After the above respective exposure, and 20 μL MTT (5 mg/mL in phosphate-buffered saline) was added and the plates were incubated for 4 hs. Solubilizing solution (500 μL) was added to wells and incubated further for 15 min. The optical density was then measured at 570 nm using spectrophotometer (Systronics, Bangalore, India). Percentage viability of the treated cells was calculated by comparing absorbance with that of untreated normal cells.

2.10. Analysis of oxidative stress parameters

Cells were first seeded in a T-75 cm^2 flask and after reaching 60% confluency, they were pre-exposed with selected concentration (100μg/mL) of AEFC then following media change, cells were treated with either H_2O_2 or AAPH or BSO at their IC_{50} values (determined in the previous experiment) for 24 h. At the end of the incubation period, the spent medium was removed and cells were washed with PBS. The cells were collected in 200μL PBS following trypsinization and were lysed by repeated freeze and thaw cycles. The cells were then centrifuged at 8000 rpm for 5 min, the supernatant was then collected [18]. Levels of various intracellular antioxidants markers such as levels of GSH [23] and thiobarbituric acid reactive substance (TBARS) [24] and activities of antioxidant enzymes such as catalase [25], glutathione peroxidase and glutathione-s-transferase [26] were determined spectrophotometrically.

2.11. Acute toxicity analysis and antioxidant activity in vivo

Acute oral toxicity analysis in Swiss albino mice for 14 days was conducted according to OECD guidelines. Twelve animals of either sex were divided into 2 groups containing 6 animals each. They were administered with single dose AEFC (5000 mg/Kg bwt) by oral gavage. Body weight, food and water consumption and behaviour changes were monitored for 14 days for any adverse effects.

in vivo, antioxidant activity was analyzed using Swiss albino mice treated with doses of 250, 500 and 1000 mg/Kg for a period of 30 days. At the end of the experiment, following overnight fasting animals were sacrificed. Liver and kidney tissues were collected and the homogenate was prepared according to the method of Narayanankutty et al. [27]. Hepatic and renal redox markers such as GSH, catalase, SOD, GPx, and TBARs were measured according to the standard methods [28].

2.11.1. Estimation of GSH level

Trichloroacetic acid (125 μL of 25%) was added to 0.5 mL of the liver homogenate to precipitate the protein. The samples were kept under cooling in ice for 5 min and 0.6 mL of 5% TCA was added to the mixture and centrifuged at 1000 rpm for 10 min. The supernatant (0.3 mL) was used for estimation and made up to 1 mL with 0.2 M phosphate buffer (pH 8.0). About 2.0 mL of freshly prepared DTNB solution (in 0.2 M phosphate buffer pH 8.0) was added to these tubes and incubated for 10 min at RT. The yellow colour formed was read at 412 nm in a UV–vis spectrophotometer. A standard graph prepared using GSH (10- 100 μM) was used for quantitative measurements in nmoles/mg protein.

2.11.2. Catalase activity

Catalase activity was measured according to the following procedure. Reaction volume consists of 2.7 mL volume consisting of catalase phosphate buffer (pH 7.4) containing 30 mM H_2O_2 and read against phosphate buffer (0.05 M, pH 7.00) as blank to set the absorbance between 0.5-0.6. Sample (30 μL) was then added to initiate the reaction. The decrease in absorbance was measured every 15 s intervals following the addition of the enzyme sample. The specific activity of catalase was expressed as U/mg protein.

2.11.3. Determination of glutathione peroxidase

The reaction mixture contained 0.2 mM GSH, 0.1 M phosphate buffer, sodium azide (25 mM), 100 μL cell lysate and H_2O_2 (1.25 mM) in a total volume of 2.5 mL. The reaction system was incubated at 37 °C for 6 min. The reaction was stopped by addition of 2 mL of 1.67% H_2PO_3, centrifuged for 15 min at 800 rpm. The supernatant (2 mL) was taken and mixed with 2 mL of 0.4 M Na_2HPO_4 and 1 mL of 1 mM DTNB. Incubation was carried out at 37 °C for 10 min. Then OD was measured at 412 nm.

2.11.4. Determination of glutathione- S- transferase

The determination is based on the rate of increase in the conjugate formation between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB), with absorbance maxima at 340 nm. The reaction mixture consisted of phosphate buffered saline at pH 6.5 (2.7 mL), 100 μL cell lysate, 100 mM CDNB in ethanol and 100 mM GSH in a total volume of 3 mL. The activity of GST was calculated from the change in absorbance at 340 nm for 3 min. The GST activity was expressed as nmoles of CDNB conjugates formed/min/mg protein using extinction coefficient of $9.3 \times 10^3\ M^{-1}\ cm^{-1}$.

2.11.5. Measurement of the extent of lipid peroxidation

The lipid peroxidation level was determined using the thiobarbituric acid reactive substance (TBARS) assay protocol. Cell lysate (200 μL) was mixed with 8% SDS. To this 1.5 mL of 20% acetic acid (pH3.5) and 1.5 mL TBA (0.8%) were added. Mixed well and incubated in a boiling water bath for 45 min. The samples were diluted with 800μL distilled water and were then centrifuged at 1000 g for 10 min and absorbance of the supernatant was measured at 532 nm. The concentration of malondialdehyde (MDA) was calculated from the standard graph.

2.11.6. Measurement of superoxide dismutase activity

The Superoxide dismutase activity was determined by the following method. The reaction system contained 100μL of tissue supernatant, Nitro blue tetrazolium (0.18 mM), riboflavin (0.12μM), NaCN/EDTA (0.3 mM NaCN in 0.1 M EDTA) and make the reaction system to 3 mL with phosphate buffer (0.06 M, pH 7.8). The tubes were illuminated for 15 min. The absorbance was measured at 560 nm before and after illumination. The control was simultaneously run without tissue homogenate. One unit of SOD activity would be defined as the amount of enzyme required to cause 50% inhibition of the NBT photo reduction rate.

Estimation of total protein was done by Lowry's method [29]. The formation of protein copper complex and the reaction of the phosphomolybdate- phosphotungstate reagent (Folin-ciocalteau phenol reagent) by the tyrosine and tryptophan residues of precipitation to form a coloured complex.

2.12. Statistical analysis

All the results were expressed as mean ± SD for each concentration

in triplicate. Statistical analysis was carried out by One-way analysis of variance followed by Dunnett's multiple comparison tests by Graph Pad Prism7 software. Values with a minimum variation of *p < 0.05; **p < 0.01; ***p < 0.001 were considered as statistically significant.

3. Results

3.1. Organoleptic characteristics and preliminary phytochemical studies of AEFC

The organoleptic characters of aqueous extract of fruit combination such as colour, odour, and the taste were carried out and the results are given in Supplementary material Table 1. Aqueous extract of fruit combination was subjected to various phytochemical tests, to determine the presence of active constituents which included qualitative tests for carbohydrates, proteins, alkaloids, amino acids, flavonoids, cardiac glycosides, saponins, phenolic compounds and tannins [30]. The tests revealed that the presence of alkaloids, flavonoids, phenolic compounds, tannins (Supplementary material Table 2).

3.2. Total phenolic content and composition of AEFC

The average yield of dried residue from 100 mL of the combination of fruit juice was found to be approximately 30%. The total phenolic content of the test compound was calculated from the standard curve plotted for gallic acid and was found to be of 47.36μg GAE/mL. The individual phenolic compounds are summarized in Table 1. They include cyanidin pentoside, pallidol 3-O- glucoside, phloridzin, delphinidin-3-O-rutinoside etc. (Fig. 1 in Supplementary material).

3.3. In vitro antioxidant activity

A dose-dependent DPPH radical reducing efficacy was observed within the range of concentrations of AEFC (0-100 μg/mL). The IC_{50} value of AEFC documented was found to be 43.63μg/mL. Vitamin C which was used as the positive control exhibited an IC_{50} value of 4.70 μg/mL. Superoxide generated during the photoreduction of riboflavin was also effectively inhibited by AEFC. The concentration of the AEFC needed to scavenge 50% superoxide anion (IC_{50}) was found to be 49.01μg/mL (Fig. 1a). However, vitamin C had an IC_{50} value of 65.31 μg/mL.

Table 1
Phenolic composition of the multi fruit combination AEFC analyzed by LC-Q-TOF.

Sl No.	RT	m/z +	m/z -	Compound
1	2.2	305	–	Epigallocatechin
2	2.4	133	–	unidentified
3	2.6	371	–	unidentified
4	3.2	449	–	Cyanidin 3- glucoside
5	3.5	151	–	unidentified
6	4.2	353	–	neo-chlorogenic acid
7	4.7	403	–	unidentified
8	5.3	280	–	unidentified
9	7.8	413	–	unidentified
10	0.6	–	191	Citric acid
11	2.3	–	417	Kaempferol-3-O-pentoside
12	2.6	–	615	Pallidol 3-O- glucoside
13	2.9	–	567	Phloretin xyloglucoside
14	3.1	–	579	Naringin
15	3.2	–	609	quercetin-3-O-rutinoside
16	3.3	–	435	Phloridzin
17	7.7	–	178	Trans caffeic acid

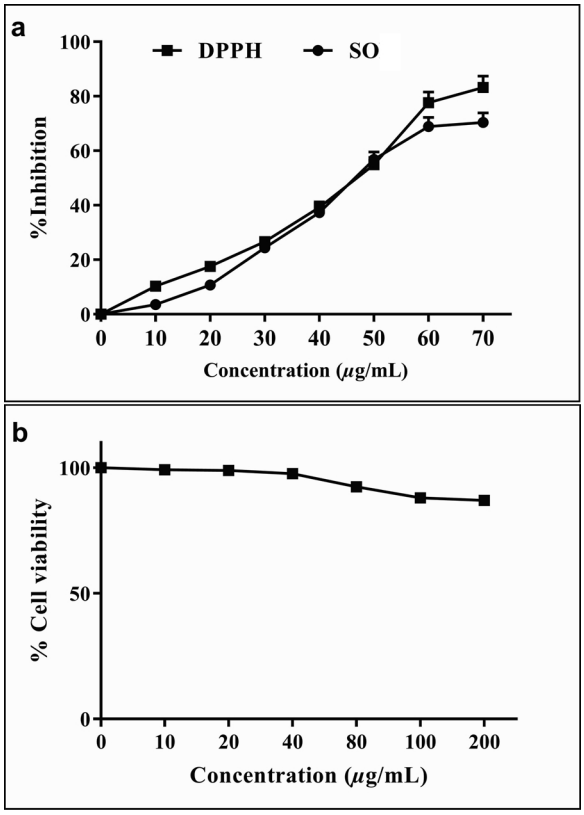

Fig. 1. (a) DPPH radical scavenging and superoxide radical generation inhibition activity of aqueous extract of fruit combination (AEFC); (b) Cytotoxicity analyses of AEFC in human colon epithelial cells (HCT-15) by MTT assay over a period of 48 h.

3.4. Evaluation of biologically safe concentrations of AEFC, H_2O_2, AAPH, and BSO

Cellular toxicity of AEFC towards HCT-15 cells was very less. Among the concentrations varying from 10-200 μg/mL, AEFC did not exert any toxicity (Fig. 1b). On the other hand, direct toxicity of H_2O_2, AAPH, and BSO was dose-dependent. The IC_{50} values of H_2O_2, AAPH, and BSO in HCT-15 cells were found to be 200 μM, 400 μM, and 2.99 mM respectively (Fig. 2a).

3.5. Cytoprotective efficacy of AEFC against H_2O_2, AAPH, and BSO induced oxidative damage

Cytoprotective efficacy of AEFC against H_2O_2, AAPH and BSO insults was investigated by MTT assay. Pre-treatment of AEFC at 40, 100 and 200 μg/mL reversed the cell death induced by H_2O_2, AAPH, and BSO at their respective IC_{50} concentrations. However, no dose-dependent protection was visible in these cells. In H_2O_2 treated cells 100 μg/mL AEFC increased the cell viability to 40.1%. The addition of AEFC at 40 and 200 μg /mL, however, did not increase cell viability to an appreciable level. The percentage of cell viability observed in cells exposed to other concentrations of AEFC (40 and 200 μg/mL) was 27.5 and 20.7%, respectively (Fig. 2b). The loss in cell viability due to AAPH exposure was improved to 72.62%by the pre-treatment of 40 μg/mL of AEFC. Similarly higher doses of AEFC (100 and 200 μg /mL) also enhanced the cell viability up to 70.98 and 64.59% respectively (Fig. 2b). In the case of BSO, all the tested concentration of AEFC was equally effective in protecting the cells from loss of viability. There were 68.42, 63.06 and 62.79% improvement in the viability at 40, 100 and 200 μg/mL BSO added cells, respectively.

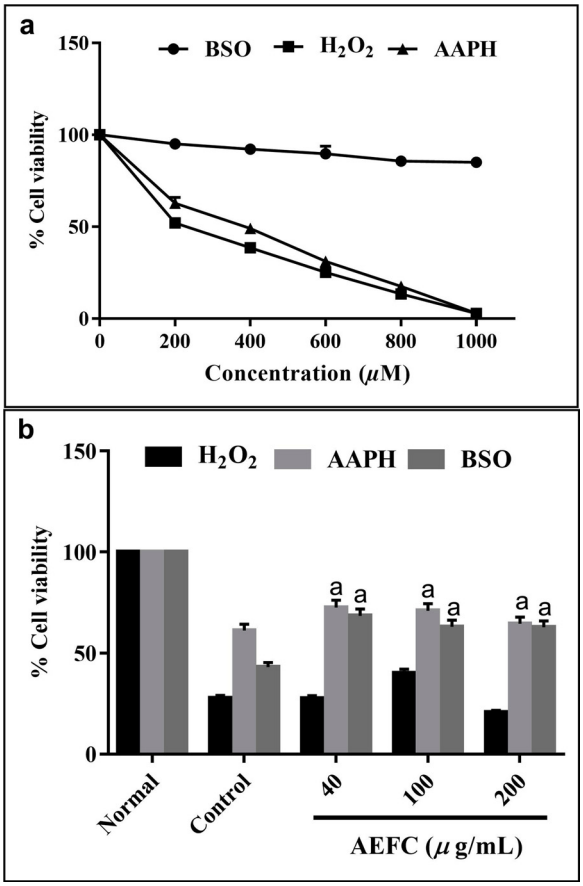

Fig. 2. (a) Cytotoxicity evaluation of BSO, H_2O_2 and AAPH in HCT-15 cells by MTT assay over a period of 48 h; (b) Protective effects of AEFC against H_2O_2, AAPH and BSO induced cell death in human colon epithelial cells. Values are represented as mean ± SD of six individual experiments. Values with a minimum variation of[a] $p < 0.0001$, [b]$p < 0.001$, [c] $p < 0.01$, [d]$p < 0.05$were considered as statistically significant.

3.6. Effect of AEFC on cellular antioxidant levels in H_2O_2 induced oxidative damage

Acute exposure to H_2O_2 is known to induce a cellular stress response in cells that involves changes in the levels of endogenous antioxidants. The intracellular GSH level in untreated cells was recorded as 57.12 ± 6.03 nmoles/mg protein. Exposure to H_2O_2 reduced the level to 30.22 ± 4.19 nmoles/mg protein. Addition of AEFC improved the reduced glutathione level to 33.84 ± 1.17 nmoles/mg protein. At the same time, H_2O_2 exposure reduced the catalase activity from 4.18 ± 0.97 (healthy cells) to 2.09 ± 0.36 U/mg protein. Under the same experimental conditions, the addition of AEFC marginally enhanced the catalase activity to 2.77 ± 0.44 U/mg protein. In corroboration with these observations, the changes in the TBARs levels were also noted. The recorded level of TBARS in untreated normal cells was 6.46 ± 0.29 nmoles/mg protein whereas H_2O_2 exposure elevated the level to 16.41 ± 0.63nmoles/mg protein. In addition to aforesaid results, treatment with AEFC also reduced the generation of TBARS to 11.67 ± 3.44 nmoles/mg protein (Fig. 3a).

3.7. Effect of AEFC on cellular antioxidant levels in AAPH induced oxidative damage

AAPH exposure also has led to the changes in the intracellular antioxidant system. The GSH level was significantly brought down by the AAPH treatment to 33.16 ± 4.35 compared to normal cells which were documented as 55.07 ± 8.2 nmoles/mg protein. At the same time, the addition of AEFC raised the GSH level to 45.62 ± 9.81 nmoles/mg protein. AAPH exposure also resulted in the reduction of catalase activity to 2.75 ± 0.33 compared to untreated cells (5.3 ± 1.48 U/mg protein). However, the addition of AEFC in cells enhanced the catalase activity to 3.87 ± 0.66 U/mg protein. In line with these, there observed an increase in TBARs in AAPH alone exposed control cells (10.22 ± 2.89 nmoles/mg protein) compared to untreated healthy cells (6.27 ± 1.16 nmoles/mg protein). On the other hand, the presence of AEFC along with AAPH addition produced no hike in TBARs level (5.5 ± 3.15 nmoles/mg protein) when compared to control cells (Fig. 3b).

3.8. Effect of AEFC on cellular antioxidant levels in BSO induced oxidative stress

Similar to the other two models, the effect of fruit combination on BSO induced oxidative stress in colon epithelial cells was also analyzed (Fig. 3c). Cellular glutathione was depleted as a result of BSO exposure to the cells and it was noted as 27.84 ± 8.4 nmoles/mg protein. In the untreated cells, GSH levels were found to be 62.22 ± 1.91 and presence of AEFC with AAPH prevented the GSH depletion (58.52 ± 2.24 nmoles/mg protein). The activity of glutathione S- transferase was found increased in BSO alone exposed group (5.4 ± 0.39 U/mg protein) when compared to normal healthy cells (4.7 ± 1.18 U/mg protein). In cells where AEFC exposed along with BSO hike in activity was maintained (5.69 ± 1.18 U/mg protein). Glutathione peroxidase (GPx) activity, another indication of redox status of cells, was increased in BSO alone exposed group (22.06 ± 1.26 U/mg protein) while in AEFC exposed cells that much increase was not observed (14.64 ± 1.08 U/mg protein) when compared to the activity of untreated cells (9.83 ± 2.07 U/mg protein). The TBARs level was found to be 4.5 ± 1.3 nmoles/mg protein in normal cells while increased in BSO alone exposed cells (11.8 ± 4.3 nmoles/mg protein**).** However, TBARS was found to be decreased in cells exposed to AEFC (7.5 ± 2.9 nmoles/mg protein) when compared to control.

3.9. Acute toxicity analysis and Effect of prolonged AEFC consumption on hepatic and renal antioxidant status

Acute toxicity experiment was conducted on male healthy mice according to OECD guidelines 423. All the animals were found to be normal during the experimental period. This observation revealed that the aqueous extract of fruit combination did not show any symptoms of toxicity and mortality up 5000 mg/kg dose. Results of acute toxicity studies were summarized in Tables 3 and 4 in the supplementary material.

There was a significant increase in the hepatic antioxidant parameters like catalase ($p < 0.001$), glutathione peroxidase ($p < 0.001$) and reduced glutathione (GSH) ($p < 0.001$) and there were no significant changes observed in lipid peroxidation and superoxide dismutase compared to normal group (Table 2). Similarly, a significant increase in redox parameters was observed in the kidney of AEFC treated animals such as catalase ($p < 0.001$), SOD ($p < 0.01$) and reduced glutathione (GSH) ($p < 0.001$) and there were no significant changes observed in GPx compared to the normal group. The level of lipid peroxidation was decreased in hepatic and renal tissues (Table 2).

4. Discussion

AEFC is expected to possess water-soluble phenolic glycosides and other phytochemicals of the constituent fruit pulps in addition to vitamins, and minerals. The phytochemical analysis reveals the presence of high amounts of polyphenols, which are a well-known class of antioxidant molecules. The compounds identified in LC–MS analysis such as cyanidin glucoside, pallidol 3-O- glucoside, phloridzin, delphinidin-3-O-rutinoside, kaempferol-3-O-pentoside, epicatechin, quercetin-3-O-

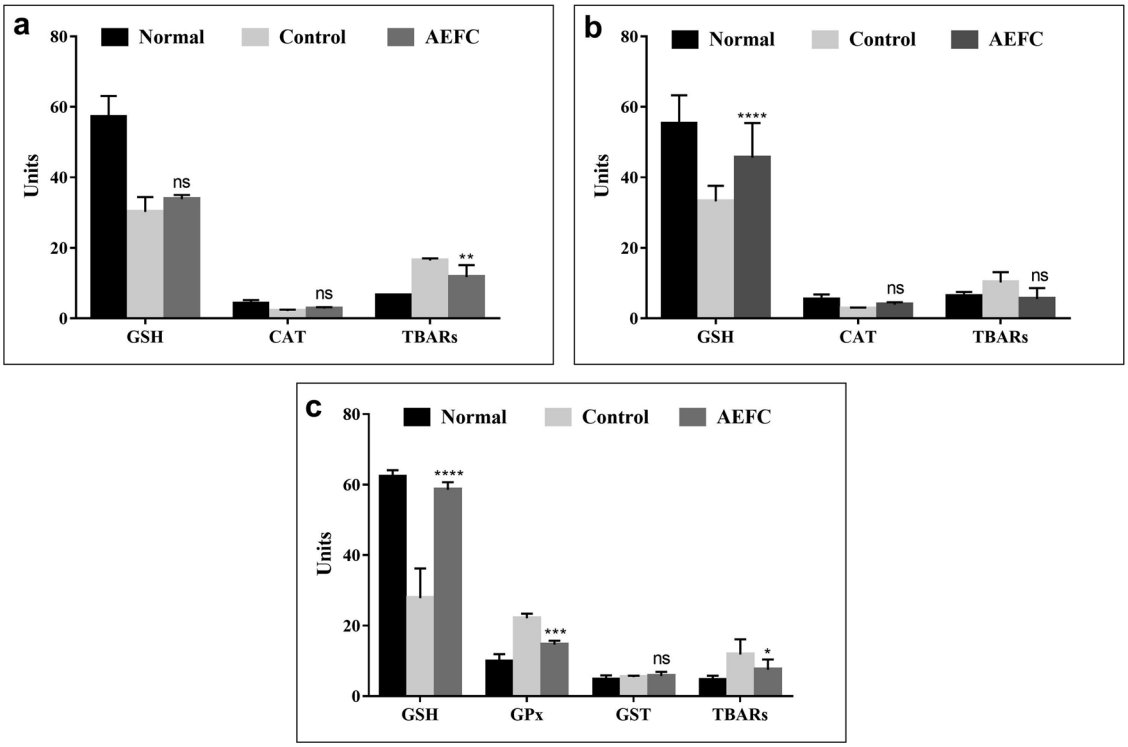

Fig. 3. Effect of AEFC on hydrogen peroxide (a), AAPH (b) and BSO (c) induced intracellular redox imbalance. Values are represented as mean ± SD of three individual experiments. GSH and TBARS are expressed as nmoles/ mg protein; Catalase, GPx and GST are expressed in terms of U/ mg protein. Values with a minimum variation of *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001 were considered as statistically significant.

rutinoside, trans-caffeic acid, naringin etc. are previously reported in all component fruits of AEFC [31–35]. Pallidol 3-O- glucoside, a derivative of resveratrol dimer pallidol, is already reported to have antioxidant and free radical scavenging activities [36]. Similarly, phloridzin which is a predominant constituent compound in apple is also documented to possess antioxidant potential [37]. In addition, apple contains another antioxidant molecule phloretin xyloglucoside [38] which is detected in the combination AEFC. Naringin which is a bioactive flavonoid compound in orange has been reported to scavenge free radicals and reduces DNA damage *in vitro* [39]. It offers protection to cardiomyocytes by downregulating ROS mediated MAPK signalling [40]. Among the compounds present in fruit combination, quercetin is a well- known bioactive molecule which is reported to possess anticancer property by inducing apoptosis *in vitro* and *in vivo* [41]. In addition, the anticancer activities of mixtures of polyphenols have been investigated. Sour orange albedo extract and its individual flavanones loaded in silica nanoparticles when administered in rats have shown to ameliorate acrylamide-induced hepatotoxicity [42]. The polyphenolic extract from the grape stem has shown to inhibit the division of colon, breast, renal and thyroid cancer cells. The cytotoxic effects are thought to be induced by the action of the polyphenols in combination [43]. In view of these reports, AEFC, which is found to be a natural combination of important polyphenols is expected to act synergistically or additively exerting a wide range of signalling that could be beneficial in many degenerative diseases.

Hydrogen peroxide, AAPH and BSO are widely used oxidative stress inducers in experimental studies. Of which, hydrogen peroxide exerts toxic signalling, AAPH spontaneously generates peroxyl radicals in aqueous medium and BSO depletes GSH in the cellular milieu [18,44]. In the present study, H_2O_2 and BSO induced cell death has been prevented by the higher concentration of AEFC added to culture; when AAPH is used, AEFC at it's lower, as well as higher concentrations, have significantly improved cell viability, but not in a dose-dependent manner. This suggests the disparity in the mode of action of AEFC on various pro-oxidant insults. Azo compounds such as AAPH undergoes spontaneous thermal decomposition to form carbon-centred radicals (alkyl and peroxyl radicals) which can initiate the production of lipid peroxides in the presence of oxygen and polyunsaturated fatty acid [45,46]. However, the presence of AEFC in the medium might have scavenged out the free radicals generated by AAPH before reaching the

Table 2
Hepatic and renal redox status of normal animals and those treated with AEFC for a period of 30 days.

	Liver		Kidney	
	Normal	AEFC	Normal	AEFC
CAT (U/mg protein)	6.98 ± 0.65	8.90 ± 0.66[a]	2.65 ± 0.27	3.85 ± 0.14 [a]
GPX (U/mg protein)	8.47 ± 0.94	11.27 ± 0.80 [a]	5.86 ± 0.05	6.01 ± 0.24
GSH (nmol/mg protein)	2.61 ± 0.04	3.03 ± 0.021 [a]	2.08 ± 0.11	2.61 ± 0.05 [a]
SOD (U/mg protein)	0.25 ± 0.02	0.32 ± 0.08[ns]	0.23 ± 0.01	0.30 ± 0.02 [b]
TBARs (nmol/mg protein)	0.58 ± 0.01	0.48 ± 0.01[ns]	0.49 ± 0.01	0.38 ± 0.01 [a]

ns- indicates non-significant.
[a] indicates P < 0.001.
[b] indicates P < 0.01.

cells. On the other hand, H_2O_2 which is an important signalling molecule in cells that is capable of diffusing through cellular membranes and BSO which inhibits GSH synthesis are non-radical species. AEFC molecules might have failed to quench these species in the extracellular medium. However, the phytochemicals present in AEFC might have induced antioxidant signalling as these molecules are largely cell impermeable. This could be the reason for the requirement for higher concentrations of AEFC in these experiments to resist H_2O_2 and BSO induced damages. There is also possible that certain phytochemicals that have entered the cells due to molecular synergism/additive action might have acted upon free radicals generated from H_2O_2 as well as radicals generated due to lowered GSH level.

In addition, it is observed that there is an increase in the antioxidant status in AEFC treated cells and a concomitant reduction in lipid peroxidation products. It may be possible that together with the reduced use of GSH for detoxification, increased biosynthesis of GSH might have contributed to this phenomenon. It has been previously reported that apple, grapes, and orange individually induces GSH biosynthesis [47,48].

L-buthionine sulfoximine (BSO) is a known inhibitor of gamma-glutamylcysteine synthetase (γGCS) and modulates the cellular redox status [49]. In this study BSO alone exposed cells exhibit decreased GSH level while enhanced GSH associated GPX and GST activities. TBARS level is also found increased in these cells. A similar increase in GPX activities in BSO exposed cells has also been reported in another study [50]. The enhanced GSH related enzyme activity could be a protective measure as GPX that detoxifies lipid peroxides and GST that utilizes GSH for removal of lipid carbonyls [51,52]. GST has been reported to protect cells by detoxifying some of the secondary ROS and lipid-derived carbonyls formed as a result of intracellular oxidative stress [53]. In addition to the reduced GSH synthesis, possibly increased GST activity might have partially contributed to the reduced glutathione status. On the other hand, the addition of AEFC reduced the TBARS, however, a concomitant decrease in BSO induced hike in GPX is observed. At the same time, AEFC exposure does not influence GST activity but the level of GSH has been brought back to normal. As a result, cellular viability also improved. This strongly suggests that AEFC protects cells from BSO induced GSH depletion and associated oxidative stress mediated cell death.

The possible reason could be the phenolic phytocompounds in AEFC that might have scavenged the ROS generated in the cells as part of BSO induced oxidative insults, thereby reducing the expense of other endogenous antioxidants. Similar observations are also reported [54]. This possibility has been supported by the observed reduction in lipid peroxidation products in AEFC exposed cells. Together with reduced lipid peroxidation products, diminished GPx activity might also have led to the maintenance of GSH pool in the cells. In addition, a marginal decrease in the GST activity is observed in BSO exposed cells compared to AEFC treated cells. GST catalyzes the conjugation of GSH with electrophilic molecules for their removal from the cell through membrane-based GSH conjugate pumps, reduction in the GST activity may also support the increase in cellular GSH levels. Overall, the results of this study indicate that in addition to GSH dependent antioxidant defence, phenolic acid-dependent free radical scavenging phenomena may also be involved in the observed antioxidant properties of AEFC.

It has been reported that polyphenols are highly unstable and can undergo oxidation to generate H_2O_2, quinones and semiquinones etc. [55]. These molecules can act as pro-oxidants generating antioxidant stress. Under these circumstances, the cellular antioxidant system can be up-regulated. In our study, this possibility has also been considered as AEFC is mostly a combination of polyphenols. However, AEFC mediates no toxic insults towards HCT-15 cells in RPMI- 1640 media and oral administration of AEFC in normal Swiss albino mice improves the antioxidant defence system and further clinical studies reported the beneficial effects of poly phenols in reducing oxidative stress [56,57]. Together, it is difficult to interpret the possibility that the polyphenols induce pro-oxidant insult and results in the up-regulation of the antioxidant system as we have not evaluated the oxidation products of AEFC in cells/ animal system. Even though, such possibility needs to be studied further.

In animals, AEFC is found to be nontoxic. The antioxidant potential of AEFC is further verified by the increase in catalase, GSH, and superoxide dismutase activities in animals administered with AEFC. Superoxide dismutase acts as the first line of defence against oxygen-derived free radicals. Improved activities of SOD are observed in AEFC given group indicating scavenging of free radicals. The level of GSH is increased in animals fed with AEFC compared to normal thus avoiding the conditions like oxidative stress. The phenolic compounds of pomegranate are reported to prevent oxidative stress by the activation of Nrf- 2 (nuclear factor erythroid 2-related factor 2) and results in increased SOD activity. Similarly, polyphenols of apple activate Nrf-2 in mice and further improves GSH and antioxidant enzyme activities [58,59]. Moreover, phytomolecules in Citrus fruits and grapes are reported as highly antioxidant and diminishes oxidative stress conditions [60,61]. Substantiating these results, catalase and GPx activities are found to be improved in mice after AEFC treatment. Further investigation in the level of lipid peroxidation verified the above said results. The bioactive compounds of fruits present in AEFC may synergistically act which might contribute to the improvement in antioxidant status in animals. Together, it seems that the AEFC possess the advantage of natural polyphenols in combination. Supporting these results, studies by Eren-Guzelgun et al. [62] have shown that compared to the individual effects of genistein, daidzein, and quercetin, their combination has been found to have better antioxidant effects. Similar studies have been conducted on the protective efficacies of strawberry [63], *Platostoma palustre* [64] and raspberry extract [65], where the concentrations used has been much higher (0.5–3.6 mg/mL). Studies by Alvarez-Suarez et al. [66] observed cytoprotective effects at a concentration of 80 μg/mL acerola (*Malpighia emarginata*) in human fibroblasts. Hence, the present study suggests that compared to the individual administration of fruits or their extracts, the combination of it might be beneficial in preventing most degenerative diseases by providing a combination of natural pharmacologically active phytochemicals as revealed in this study and also other nutrients including fibres in adequate quantity.

5. Conclusion

The results obtained are promising as they document the ability of natural phenolic glycosides offering protection against oxidative insults to cells and improve enzymatic and non-enzymatic antioxidant system upon oral administration in normal mice. As fruits are a rich source of antioxidant molecules, a combination of fruit intake can provide natural bioactive phytocompounds, vitamins, minerals as well as fibres and other nutrients which may synergize with each other in order to elicit promising health benefits. Hence the combination of these fruits could be effective in improving the natural antioxidant defence in the body and resisting various oxidative insults.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2019.07.005.

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