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Aqueous areca nut extract induces oxidative stress in human lung epithelial A549 cells: probable role of p21 in inducing cell death.

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Abstract

Areca nut a well-known masticator used across globe. Habitual chewing of areca nut is associated with serious oral health effects. However, the role of areca nut in oxidative stress induction and cell death is less understood. Hence, in the present study we aimed to evaluate the toxic mechanism of areca nut extract on human lung epithelial A549 cells. Cells were treated with or without aqueous areca nut extract and cell viability was measured by MTT assay. Cells treated with areca nut extract show reduced viability in a dose dependent manner with the IC_{50} of 0.5 % concentration. Areca nut extract induced the reactive oxygen species (ROS), lipid peroxidation followed by membrane damage with leakage of lactate dehydrogenase (LDH) enzyme. Cells with continuous exposure of areca nut extract depletes the free radical neutralizing anti-oxidant enzymes like superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and Glutathione-S-transferase (GST). Further, the analysis of mRNA expression of apoptotic genes and cell cycle regulators show decreased expression of anti-apoptotic gene (Bcl-2), Cyclin E1, CyclinD1, CDK4, Rb and p53 whereas induced expression of p21 and marginal increase of pro-apoptotic gene (Bax) confirms the toxic nature of areca nut. Thus, cell death due to areca nut exposure may be through different mechanism rather than the conventional apoptotic pathway, where p21 induction might be independent of p53 action, which possibly suggests that there may be a role of p21 in oxidative stress induced cell death. Further FACS analysis confirms cell death in areca nut treated cells.

Key Words: Areca nut extract, toxicity, ROS, GSH-Px, p21, cell death.

Highlights:

Aqueous extract of areca nut induces oxidative stress.

p21 gene expressions were induced on areca nut extract treatment which is independent of p53 action.

Differential role of p21 in causing cell death by inhibiting the major cell cycle regulators might not be through the conventional apoptotic pathway.

Abbreviations:

RPMI, Rosewell Park Memorial Institute media, **FBS** , Fetal bovine serum, **PBS**, phosphate buffered saline, **ROS**, reactive oxygen species, **LPO**, Lipid peroxidation, **LDH**, Lactate dehydrogenase, **GSH-Px**, Glutathione peroxidase,

GST, Glutathione S transferase, **SOD**, Superoxide dismutase, **OSF**, Oral sub mucosal fibrosis, **OSCC**, Oral squamous cell carcinoma.

1. Introduction

Areca nut, the hard edible endosperm of the palm tree *Areca catechu Linn.* (Family: *Palmaceae*), is been cultivated all over South India, Southeast Asia and in various islands of Pacific Ocean. Areca nut a common masticator, used alone or as a component of betel quid along with betle leaf, slaked lime and tobacco. In many Southeast Asian countries areca nut chewing is becoming a popular oral habit (IARC, 1985; Thomas and Kearsley, 1993) (Sharan, 1996; Jeng et al., 2001). About 200–600 million people in Southeast Asia are habituated of chewing betel quid in combination with areca nut and other constituents (Chang et al., 2004; Chen et al., 2004). There is substantial evidence for chewing areca nut often causes severe oral health effects. Although oral cancer is one of the most common cancers, nearly 275,000 cases are reported yearly worldwide (Warnakulasuriya, 2009). In India it was projected that around 75000 new cases of oral cancer were added annually and the incidence rate of oral cancer appears to be highest in the world (Nair et al., 2004). Areca nut chewing along with betle leaf and tobacco has an added risk of evoking serious disorders such as cancer of mouth and esophagus (Javed et al., 2010; Garg et al., 2014). An elevated risk of pre-invasive lesions, the leukoplakia with oral sub mucosal fibrosis (OSF) and oral cancer are in close concomitant with habitual chewers of betel quid. Commonly observed signs in betel quid chewers were histologically swelling, vacuolated cells, round nuclear fragments or pyknotic nuclei, substantial inflammatory intrusion and derestricted epithelial growth leading to OSF (Reichart and Phillipsen, 1998; Trivedy et al., 2002; Chiu et al., 2008; Rajendran, 2009). Recent studies in United Kingdom (UK) on Asian migrants revealed clear relation about areca nut chewing and incidence of OSF, 70 % of the cases were found at an age group less than 35 yr. (Gupta and Ray, 2003). International Agency for Research on Cancer (IARC), the World Health Organization reached to a conclusion that there is adequate evidence of chewing betel quid with or without tobacco is carcinogenic to humans (Chou et al., 2009).

Areca nut comprises of 5-40 % polyphenols and numerous alkaloids such as arecoline, arecaidine, guvacine and guvacoline. The presence of arecoline was found to be 1 % of the dry weight of areca nut and is known to be genotoxic (Dave et al., 1992). Salmonella typhimurium strains were found to be mutated on areca nut extract exposure (Shirname et al., 1983; Shirname et al., 1984). Aqueous extract of areca nut incited mitotic gene conversion (Rosin et al., 2002) and has been categorized as carcinogenic to humans (IARC, 2004). Even though

there are several confirming evidences for the causative act of areca nut in developing OSF, However the mechanism by which it occurs are not that evident. Arecoline and its hydrolyzed product arecaidine elicit proliferation/collagen synthesis in *in vitro* cultured fibroblast (Canniff and Harvey, 1981; Harvey et al., 1986). Arecoline at higher concentration are known to be cytotoxic (van Wyk et al., 1994; Jeng et al., 1996). Areca nut extract treated cells induce reactive oxygen species (ROS) that causes morphological changes leading to the retraction and autophagosome like vacuoles (Chang et al., 2001; Lu et al., 2010). Anti-oxidant enzymes like Glutathione S- transferase, known to detoxify ROS. Arecoline treated human oral keratinocytes and fibroblasts show exhaustion in the activity of cellular anti-oxidant glutathione and reduced glutathione S-transferase (Jeng et al., 1996; Chang et al., 2001).

In response to genotoxic stress cells naturally display cell cycle arrest and allowing it for DNA repair (Smith and Fornace, 1996; Shackelford et al., 1999; Shackelford et al., 2000). Though, growth of cells is rigorously controlled by cell cycle progression. Exposure of lethal compound causes impairment of the cell cycle which in turn leads to growth retardation, cytotoxicity and apoptosis (Hickman and Boyle, 1997; Laird and Shalloway, 1997; Eastman and Rigas, 1999; Li and Brooks, 1999; Shackelford et al., 1999). However, it is interesting to know the possible outcome of areca nut extract on induction of cell cycle arrest or apoptosis and its action on the regulatory proteins of cell cycle.

The cyclin-dependent kinase (CDK) inhibitor p21 mediates several biological events and is well-known as a negative regulator of cell proliferation in response to many stimuli (Gartel et al., 1996). Normal oral mucosal epithelium shows undetectable levels of p21 waf1 protein, whereas in pre-malignant lesions of betel quid chewers show significantly elevated levels of p21 waf1 (Agarwal et al., 1998). In case of Oral Squamous Cell Carcinoma *(*OSCC) there exists a correlation between the accumulation of nuclear p21 with poor prognosis and disease progression (Schoelch et al., 1999; Nemes et al., 2005) (Nemes et al 2005, Schoelch et al 1999). While, G1/S transition of cell cycle is facilitated by p21 which enables assembling of CCND1/Cdk2/p21/PCNA complexes unless cyclin E/Cdk2 is sequestered by excessive p21 proteins (Law et al., 2006; Masamha and Benbrook, 2009). Areca nut extract induced p21 retards cell cycle; cells may even continue proliferation once areca nut is removed after chewing. Increased level of p21 protein expression is also found in association with well-differentiated SCC (squamous cell carcinoma) while poorly differentiated SCCs do not (Agarwal et al., 1998).

Studies from times have shown the consequence of constant usage of areca nut leads to oral cancer, but not much is known about the exact mechanism of toxicity and the genes that are involved in triggering cytotoxicity. Hence in the present study human lung epithelial A549 cells were used as an *in vitro* model system to understand the toxic effect of aqueous areca nut extract on ROS, release of anti-oxidant enzymes and its effects on mRNA levels of cell cycle regulators and apoptotic genes. Further, FACS analysis was carried out to confirm the act of areca nut extract in treated cells.

2. Materials and Methods

A549 cells (human lung epithelial cells) were purchased from NCCS (Pune, India); TRIzol, Lithium lactate, iodonitrotetrazolium chloride (INT), phenazine methosulphate (PMS), malondialdehyde (MDA), glutathione reductase, reduced glutathione and Oligos forward and reverse primers for cell cycle regulator and apoptotic genes (Table 1) 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). Oligo dTs and superscript reverse transcriptase were obtained from Invitrogen Bio Services India Pvt. Ltd (Bangalore, India), Taq DNA polymerase (1 U/µl) was purchased from Merck-Millipore (Mumbai, India).

2.1. Culturing of A549 cells

A549 cells were grown in 25cm^2 culture flask using RPMI 1640 medium with 10 % FBS, 100 U/ml penicillin and 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 cell were trypsinised and subcultured in 96 or 6 well plate for treatments. (Patil et al., 2015)

2.2. Preparation of aqueous extract of areca nut

Areca nut was collected from Uttara Kannada district, Karnataka, India. 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 to treat cells at different concentration or stored at -20 °C until further use.

2.3. Cell viability Assay

Cell viability was carried out using MTT assay as per the protocol described earlier (Babu et al., 2013) with slight modifications. In brief A549 cells (3×10^3cells/well) in RPMI-1640 medium with a final volume of 200 µl were seeded into 96-wells culture plate and incubated overnight at 37 °C with the supply of 5 % CO_2. The cells were treated with or without different concentration of aqueous areca nut extract (0.1, 0.25, 0.5, 0.75 and 1.0%) and further incubated for 48 h. The cells were washed with PBS, treated with 50 µl of MTT (5 mg/ml) and incubated for 4 h at 37 °C in a CO_2 incubator. 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).

2.4. Reactive Oxygen Species (ROS) Assay

ROS generated in cells by the action of toxic substances were measured by a real time assay as per the protocol described earlier (Periyakaruppan et al., 2009). A549 cells (3×10^3 cells/ well) were cultured overnight in a black colored 96-wells plate, washed with phosphate buffered saline (PBS) and treated with 10 µM DCF in 1N NaOH for 3 h. Further, cells were washed with PBS and incubated with different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) in medium for different time interval. Fluorescence intensity was recorded using a multimode plate reader (Perkin Elmer) at excitation wavelength of 485 nm and emission of 527 nm.

2.5. Lipid peroxidation (LPO) Assay

Toxic effect of compound on cells may cause membrane damage sequentially leads to lipid peroxidation. Effect of aqueous extract of areca nut on lipid peroxidation was analyzed as per the method described earlier (Wise et al., 2005). It depends on the detection of thiobarbituric acid-reactive malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids or related esters. A549 cells were cultured overnight and treated with or without different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) and incubated for 24 h. The cell lysate was prepared using lysis buffer (0.5 mM Tris HCl, 150 mM NaCl, 0.1 % SDS and 0.5 mg/ml

Proteinase K) and lysate containing equal amount of protein was made up to 100 µl followed by addition of 800 µl of lipid peroxidation assay mix (0.8 % thiobarbituric acid, 8.1 % sodium dodecyl sulfate and 20 % acetic acid) and incubated at 95 °C for 1 h, Samples were cooled to room temperature, 1 ml of distilled water and 5 ml of butanol pyridine mixture, (15:1) was added and centrifuged at 4,000 rpm for 10 min. The absorbance of upper orange layer was read at 532 nm using spectrophotometer.

2.6. Lactate Dehydrogenase (LDH) Assay

The cytotoxic effect of aqueous areca nut extract on A549 cells was measured by LDH assay as described earlier with slight modification (Decker and Lohmann-Matthes, 1988; Ramirez et al., 2014). 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 that can be measured at 490 nm. The formazan product formed is directly proportional to the amount of LDH released into the medium, which reflects the cytotoxic effect. Cells were cultured in a 6-wells plate and incubated overnight, further treated with or without different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) incubated for 24 h. Cell-free supernatant (50 µl) from each well was transferred in to a 96-wells plate in triplicate and, 150 µl of LDH-assay reaction mixture was added. Once the color was developed the optical density was measured at a wavelength of 490 nm using a multimode plate reader (Perkin Elmer).

2.7. Superoxide dismutase (SOD) Assay

Total SOD activity was determined according to the method described earlier (Kankofer, 2002b). A549 cells were cultured overnight and treated with or without different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) for 24 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 100 µl epinephrine in 0.1 M 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 aqueous extract treated samples against control.

2.8. Glutathione peroxidase (GSH-Px) Assay

GSH-Px assay was carried out as per the protocol described earlier (Kankofer, 2002a). A549 cells (5×10^5 cells/well)

were cultured in a 6-wells plate and incubated overnight. Cells were treated with or without different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) and further incubated for 24 h. Cell lysate was prepared using 200-400 µl of lysis buffer .To 100 µl of cell lysate 2.8 ml of glutathione peroxidase assay mixture containing 100 µl of 8.4 mM NADPH, 10 µl glutathione reductase (GSSG-R, 100 U/mg protein/ml), 10 µl of 1.125 M sodium azide, 100 µl of 0.02 M reduced glutathione in 0.05 M phosphate buffer of pH 7.0 was added. The enzymatic reaction was initiated by the addition of 100 µl of 0.02 mM H_2O_2 to the GSH-Px reaction mixture. Rate of GSSG formation was measured by decrease in the absorbance at 340 nm, as NADPH oxidized to NADP+, using spectrophotometer.

2.9. Glutathione S-transferase (GST) Assay

GST assay was carried out as per the protocol described earlier (Mannervik, 1985). A549 cells (5×10^5 cells/well) were cultured in a 6-wells plate and incubated overnight. Cells were treated with or without different concentration of aqueous extract of areca nut (0.1, 0.25, 0.5, 0.75 and 1 %) and further incubated for 24 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.

2.10. RNA isolation and semi-quantitative RT-PCR analysis

A549 cells (5×10^5 cells/well) were cultured overnight in 6-wells plate for attachment and treated with or without aqueous extract of areca nut (0.25 and 0.5 %). Further, the cells were incubated for 48 h and were processed for RNA isolation as per the protocol described earlier (Sharma and Richards, 2000). In brief, the cells were washed with ice cold PBS and lysed with 1 ml of TRI Reagent. The lysate was then transferred to an Eppendorf tube and allowed to stand at room temperature for 10 min. To this 100 µl of chloroform was added and mixed with vigorous shaking and allowed to stand at room temperature for 15 min, centrifuged at 12,000 rpm for 10 min at 4 °C. Upper aqueous phase containing RNA was precipitated by adding equal volume of isopropyl alcohol and centrifuged at 12,000 rpm for 10 min. RNA pellet was re-precipitated with 80 µl of 3 M sodium acetate and 2.5 volume of chilled ethanol, centrifuged at 12,000 rpm for 10 min following 70 % ethanol wash. The RNA pellet was dissolved in 50 µl of RNase free water. Concentration of RNA was determined by measuring the absorbance at 260 nm and purity was

assessed by measuring the absorbance at 260/280 nm. The quality of the isolated total RNA was also analyzed using 1 % MOPS-formaldehyde agarose gel.

Reverse transcription of RNA and PCR analysis of cell cycle regulators and apoptotic genes were carried out as per the protocol described earlier (Sharma et al., 1999) **.** In brief total RNA (2 µg) was reverse transcribed using Superscript III First strand synthesis kit (Invitrogen). The cDNA was subjected to 30 cycles of PCR using different forward and reverse primers of cell cycle regulators and apoptotic genes using appropriate annealing temperatures as indicated in Table 1 in a gradient Eppendorf thermo cycler. Amplified PCR products were analyzed on 1 % agarose gel using 1X TAE buffer. The expression of β-actin mRNA was used as a positive control and for normalization. The relative mRNA levels were quantified using image analysis software (Image J)(Patil et al., 2016)**.**

Table 1: Sequence of primers used for the PCR amplification

Gene	Primer sequence (5′ - 3′)	Annealing temp. (°C)	Product size (bp)
Cyclin E1	F: GTCCTGGCTGAATGTATACATGC R: CCCTATTTTGTTCAGACAACAT	60	415
Cyclin D1	F: AGACCTGCGCGCCCTCGGTG R: GTAGTAGGACAGGAAGTTGTTG	58	574
CDK 4	F: AGTGGCGGATCCATGGCTACCTCTCGATAT R: TCTCGGAAGCTTTCACTCCGGATTACCTTCA	60	912
Rb	F: AAGTACCCATCTAGTACT R:AAGTTACAGCATCTCTAAA	58	547
p53	F: GAGCCCCCTCTGAGTCAG R: GCAAAAACATCTTGTTGAG	56	375
p21	F : GATCACAAGCATGGGGTGA R : CTGAGTGACTGCACGACCTT	58	160
Bcl-2	F: AGATGTCCAGCCAGCTGCACCTGAC R:AGATAGGCACCCAGGGTGATGCAAGCT	62	365
Bax	F: AAGCTGAGCGAGTGTCTCAAGCGC R: TCCCGCCACAAAGATGGTCACG	61	366
β-actin	F: TACCACTGGCATCGTGATGGACT R: TCCTTCTGCATCTGTCGGCAAT	62	516

Columns 3 and 4 *show different annealing temperatures used and the size of the amplified products.* ***F*** *forward,* ***R*** *reverse*

2.11. Apoptosis Detection by Annexin V-FITC and Propidium Iodide Staining

Annexin V-FITC apoptosis detection kit I (Thermo Fisher, Oregon, USA.) was used to sort live and apoptotic cells by Flow Cytometry as per the protocol described earlier (Allen et al., 1997). Briefly, A549 cells (5×10^5 cells/well) in a 6-wells plate were treated with or without different concentration of aqueous extract of areca nut (0.25 and 0.5%) for 24 h. After incubation, cells were harvested by trypsinization, washed with PBS and were suspended in

300 µl of Annexin-V/PI reaction mixture for 15-20 min in dark and further analyzed using a Becton Dickinson FACS Aria III flow Cytometry (Scientific Instruments Centre, Indian Institute of Science, Bengaluru).

2.12. 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 treated cell samples were considered significant if the level was *****P < 0.05, ******P < 0.005.

3. *Results:*

3.1. Aqueous extract of areca nut decreases the cell viability of A549 cells

A549 cells were treated with or without different concentration of aqueous areca nut extract for 48 h and the cell viability was determined by MTT assay. Results show that the aqueous extract of areca nut decreased the cell viability by 48 h in a dose dependent manner. More than 50 % decrease in cell viability was found at 0.5 % aqueous extract of areca nut (Fig. 1).

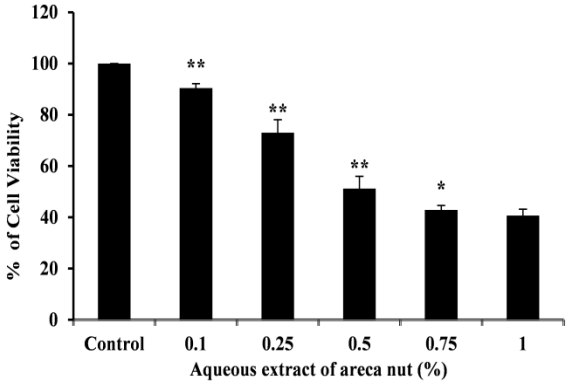


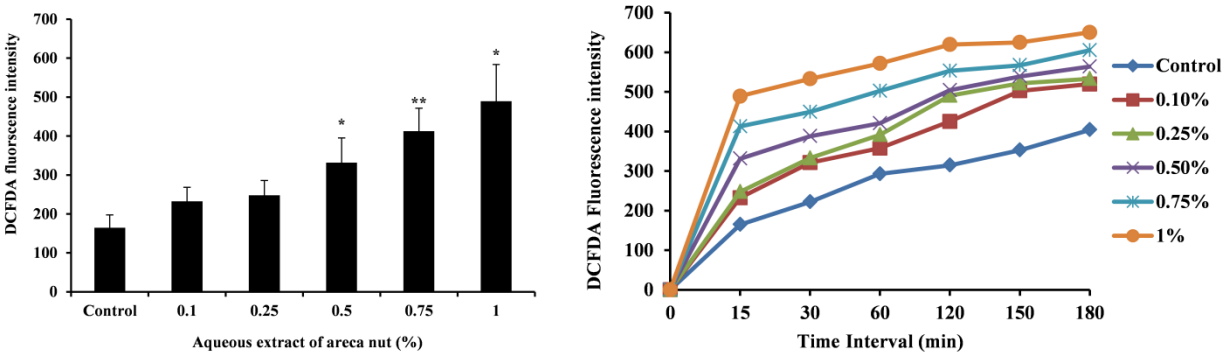
Fig. 1: Effect of aqueous extract of areca nut on the cell viability of A549 cells

*A549 cells (3 × 10^3cells/well) were treated with or without different concentration of aqueous extract of areca nut and incubated for 48 h and cell viability was measured by MTT assay. 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 by using student t-test and one-way ANOVA followed by post hoc tukey test. The results were shown was a*

representative of three independent experiments

3.2. Aqueous extract of areca nut induces Reactive Oxygen Species (ROS) in A549 cells

A549 cells were treated with or without different concentration of aqueous extract of areca nut and for different time interval (0 - 180 min). Total ROS produced in cells were measured by real time assay. Results show that aqueous extract of areca nut induces ROS in a dose dependent manner Fig. 2. (a) And also induces the production ROS as a function of time Fig. 2. (b). More than 60 % increase in production of ROS at 1 % aqueous areca nut extract treated cells. Further the increase in ROS was found to be significantly increased by as early as 15 min and increased with time at all treated concentrations of areca nut extract.



(a) **(b)**

Fig. 2: Effect of aqueous extract of areca nut on ROS production at (a) different concentration (b) different time intervals in A549 cells.

A549 cells (3×10^3cells/well) were treated with 10 µM DCF in 1N NaOH and incubated for 3 h. Cells were incubated with different concentration 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 as measured after each time interval as shown (mean±SD, n=4). Values are significantly different from control if ***P<0.05,*** ****P<0.005** *by using student t-test and one-way ANOVA followed by post hoc tukey test. The results were shown as representative of three independent experiments.*

3.3. Aqueous extract of areca nut induces lipid peroxidation in A549 cells

Aqueous extract of areca nut induces ROS and oxidative stress and such an event could induce lipid peroxidation (LPO) in cells. Therefore investigation for malondialdehyde (MDA) levels in A549 cells treated with or without different concentration of aqueous extract of areca nut for 24 h show increased levels of MDA which reflects the degree of LPO. LPO was found to be significantly increased with different concentration of aqueous extract of areca nut compared to control in a dose dependent manner. Maximum lipid peroxidation of more than 1.9 fold increase was observed in the cells treated with 1 % extract of areca nut compared to control (Fig. 3).

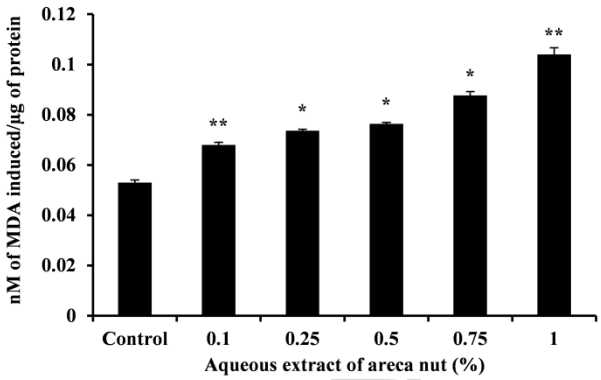


Fig. 3: Effect of aqueous extract of areca nut on lipid peroxidation in A549 cells.

A549 cells (5×10^5 cells) were treated with different concentration of aqueous extract of areca nut for 24 h and lipid peroxidase enzyme product MDA was assayed. Values are significantly different from control if ***P<0.05,*** ****P<0.005 by using student t-test and one way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.*

3.4. Aqueous extract of areca nut induces LDH leakage into medium in A549 cells

A549 cells were treated with or without different concentration of aqueous extract of areca nut for 24 h, and leakage of LDH from cells into the spent medium was analyzed. The results show that the treatment of cells with areca nut show the leakage of LDH into medium suggested that the components present in arecanut were toxic to cells. The leakage was found to be dose dependent and more than 3.2 folds increase in LDH leakage was observed with 1 % areca nut extract treated cells when compared to control. (Fig. 4)

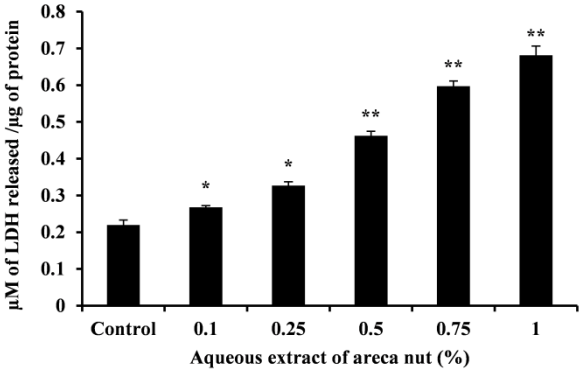


Fig.4: Effect of aqueous extract of areca nut on LDH leakage from A549 cells.

A549 cells (5×10^5 cells/well) in a 6-wells plate were treated with different concentration of aqueous extract of areca nut and LDH leakage in the medium was measured. Values are significantly different from control if ***P<0.05,*** ****P<0.005 by using student t-test and one-way ANOVA followed by post hoc tukey test. The results were shown as representative of three independent experiments.*

3.5. Aqueous extract of areca nut decreases SOD activity in A549 cells

A549 cells were treated with or without different concentration of aqueous extract of areca nut for 24 h. Cell lysates were prepared and subjected for total SOD analysis. Results show that the cells treated with aqueous extract of areca nut decreases the SOD activity in a dose dependent manner and maximum of 24 % decrease in SOD enzyme activity was observed at 1 % areca nut extract treated cells when compared to control (Fig. 5).

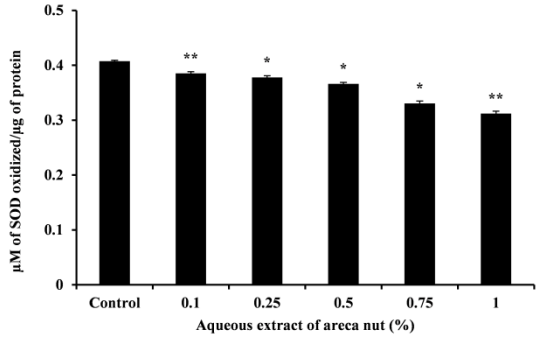


Fig.5: Effect of aqueous extract of areca nut on SOD activity in A549 cells.

A549 cells (5×10^5 cells/well) were treated with different concentration of aqueous extract of areca nut *for 24 h. The amount of SOD enzyme activity present in the cell lysate was measured. Values are significantly different from*

*control if **P<0.05, ***P<0.005 by using student t-test and one way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.*

3.6. Aqueous extract of areca nut depletes Glutathione peroxidase (GSH-Px) enzyme activity in A549 cells

A549 cells were treated with or without different concentration of aqueous extract of areca nut for 24 h and cell lysates were analyzed for the GSH-Px activity. Results show that GSH-Px enzyme activity was significantly decreased in cells treated with extract of areca nut in a dose dependent manner (Fig. 6). Cells treated with 1 % aqueous extract decreases the GSH-Px enzyme activity by more than 15 % (Fig. 6).The decrease in GSH-Px enzyme activity may be one of the major factor responsible for the oxidative stress due to areca nut treatment.

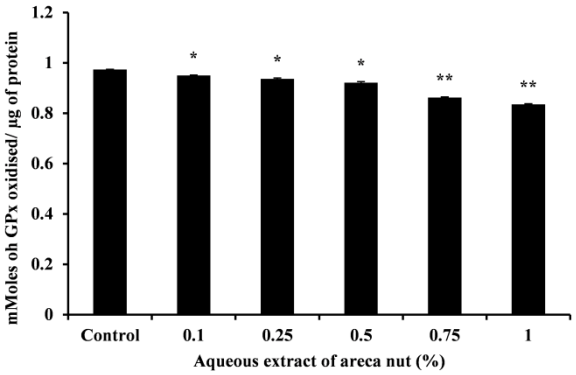


Fig.6: Effect of aqueous extract of areca nut on Glutathione peroxidase enzyme activity in A549 cells.

*A549 cells (5 × 10^5 cells/well) treated with different concentration of extract of areca nut and GSH-Px assay was carried out by measuring total 2-Nitro-5-ThioBenzoic acid (TNB). Values are significantly different from control if **P<0.05, ***P<0.005 by using student t-test and one way ANOVA followed by post hoc Tukey test. The results were shown as a representative of three independent experiments*

3.7. Areca nut extract decreases Glutathione S-transferase activity in A549 cells.

A549 cells were treated with or without different concentration of aqueous extract of areca nut for 24 h and GST enzyme activity were assayed. Results show that the cells treated with aqueous extract of areca nut decreases the GST enzyme activity in a dose dependent manner. Cells treated with 1 % aqueous extract of areca nut show 15 % decrease in GST enzyme activity compared to control. (Fig. 7)

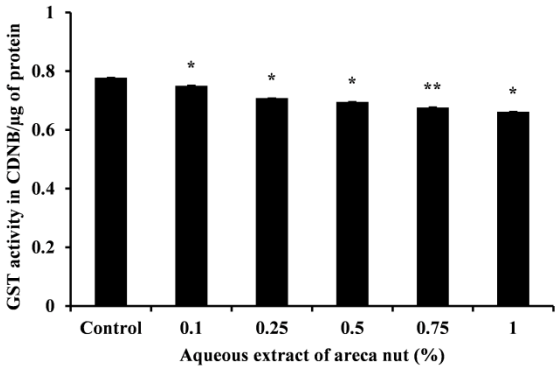


Fig.7: Effect of aqueous extract of areca nut on GST activity in A549 cells.

A549 cells (5 × 10^5cells/well) were treated with different concentration of aqueous extract of areca nut for 24 h. The GST enzyme activity present in cell lysate was measured. Values are significantly different from control if ***P<0.05,*** ***P<0.005 by using one way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.*

3.8. Aqueous extract of areca nut decreases anti apoptotic Bcl-2 gene expression in A549 cells

A549 cells were treated with or without aqueous extract of areca nut for 48 h and the mRNA expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 genes were analyzed by semi-quantitative RT-PCR. Results show that there was a marginal increase in mRNA levels of Bax by 1.1 and 1.2 fold with 0.25 and 0.5 % aqueous areca nut extract treated cells respectively when compared to control, while the mRNA expression of Bcl-2 was decreased by 32 and 72 % with 0.25 and 0.5 % areca nut extract treated cells respectively. Results suggested that areca nut induces apoptosis of A549 cells (Fig. 8).

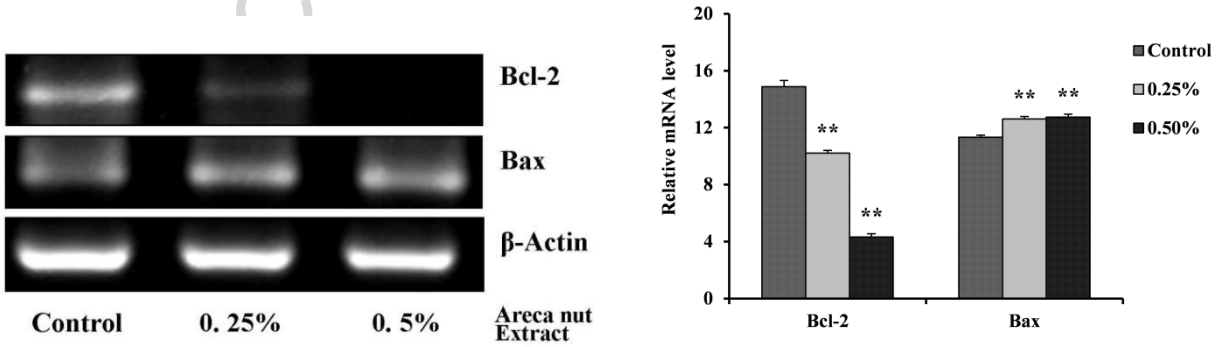


Fig. 8: Effect of aqueous extract of areca nut on mRNA levels of Apoptotic genes.

Cells were treated with or without aqueous extract of areca nut for 48 h. The mRNA expression of Bcl-2 and Bax genes were carried out by semi quantitative RT-PCR and were analyzed on 1 % agarose gel. The band intensity of the experimental samples was compared with the control and β-actin was used as a positive control and for normalization. Differences in the expression of apoptotic gene mRNA levels are statistically significant if ***P < 0.05** *or* ****P< 0.005** *compared with control value using one way ANOVA followed by Post-hoc Tukey test. The results were shown as representative of three independent experiments. The bar graph represents the densitometric analysis of mRNA level of pro and anti-apoptotic genes.*

3.9. Aqueous extract of areca nut decreases the mRNAs expression of Cyclin E1, Cyclin D1, CDK4, Rb and p53 but increases p21 transcript in A549 cells.

Cells treated with or without aqueous extract of areca nut for 48 h were analyzed for the expression of mRNA levels of cell cycle regulators (Cyclin E1, Cyclin D1, CDK4, p53, Rb and p21). The aqueous areca nut extract treated cells show dose dependent decrease in the expression of Cyclin E1 by 16 and 43 %, Cyclin D1 by 81 and 90 %, CDK4 by 64 and 91 %, Rb by 50 and 73 % and p53 by 54 and 87 % with 0.25 and 0.5 % areca nut extract respectively whereas significant increase in p21 mRNA expression by 4.1 and 4.8 fold with 0.25 and 0.5 % areca nut extract respectively suggested that aqueous extract of areca nut show varied effect on cell cycle regulators. (Fig. 9)

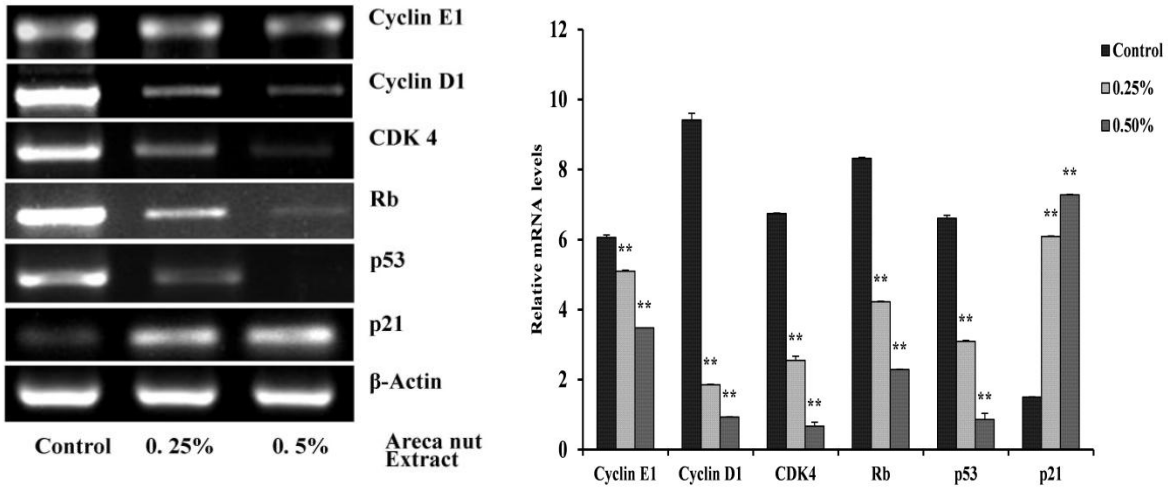
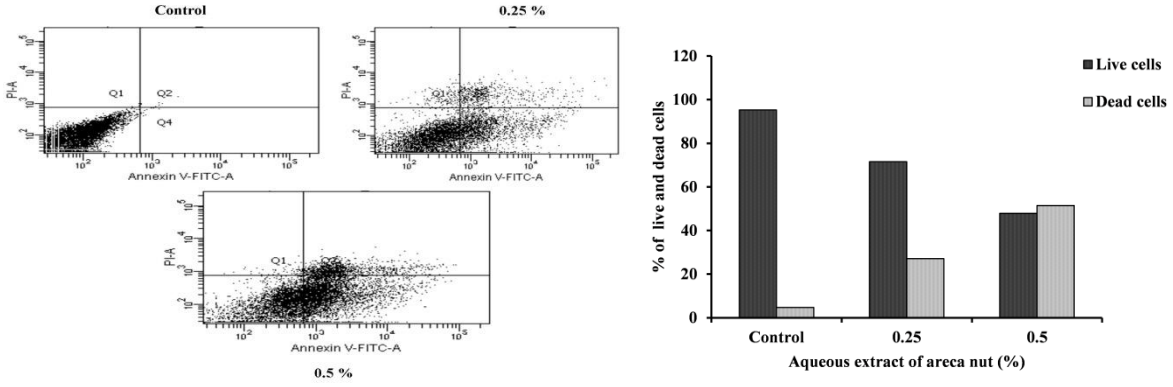


Fig. 9.Effect of aqueous extract of areca nut on mRNA levels of cell cycle regulators

*Cells were treated with or without aqueous extract of areca nut for 48 h. The mRNA expression of cell cycle regulators were carried out by semi quantitative RT-PCR and were analyzed on 1 % agarose gel, and the band intensity of the experimental samples was compared with the control. β-actin was used as a positive control and for normalization. Differences in the expression of cell cycle regulators mRNA levels are statistically significant: if *P < 0.05 or **P< 0.005 compared with control value using one way ANOVA followed by Post-hoc Tukey test. The results were shown as representative of three independent experiments. The bar graph represents the densitometric analysis of the mRNA levels of cell cycle regulator.*

3.10. Aqueous extract of areca nut induces apoptosis in A549 cells

A549 cells were cultured overnight and then incubated with and without different concentration of (0.25 and 0.5 %) aqueous areca nut extract for 24 h. To assess apoptosis the cells were subjected to Annexin V-FITC assay followed by FACS analysis. Results show that the treatment of cells with aqueous areca nut extract for 24 h induces apoptosis in A549 cells. Control cells show little or no apoptosis Fig. 10(a), while treatment with 0.25 % extract's dead cell accounts for more than 27 % and there was an increase by 52 % in cell death with 0.5 % extract. Fig. 10(b) shows the bar graph representation of FACS analysis of control and aqueous areca nut extract treated A549 cells.



(a) **(b)**

Figure 10(a and b): FACS analysis of A549 cells treated with aqueous extract areca nut.

A549 cells were treated with or without aqueous extract of areca nut (0.25 and 0.5%) for 24 h followed by Annexin V-FITC/PI staining to observe the induction of apoptosis. Representative results ***Fig. 10(a)*** *of the flow cytometric analysis of control, 0.25 and 0.5 % 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;* ***Fig 10b*** *shows the graphical representation of apoptotic levels (early plus late apoptosis) after treatment with aqueous areca nut extract for 24 h. Q, Quadrant*

4. Discussion

Betel quid chewing is a popular, yet harmful oral habit. Areca nut recognized as a psychoactive element next to nicotine/alcohol/caffeine. OSCC has been the third most common malignancy found in developing countries (Chang et al., 2004; Chen et al., 2004). Areca nut in betel quid has been recognized as a Group I carcinogen to humans by the International Agency for Research on Cancer (IARC) and World Health Organization (IARC, 2004). Epidemiological studies have elucidated a strong correlation between the incidence of oral leukoplakia, sub mucosal fibrosis and oral cancer by the heavy consumption of betel quid (IARC, 1985). However, the mechanism underlying these oral mucosal diseases is not fully understood.

By using various experimental models it was demonstrated that the areca nut causes mutagenicity, genotoxicity and cytotoxicity (Chiang et al., 2007; Lin et al., 2009). In the present study human lung epithelial A549 cells were used as an *in vitro* model system to understand the toxic mechanism of aqueous extract of areca nut. A549 cells treated with different concentration of areca nut extract show decreased cell viability confirming the previous studies that areca nut were found to be genotoxic to oral epithelial cells. Treatment of areca nut extract induces reactive oxygen species (ROS) and morphological changes in cultured cells (Chang et al., 2001; Lu et al., 2010). Results of our studies also show that areca nut extract induces reactive oxygen species in a dose dependent manner and with significant increase with increase in time. Cells exposed to areca nut extract increases the release of free radicals which may further lead to loss of membrane integrity, results in increased levels of lactate dehydrogenase enzyme.

Areca nut extract treated cells lead to loss of membrane integrity with the leakage of LDH into the medium confirms the toxicity of areca nut. Similar results were shown with loss of membrane integrity and release of LDH due to constant pan masala exposure (Kumari and Dutta, 2013). Increased ROS and LDH leakage followed by formation of acid-reactive substance thiobarbituric acid in human oral keratinocytes when exposed to gutkha and pan masala extracts (Bagchi et al., 2002). Results of our study suggested that the extract of areca nut induces elevated levels of enzyme lipid peroxidase that cause oxidative degradation of membrane lipid leading to cell membrane damage and cell death reflected the decreased cell viability as measured by MTT assay.

In order to provide a defense mechanism besides the excessive production of ROS, the cells exert non-enzymatic and enzymatic systems to neutralize the released free radicals. Non-enzymatic involves the use of Glutathione (GSH), NADPH, cysteine etc., (Amstad and Cerutti, 1990; Kehrer, 1993). The superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) are the three major enzymatic anti-oxidant defense systems responsible for scavenging free radicals and nascent oxygen(Manoharan et al., 2005)**.** 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**.** Result of our present study show that there was decreased enzyme activity of SOD, GPx and GST in areca nut treated A549 cells, is also in agreement with the earlier results in oral cancer patients who were constantly exposed to areca nut where there was decline in the levels of the above enzymes (Gurudath et al., 2012). Further significant decrease in the levels of GST was observed in human buccal mucosal fibroblast with arecoline treatment (Chang et al., 2001).

Although toxicants may initiate cell damage or stress, the cellular proteins that are involved in control of cell cycle and apoptosis are the final arbiters of cell fate. $BCl_{2,}$ the member of a larger family of related proteins prevent cell death, while others like Bax; accelerate it (Duvall and Wyllie, 1986). Results of our study show that there was a significant decrease in the expression of anti-apoptotic gene BCl_2 and marginal increase in the pro-apoptotic Bax gene in areca nut treated A549 cells compared to control. Similar results were shown in human hepatoma cells when treated with arecoline a major alkaloid present in areca nut (Cheng et al., 2010).

DNA damage elicited due to exposure to toxic compound activates repair or stress responsive systems which might trigger cell cycle arrest or apoptosis (Barzilai and Yamamoto, 2004). Cyclin E1, Cyclin D1, CDK4, p53 and Rb are the key regulators of cell cycle, appears to play a major role in cell cycle transition. Results of our study show increased expression of p21 mRNA levels in areca nut extract treated cells. p21 known to regulate the cyclin and

cyclin-dependent kinase activity and also involved in the assembly of CDK4/6-Cyclin D and CDK2-Cyclin E complexes for the transition of cells from G1 to S phase of cell cycle. Further treated A549 cells show decreased mRNA expression of cyclin E1, cyclin D1, CDK4, Rb and p53 when compared control.

Transition of cells from G1 to S phase involves the sequential phosphorylation of Rb in co-ordination with CDK4/6-cyclin D complex for downstream transcriptional activation. Since the levels of cyclin E1, cyclin D1, CDK4 and Rb are down regulated and the levels of p21 are substantially upregulated in areca nut treated cells. The upregulated of p21 could also be an important element underlying the G1 arrest. Probably in our studies the A549 cells try to undergo G1 arrest, where the p21 protein levels is usually undetectable in normal oral mucosa epithelium, whereas its expression is significantly elevated in pre-malignant lesions of betel quid chewers in India (Agarwal et al., 1998). DNA damage can activate p53 to protect cells by inducing target genes. The molecules activated by p53 induce apoptosis, cell cycle arrest and DNA repair to conserve the genome (Blagosklonny, 2002). However, the activation of p53 is reflected by the amount of protein or the phosphorylation level of Ser-15, was not in case of areca nut treatment. Therefore, areca nut damage may bypass the surveillances of p53 and undergo genetic pathogenesis predisposing tumorigenesis also suggested that the activation of p21 by areca nut exposure is p53-independent (Lu et al., 2006). On the other hand, recent studies have shown increased expression of p21 could lead to cell death in certain models, but the mechanism involved in this process are poorly understood. Further certain studies pronounce the induction of apoptosis by p21 in sarcoma cell lines that is p53-independent and can be ameliorated with anti-oxidants (Masgras et al., 2012). Flow cytometric analysis of A549 cells treated with aqueous extract of areca nut show increased apoptosis in a dose dependent manner. Increased expression of p21 which is independent of a p53 probably triggers apoptosis that needs to be investigated. Thus, cell death caused due to areca nut exposure may be through different extrinsic apoptotic pathway via oxidative stress induced p21 which needs to be explored.

To conclude, A549 cells treated with areca nut extract induces ROS and causes membrane damage with the leakage of LDH. Oxidative stress subsequently triggered apoptosis due to depletion in free radical scavenging anti-oxidant enzymes. Further changes in key cell cycle regulators expression in areca nut extract treated cells showed the status of cell cycle processes in toxic environment. Thus our study provides an *in vitro* model system to analyze the role of anti-oxidant molecules such as GSH, N-acetyl cysteine, vitamin C etc. to understand the counter activity and the protagonist of p21 in induction of cell death.

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Conflict of Interest

Authors declare that there are no conflicts of interest.

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Abbreviations:

RPMI, Rosewell Park Memorial Institute media, **FBS** , Fetal bovine serum, **PBS**, phosphate buffered saline, **ROS**, reactive oxygen species, **LPO**, Lipid peroxidation, **LDH**, Lactate dehydrogenase, **GSH-Px**, Glutathione peroxidase, **GST**, Glutathione S transferase, **SOD**, Superoxide dismutase, **OSF**, Oral sub mucosal fibrosis, **OSCC**, Oral squamous cell carcinoma.