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OXIDATIVE STRESS INDUCED G_1/S PHASE CELL CYCLE ARREST AND CELLULAR APOPTOSIS IN CULTURED BUCCAL KB EPITHELIAL CELLS ON EXPOSURE OF AQUEOUS EXTRACT OF ARECA NUT

Rashmi Nagesh[1], Kiran Kumar K. M[1], Naveen Kumar M.[1], S. Chidananda Sharma[1†] and M. Sreepriya[1*]
[1]Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi, Bengaluru-560 056, Karnataka, India

***Corresponding Author Email: mpriya7@yahoo.com**

ABSTRACT

Areca nut chewing is often associated with incidence of severe oral cancer, leukoplakia and oral submucous fibrosis (OSF). Areca nut and its components are the major etiological factors that are responsible for the causation of oral cancers. Although there exist numerous evidences on harmful effects of areca nut chewing, the present study shows the effect of aqueous extract of areca nut on human buccal KB epithelial cells on growth and viability followed by biochemical analysis of oxidant and anti-oxidant levels that are known to alter the cell cycle progression. Buccal KB cells on treatment with aqueous areca nut extract showed reduced viability by 48 h with IC_{50} value of 0.35 %. Areca nut extract induced the production of reactive oxygen species (ROS) which in turn react with lipid moiety on plasma membrane and cause loss of membrane integrity with leakage of lactate dehydrogenase enzyme into culture supernatant. The excessive production of oxidants with decreased anti-oxidant levels of superoxide dismutase (SOD) and glutathione S transferase (GST) which leads to altered redox homeostasis. Further, the results of cell cycle analysis by flow cytometry showed G_1/S phase cell cycle arrest on extract exposure. Additionally, annexin V FITC/PI staining was carried out to assess cellular apoptosis on exposure of areca nut extract which showed induction of apoptosis by 48 h. Hence, the present study helps in better understanding the role of oxidative stress in altering cell cycle progression and apoptosis pathway in aqueous areca nut extract treated KB cells.

KEY WORDS

Areca nut extract, ROS, OSF, cell cycle arrest, apoptosis, flow cytometry.

INTRODUCTION

Betel quid chewing is the fourth most common oral habit in the world. There are about 600 million betel quid chewers in the world [1]. Areca nut chewing has shown a strong association with the occurrence of oral cancer and oral submucous fibrosis (OSF) [2, 3]. Recently, reports have shown that areca nut chewing has caused keratinocyte inflammation in turn to be decisive for tissue fibrosis and chemical carcinogenesis [4]. Areca nut and its component arecoline are known to be the major etiological factors in the pathogenesis of oral cancer and OSF [3, 5] . Studies have reported that areca nut extract and arecoline are highly cytotoxic and genotoxic on bacterial test system as well as on cultured human oral epithelial cells [6, 7]. Further, International Agency for Research on Cancer (IARC), the World Health Organization (WHO) reached to a conclusion that there is adequate evidence for chewing betel quid with or without tobacco is carcinogenic to humans [8]. Areca nut extract are known to induce DNA breaks, unscheduled DNA synthesis and differentiation in oral keratinocytes [7, 9]. The production of reactive oxygen species are due to auto-oxidization of areca components at alkaline condition [10]. Several cell types

on areca nut extract exposure have shown the induced production of ROS [11, 12]. The produced ROS are detoxified by scavengers known as anti-oxidants like super oxidase dismutase, glutathione S transferase, catalase and many lower molecular weight compounds like lipoic acid. Arecoline treated human oral keratinocytes and fibroblasts show exhaustion in the activity of cellular anti-oxidant glutathione and reduced glutathione S-transferase [11, 13]. Recently, production of ROS depletion [14], cellular glutathione [15] and regulation of mitochondrial functions [16] have been shown to influence on cell cycle progression, apoptosis on chemical toxicity.

In response to genotoxic stress cells naturally display cell cycle arrest and allowing it for DNA repair [14, 17, 18]. Although, growth of cells is strictly regulated by numerous cell cycle regulators which aids the transition of cell cycle from one phase to another. Impairment in cell cycle may be due to exposure of lethal compound which in turn leads to growth retardation, cell cycle arrest, cytotoxicity and apoptosis [17, 19-22]. However, it is of interest to know the effect of areca nut extract on induction of oxidative stress and their influence on cell cycle progression and cell death. Earlier reports have shown the deleterious effects of chewing areca nut in causation of numerous oral disorders but few studies have addressed it with respect to the biochemical players, the oxidants and the anti-oxidants that play a vital role in deciding the cellular fate. Hence in the present study human buccal KB epithelial cells were used as an *in vitro* model system to understand the toxic effect of aqueous areca nut extract on viability, production of oxidants and the levels of anti-oxidants and in turn their effects on cell cycle progression and apoptotis.

MATERIALS AND METHODS

Human buccal adenocarcinoma KB cells were purchased from NCCS (Pune, India); TRIzol, Lithium lactate, iodonitrotetrazolium chloride (INT), phenazine methosulphate (PMS), malondialdehyde (MDA), glutathione reductase, reduced glutathione were purchased from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), fluorescein diacetate, sodium dodecyl sulphate, acetic acid, sodium carbonate, epinephrine, NADH, sodium azide, NADPH, sodium acetate were purchased from Himedia (Mumbai, India). Annexin V-FITC apoptosis detection kit I (Thermo Fisher, Oregon, USA). *Areca catechu*, the processed form of areca nut was purchased, the variety often used in betel quid preparation from Sirsi market, Uttara Kannada district, Karnataka.

Culturing of human buccal KB cells

KB cells were grown in 25 cm^2 culture flask using RPMI 1640 medium with 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37°C by supplying 5% CO_2 in an incubator. The 80-90 % confluent flask containing cells were trypsinised and sub cultured in 96 or 6 well plate for treatments.

Preparation of aqueous extract of areca nut

Areca nut was finely powered using pestle and mortar, 1 g of powdered areca nut was suspended in 10 ml of sterile water and placed in orbitary shaker at room temperature for 24 h and the aqueous extract was filtered using Whatmann no. 1 filter paper and filtered extract was used for treatments or stored at -20 °C until further use [23].

Cell viability Assay

Cell viability was carried out using MTT assay as per the protocol described earlier [24]. KBcells (3×10^3cells/well) in RPMI-1640 medium with a final volume of 200 µl were seeded into 96-well plate and incubated overnight at 37°C with the supply of 5% CO_2. The cells were treated with different concentrations of aqueous areca nut extract (0.1-1.0 %) and further, incubated for 48 h. The cells were washed with PBS, treated with 20 µl of MTT (0.5 mg/ml) and incubated for 4 h. The formazan product formed in cells were dissolved in DMSO (100 µl) and absorbance was measured at 540 nm using multimode plate reader (Perkin Elmer).

Reactive Oxygen Species (ROS) Assay

ROS generated in cells by the action of toxic substances were measured as per the protocol described earlier [25]. Overnight cultured KB cells were incubated with 10 µM DCFDA for 3 h in a CO_2 incubator. Further, cells were washed with PBS and treated with different concentrations of aqueous extract of areca nut in media for 15 min. Fluorescence intensity of DCFDA was recorded using a multimode plate reader at excitation wavelength of 485 nm and emission of 527 nm.

Lactate Dehydrogenase (LDH) Assay

The cytotoxic effect of aqueous areca nut extract on KB cells was measured by LDH assay as described earlier [26]**.** LDH in media was quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product measured at 490 nm. Cells were treated with different concentrations of aqueous extract of areca nut and incubated for 48 h. Cell-free supernatant (50 µl) from each well was transferred in to a 96-well plate in triplicate and 150 µl of LDH reaction mixture was added. The formazan product formed is directly proportional to the amount of LDH leaked into the media, which reflects the cytotoxic effect.

Superoxide dismutase (SOD) Assay

Total SOD activity was determined according to the method described earlier [27]. KB cells were treated with different concentrations of aqueous extract of areca nut for 48 h. Cell lysates were prepared using lysis buffer. To 100 µl of cell lysate 880 µl carbonate buffer (0.05 M Na_2CO_3, 0.l mM EDTA of pH 10.2) and epinephrine (100 µl) in 0.1 N HCl was added and the absorbance was measured spectrophotometrically at 340 nm. The amount of enzyme that results in 50 % inhibition of epinephrine auto oxidation is defined as one unit of enzyme activity. Results were plotted as µM of SOD oxidized/µg of protein in areca nut extract treated samples against control.

Glutathione S-transferase (GST) Assay

GST assay was carried out as per the protocol described earlier [28]. KB cells (5×10^5 cells/well) in a 6-well plate and were treated with different concentrations of aqueous extract of areca nut for 48 h. Cell lysate was prepared using 200 µl lysis buffer/well. 100 µl cell lysate was added to 900 µl enzyme cocktail containing PBS of p^H 6.5, 100 mM CDNB in ethanol and 100 mM reduced glutathione in ethanol. Reaction mixture was incubated at room temperature for 5 min and absorbance was measured spectrophotometrically at 340 nm.

Aqueous areca nut extract induces G_1/S phase cell cycle arrest in KB cells

To measure the number of cells residing at different cell cycle phases by flow cytometry the KB cells in a 6 well plate were treated with different concentrations of areca nut extract (0.25 and 0.35 %) for 48 h. Cells were washed with PBS and trypsinized. Further, the cells were fixed in 70 % ice-cold ethanol containing 2 mg/ml RNase for 30 min followed by PBS wash and finally stained with propidium iodide (50 µg/ml) for 10 min at room temperature. The cell samples were analyzed by FACS Calibur Flow Cytometer (Becton Dickinson). The percentage of cells residing in Sub G_0, G_1/ S and G_2/M-phase were analyzed by BD CSampler software.

Apoptosis Detection by Annexin V-FITC and Propidium Iodide Staining

Annexin V-FITC/PI apoptosis detection kit I (Thermo Fisher, Oregon, USA) was used to detect apoptotic cell number by Flow Cytometry as per manufacturer's instruction. Briefly, KB cells in a 6-well plate were treated with different concentrations of aqueous extract of areca nut (0.25 and 0.35%) for 48 h. After incubation, cells were harvested by trypsinization, washed with PBS and were resuspended in 100 µl of 1X binding buffer for about 15 min and then were treated with Annexin-V FITC/PI mixture for 15-20 min in dark and volume was made up to 400 µl by adding 1X binding buffer and further, analyzed using a Becton Dickinson FACS Aria III flow Cytometry (Scientific Instruments Centre, Indian Institute of Science, Bengaluru).

Statistical Analysis

Experimental data shown as mean ± standard deviation from three independent experiments. Statistical analysis was done by Student's t-test and one-way ANOVA followed by post hoc Tukey test. Difference between control and areca nut extract treated cell samples were considered significant if the level was *P < 0.05, **P < 0.005, ***P < 0.001.

RESULTS

Aqueous extract of areca nut decreased the viability of KB cells

KB cells treated with different concentrations of aqueous areca nut extract for 48 h and the viability was determined by MTT assay. Results show that the aqueous areca nut extract decreased the cell viability by 48 h in a dose dependent manner. More than 50 % decrease in viability was found at 0.35 % extract and a maximum of 90 % reduction in the viability was observed at 1.0 % aqueous areca nut extract as compared to control (Fig.1).

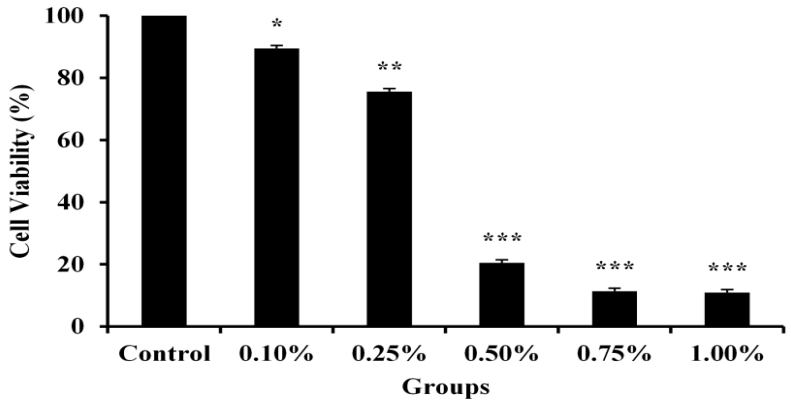

Fig. 1: Effect of aqueous extract of areca nut on the viability of KB cells

*Results were expressed as % viability of cells compared to control (mean±SD, n=4). Values are significantly different from control if *P<0.05, **P<0.005 and ***P< 0.001 by using student t-test and one-way ANOVA followed by post hoc tukey test.*

Induction of Reactive Oxygen Species (ROS) in KB cells on areca nut extract exposure

Cells were treated with different concentrations of aqueous extract of areca nut and were assayed for ROS production by DCFDA method. Results show that aqueous extract of areca nut induces ROS in a dose dependent manner with increase in concentration. As low as 0.1 % areca extract significantly increased ROS by 1.3-fold and maximum of 2.6-fold increases was observed at 1.0 % areca nut extract in KB cells as compared to control (Fig.2).

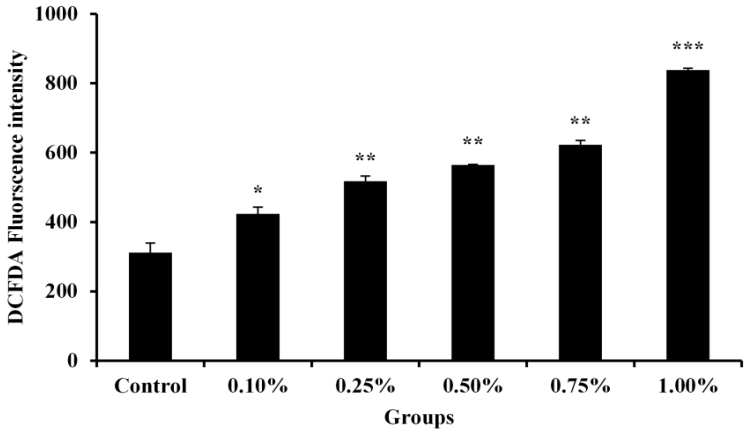

Fig. 2: Effect of aqueous extract of areca nut on ROS production in KB cells

*Cells were incubated with different concentrations of aqueous extract of areca nut in RPMI media. The fluorescence was read at different time intervals. Results were expressed as the change in DCF fluorescence Intensity with different concentrations (mean±SD, n=4). Values are significantly different from control if *P<0.05, **P<0.005 ***P< 0.001 by using student t-test and one-way ANOVA followed by post hoc tukey test.*

Aqueous extract of areca nut induces LDH leakage into culture supernatant in KB cells

Cells were treated with different concentrations of aqueous extract of areca nut for 48 h, and leakage of LDH into the culture supernatant was analyzed at 490 nm. The increase in leakage of LDH into culture supernatant is directly proportional to the number of cells lysed. The results show that the treatment of cells with areca nut extract showed the leakage of LDH into spent media by more than 3.5-fold at 1 % areca nut extract as compared to control (Fig.3). Further, it confirms that areca components exhibited cytotoxicity.

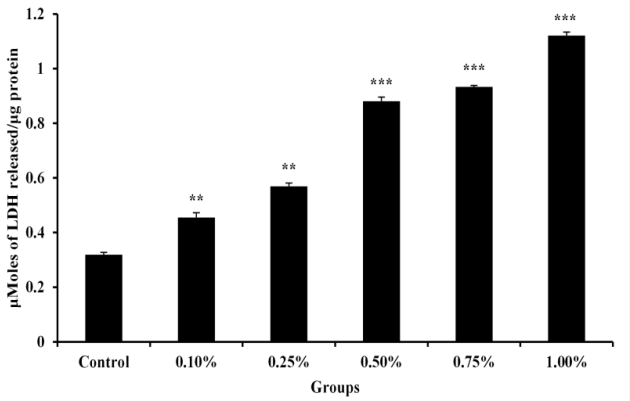

Fig.3: Effect of aqueous extract of areca nut on LDH leakage from KB cells.

*Results were expressed as µM of LDH released/µg of protein in areca nut extract treated KB cells as compared to control (mean±SD, n=4). Values are significantly different from control if *P<0.05, **P<0.005 and ***P<0.001 by using student t-test and one-way ANOVA followed by post hoc tukey test.*

Decreased SOD enzyme activity on aqueous areca nut extract treated KB cells

Cells were treated with different concentrations of aqueous extract of areca nut for 48 h. The cell lysate were prepared and subjected for total SOD analysis. Results show that the cells treated with aqueous extract of areca nut decreased the SOD activity in a dose dependent manner and maximum of 32 % decrease in SOD enzyme activity was observed at 1 % areca nut extract treated KB cells as compared to control (Fig. 4).

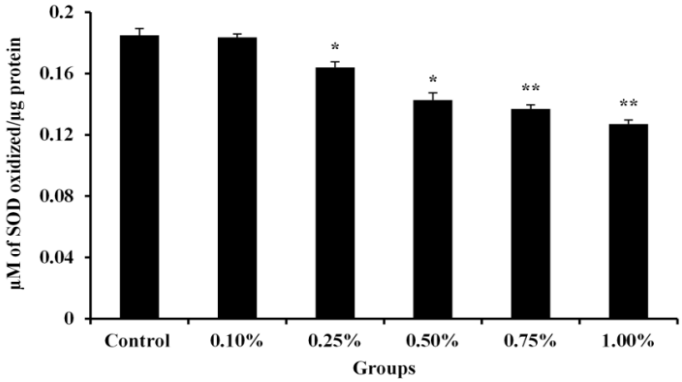

Fig.4: Effect of aqueous extract of areca nut on SOD activity in KB cells.

*Results were expressed as µM of SOD oxidised/µg of protein in areca nut extract treated KB cells as compared to control (mean±SD, n=4). Values are significantly different from control if *P<0.05, **P<0.005 and ***P<0.001by using student t-test and one-way ANOVA followed by post hoc Tukey test.*

Areca nut extract reduced glutathione S-transferase enzyme activity in KB cells

Cells treated with different concentrations of aqueous extract of areca nut for 48 h and GST enzyme activity was assayed. Results show that the cells treated with aqueous extract of areca nut decreased GST enzyme activity in a dose dependent manner. Maximum reduction of GST enzyme activity by 41 % was observed at 1 % aqueous areca nut extract as compared to control (Fig.5).

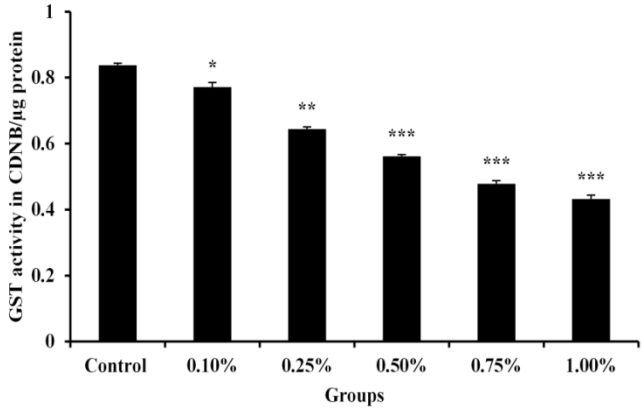

Fig. 5: Effect of aqueous extract of areca nut on GST activity in KB cells.

*The GST enzyme activity present in cell lysate was measured. Values are significantly different from control if *P<0.05, **P<0.005 and ***P<0.001by using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.*

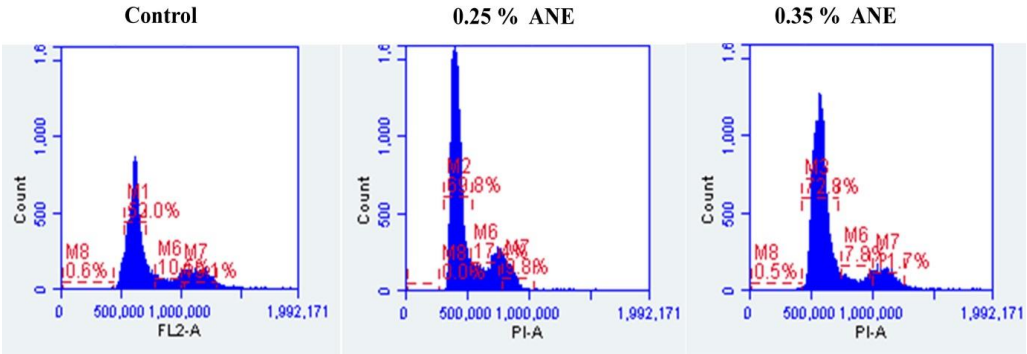

Fig.6: Flow cytometric analysis of cell cycle in areca nut extract treated KB cells

KB cells were treated with aqueous extract of areca nut (0.25 and 0.35%) for about 48 h followed by PI staining to analyses number of cells residing in different phases of cell cycle. Representative results of flow cytometric analysis of control, 0.25 and 0.35 % areca nut extract treated KB cells.

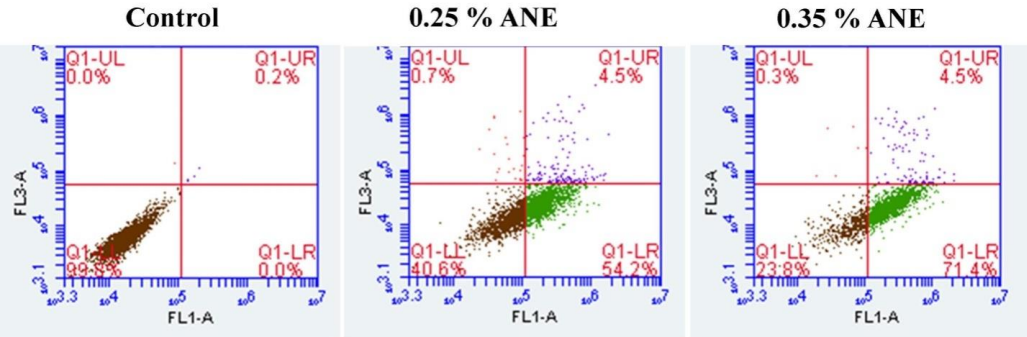

Fig.7: FACS analysis of apoptosis in KB cells treated with aqueous areca nut extract

Representative results of the flow cytometric analysis of control, 0.25 and 0.35 % areca nut extract treated cells. Cells in the lower right quadrant indicate the percentage of Annexin-positive, early apoptotic cells. Cells in the lower left quadrant indicate the percentage of Annexin-negative/PI-negative, viable cells. Cells in the upper right quadrant indicate the percentage of Annexin-positive/PI-positive, late apoptotic cells. Cells in the upper left quadrant indicate the percentage of PI-positive, necrotic cells, Q, Quadrant

Aqueous areca nut extract induces G_1/S phase cell cycle arrest in KB cells

Cells treated with different concentrations of aqueous areca nut extract to analyze different phases of cell cycle by flow cytometry. Results of the study showed increase

in number of cells residing at G_1 phase by 69.8 to 72.1 % and decrease in number of cells residing at S phase by 17 to 7.8 % with 0.25 and 0.35 % areca nut extract respectively by 48 h. Therefore, results of the study suggested that cells treated with areca nut extract induced G_1/S phase cell cycle arrest. Further, the result confirms that areca extract affects at the levels of DNA replication.

Aqueous extract of areca nut induces apoptosis in KB cells

Cells were treated with different concentrations of aqueous areca nut extract to assess apoptosis by Annexin V-FITC/PI staining by FACS analysis. Results of the study showed aqueous areca nut extract treated KB cells induced apoptosis by 48 h. KB cells treated with extract showed increased number of apoptotic cells by 59 and 76 % at 0.25 and 0.35 % areca nut extract respectively as compared to control.

DISCUSSION

Oral squamous cell carcinoma (OSCC) accounts for more than 90 % of oral cancer incidence and is frequently found at tongue, buccal, and gingival areas that are often associated with constant betel quid chewing [5]. Recent studies have demonstrated that areca nut extract and arecoline exhibit cytotoxicity and inhibited the growth of oral fibroblasts and keratinocytes [29]. Aqueous extract of areca nut effects the growth, differentiation, morphology and elicit DNA damage in cultured human buccal epithelial cells [9, 30]. Although there exist numerous evidences on the destructive effects of areca nut chewing, however the effects of areca nut extract on cell growth and proliferation, their influence on biochemical players like oxidants and anti-oxidants levels which would take part in maintaining the cellular homeostasis, and their impact on various biological macromolecules are not well understood. Additionally, a cascade of altered events like cell cycle progression and cell death are studied. Hence in the present study human buccal KB cells were used as *in vitro* model system to study the effect of aqueous areca nut extract.

Areca nut extract were found to be cytotoxic and genotoxic to oral mucosal fibroblasts and keratinocytes and found to inhibit the growth of human gingival fibroblasts [3]. KB cells on treatment with aqueous areca nut extract showed reduced viability in a dose dependent manner by 48 h. IC_{50} value of 0.35 % extract was calculated based on MTT assay. The results are in agreement with previous report that showed cytotoxic effect of areca nut extract on oral epithelial cells [9]. Thereby confirms the toxic nature of areca nut extract on human buccal KB cells. Several *in vitro* studies have pointed out that areca nut extracts and other betel quid ingredients can generate ROS and induce oxidative damage [18–23]. Free radicals which are highly unstable are known to cause adversarial effect on various biological macro molecules such as nucleic acids, lipids, and proteins, thereby altering the normal redox status [31]. Aqueous areca nut extract treated KB cells showed increasing DCFDA fluorescence as an indication of Induced ROS production in a dose dependent manner as compared to control. Excessively produced free radicals tend to reactive with lipids molecules of the plasma membrane and produces peroxide radicals and brings about loss of membrane integrity and leads to leakage of lactate dehydrogenase (LDH), a cytoplasmic enzyme, which results of membrane damage with decreased viability. KB cells treated with aqueous areca nut extract showed increased leakage of LDH into culture supernatant as a result of membrane damage along with decreased viability as of MTT assay confirms the toxic nature of extract. The above stated effects are totally in acceptance with loss of membrane integrity followed by leakage of LDH on constant pan masala exposure [32].

Due to persistent exposure of cytotoxic substances there may be a loss in balance between oxidants and anti-oxidants which would result in the development of oxidative stress in cells**.** In order to provide defense mechanism besides the excessive production of free radicals, the cells exert anti-oxidants to neutralize the released free radicals [33, 34]. The SOD, GSH-Px, and Catalase are the major enzymatic anti-oxidant defense systems responsible for scavenging free radicals and nascent oxygen [35]**.** Results of the present study showed decreased enzyme activity of SOD and GST in areca nut extract treated KB cells as compared to control. Previous reports showed oral cancer patients who were constantly exposed to areca nut chewing exhibited decline in the levels of the anti-oxidant enzymes [36]. Likewise arecoline treated human buccal mucosal fibroblast cells showed significant decrease in the levels of GST [11]. Further, results of the study states that altered cellular redox homeostasis thereby bring

about cellular variation that would lead to disorderness within the cell.

Impairment of cell cycle by toxic chemicals usually leads to growth retardation, cytotoxicity and apoptosis [18, 22]. Deregulation of cell cycle control is one of the major causes of cancer induction [19]. Therefore, it is of interest to know whether areca nut extract affects cell cycle progression and apoptosis. Results of the study showed increased number of cell residing in G_1 phase and decreased cell number in S phase showed G_1/S phase cell cycle arrest in aqueous areca nut extract treated KB cells. Similar results were observed in oral carcinoma 3 cells on areca nut extract exposure showed G_1/S phase cell cycle arrest [37]. Results of the study further, confirm the deadly effect of areca nut on cellular DNA which thereby triggers cell cycle arrest in human KB cells.

Although toxicants may initiate cell stress or damage, the cellular proteins that are involved in control of cell cycle and apoptosis are the final arbiters of cellular fate. DNA damage elicited due to exposure to toxic compound activates repair or stress responsive systems which might trigger cell cycle arrest or apoptosis [38]. Results of the study showed induction of apoptosis on exposure of aqueous areca nut extract in KB cells and the results were confirmed by flow cytometric analysis by annexin V FITC/PI staining. Studies carried out in human oral and tongue squamous cell carcinoma (HSC-2 and HSC-3) showed the induction of apoptosis on areca nut extract exposure over 24 and 48 h [39]. Further, the results of the study suggest that areca components exert toxic effect on cells thereby inducing cell death.

Hence, the present study helps in better understanding the effect of aqueous areca nut extract on growth and viability on human buccal KB cells and their implications on biochemical defense players i.e. the oxidants and the anti-oxidants that play a crucial role in maintaining the cellular homeostasis.

Conclusion

Imbalance between oxidants and anti-oxidants brings about oxidative stress status that would alter numerous biological processes and would cause serious health effects like cancers and so on. Thus, the study provides better elucidation of redox homeostasis which facilitates in designing effective therapeutic modalities to overcome the redox status and its deleterious effects on cell system.

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***Corresponding Author:**
M.Sreepriya*
Email: **mpriya7@yahoo.com**