Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

Regulation of Jun and Fos AP-1 transcription factors by JNK MAPKs signaling cascade in areca nut extract treated KB cells

Rashmi Nagesh [a,b,*], K.M. Kiran Kumar [d], M. Naveen Kumar [b], Rajeshwari H. Patil [c], S. Chidananda Sharma [a,1]

[a] *Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi, Bengaluru, 560 056, Karnataka, India*
[b] *Department of Biotechnology and Genetics, M S Ramaiah College of Arts Science and Commerce, Bengaluru, 560 054, Karnataka, India*
[c] *Department of Molecular Reproduction Development and Genetics, Indian Institute of Science, Bengaluru, 560 012, Karnataka, India*
[d] *Department of Biotechnology, The Oxford College of Science, Bengaluru, 560 102, Karnataka, India*

A R T I C L E   I N F O

A B S T R A C T

The edible endosperm of *Areca catechu* is recognized as a potent carcinogenic agent either consumed alone or in combination with tobacco. Habitual chewing of areca nut leads to orally potential malignant disorders which are highly effective in malignant transformation and thereby lead to oral carcinogenesis. Human buccal epithelial KB carcinoma cells were used as an experimental cell system to inspect the mechanistic act of aqueous extract of areca nut on biochemical status and their implications on transcriptional activation of cancer signaling cascade that could possibly trigger numerous oncogenic players and finally decides the cells fate. Extract treated cells showed reduced viability with altered balance between oxidants and antioxidants which lead to redox status and which is known to distort various biological processes within the cell system. Results of RT-PCR demonstrated decreased expression of BCl2, cell cycle regulators along with Activator Protein −1 (AP-1) components. While Bax, p16 and p21 mRNAs showed increased expression in extract treated KB cells. Likewise, the translational levels of proliferation cell nuclear antigen (PCNA), tumor suppressor p53, retinoblastoma (Rb) and cyclin dependent kinase 4 (CDK4) were decreased along with AP-1 subunits (c-Jun/c-Fos) with increased protein levels of p21 in extract treated KB cells. Further, the downstream activation and regulation of AP-1 transcription factors could be through stress activated c-Jun – N terminal Kinase (JNK) Mitogen Activated Protein Kinases (MAPKs) which downregulated both Jun and Fos mRNA transcripts in areca nut extract exposed KB cells. Thus, outcome of the study provides insights into mechanistic path of pathogenesis of areca related disorders. Further, it could aid in designing new therapeutic modalities that specific targets these oncogenic players and help in disease management.

1. Introduction

Mitogen-Activated Protein Kinases (MAPKs), a class of protein kinases that auto phosphorylate their own serine and threonine residues or phosphorylate their transcriptional substrates, to activate or to suppress their downstream target genes which are actively involved in modulation of numerous biological progression [1]. There are ubiquitously expressed and evolutionarily conserved in eukaryotes and are involved in signaling cascade that modulate physiological and pathophysiological cellular responses [2]. MAPKs are known to regulate key cellular processes such as proliferation, stress responses, apoptosis, and immune defense [3]. Extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 isoforms of MAPKs are grouped according to their activation motif, structure, and function [4]. Growth factors, hormones and proinflammatory cytokines activate ERK, whereas JNK and p38 MAPKs are activated by cellular and environmental stressors [4, 5]. Further MAP Kinases also regulate numerous transcription factor that are stimulated either by mitogens, growth factor, cellular or

---

environmental stress factors.

Likewise, Activator Protein −1 (AP-1) is one such transcription factor that is activated and regulated by MAP Kinases. AP-1, a mammalian dimeric transcription factor composed of Jun, Fos or Activating Transcription Factor (ATF) subunits, often binds to 12-O-tetradecanoyl-phorbol 13-acetate (TPA) responsive elements as homo or hetero dimers and brings about various biological processes [6]. AP-1 activity is regulated by a broad range of physiological and pathological stimuli, including cytokines, growth factors, stress signals, infections, as well as on oncogenic stimuli [7]. The oncogenic subunit of AP-1 gets activated by upstream mitogen activated protein kinases upon mitogenic activation, growth factors, stress and so on [8]. Further components of MAPKs i.e., ERK regulate the expression of c-Fos [9], JNK tightly regulates the levels of c-Jun [10] and p38 MAPKs are known to modulate the act of Jun and Fos subunits of AP-1 [11,12]. Since AP-1 and its upstream regulator mitogen activated protein kinase are activated upon cellular stress. The present investigation discusses about the regulatory activation of AP-1 via MAPKs on treatment with aqueous areca nut extract.

Areca nut is a common masticator which is often used alone or in combination with betel leaf, slaked lime with or without tobacco [13]. Habitual chewing of areca nut with or without tobacco is known to cause harmful effects on tissues of oral cavity. Chronic usage of areca nut will lead to infectious lesions that lead to various oral disorders and could transform to oral carcinogenesis, collectively called as orally potential malignant disorders (OPMDs) [14,15]. Areca alkaloid arecoline is a significant etiological factor that is responsible to cause cytotoxicity and genotoxicity in various cell system [16,17]. Various active constituents of areca nut extract have been reported to cause decreased viability with increased generation of free radicals [18]. This would cause imbalance in cellular homeostasis and could disrupt the normal functioning of various cellular processes like proliferation, growth and transformation. The distortion in the activity of oxidants and antioxidants which further influences the downstream stimulation of transcription factors that modulate numerous cellular acts that finally decides the cells fate are to be studied in detail. Hence, the present investigation helps in better understanding the lethal effects of aqueous areca nut extract on the human buccal KB cells. Further, the toxic implications caused on treatment of areca nut extract on the biochemical status of *in vitro* cells are to be addressed. Additional, the study aids to comprehend the influence of stress on treatment of areca nut extract on downstream activity of AP-1 subunits by using specific inhibitors of MAPK components to elucidate the signaling cascade that could possibly be involved in pathological status of numerous oral disorder which could contribute to the incidence of oral malignancy.

2. Material and methods

2.1. In vitro cell culturing of human buccal cancer cells

Buccal KB cells were procured from National Centre for Cell Science, Pune and were sub-cultured into roux cell culture flask in 1:3 spilt ratio and were placed in CO_2 incubator at 37 °C as per standardized culture conditions described in earlier protocol [19]. The confluent flask containing cells was further sub-cultured into 96 and 6 well culture dish for performing other experiments on treatment with aqueous areca nut extract (ANE).

2.2. Preparation of stock solutions of ANE

Commercially available processed nut was purchased from Sirsi, Uttar Kannada district, Karnataka (Largest producer of areca nut in India). Finely powdered nuts were subjected for extraction of aqueous extract as per earlier protocol [20]. The concentration of prepared areca nut extract was 10 % (main stock) which was further diluted to obtain different concentrations (0.1–1.0 %) which were used for treatment.

2.3. Cell viability (MTT) and Reactive Oxygen Species (ROS) assay

Briefly 3×10^3 cells/well of buccal KB cells were cultured in a 96-well culture plate and incubated with different concentration of ANE (0.1–1.0 %) for 48 h to determine the cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Himedia, India) [21]. The percentage of viable cells was directly proportional to the optical density (at 540 nm) of purple colored formazan formed. Estimation of reactive oxygen species was carried out by dichlorofluorescin diacetate (DCFDA) method (Himedia, India) from 0 to 15 min soon after the treatment of areca nut extract as per earlier protocol [22]. Free radicals produced in cells on exposure of ANE was represented as percentage of fluorescent intensity at excitation (485 nm) and emission (527 nm) wavelength.

2.4. Lipid peroxidation (LPO) and Glutathione Peroxidase (GSH-Px) assay

KB cells on treatment with aqueous areca nut extract of different concentrations (0.1–1.0 %) for 24 h were subjected for assessment of acid reactive malondialdehyde (MDA) as per earlier protocol [50] and for the enzyme activity of GSH-Px as per standard procedure [23]. The extract treated KB cells were lysed and equal amount of protein in lysate was further subjected to assess LPO and GSH-Px activity. The optical density of acid reactive MDA was read at 532 nm. Further, the rate of formation of oxidized glutathione (GSSG) was analyzed by reduction in the optical density at 340 nm using spectrophotometry.

2.5. Lactate dehydrogenase (LDH) and Glutathione S Transferase (GST) analysis

Leakage of lactate dehydrogenase to the cell free supernatant was evaluated as per earlier protocol [24]. The spent media from extract treated cells was collected and analyzed for quantifying LDH at 490 nm. The absorbance of red formazan formed was directly proportional to the amount of LDH released into the culture media. GST, potent antioxidant often engaged in reducing the excessively liberated free radicals and counteract against the oxidative burden. GST enzyme activity was estimated as per standard procedure [25]. The cell lysates of areca nut extract treated cell samples were assessed for GST activity and the reduction in activity of GST were read at 340 nm. GST enzyme activity is expressed as μM of GST enzyme conjugates/min/mg protein.

2.6. RNA extraction and RT-PCR studies

Buccal cells were cultured in a 6-well plate and was incubated with or without ANE (0.35 %) or inhibitors of ERK, JNK and p38 in combination with ANE for 48 h. Further, RNA was extracted from control and treated cell samples using TRIzol reagent as per manufacturer's instructions (Sigma Aldrich, USA). The obtained RNA samples were quantified and subjected to reverse transcription and PCR analysis as per earlier protocol [26]. 2 μg of RNA from different samples were reverse transcribed by using superscript reverse transcriptase (Invitrogen, India). The synthesized cDNA was subjected to 30 cycles of PCR in a gradient Eppendorf thermo cycler using different forward and reverse primers of apoptosis, cell cycle regulators [19] and AP-1 factors [20]. β-actin was used as a house keeping gene and a positive control for normalization. Amplified PCR products were analyzed on agarose gel electrophoresis.

2.7. Western blotting

Total protein samples from ANE treated and control cells were extracted and assessed by Western blotting as per the earlier protocol [27]. The extracted protein's concentration were determined as per standard procedure [28]. Further, 60 μg of protein sample from different
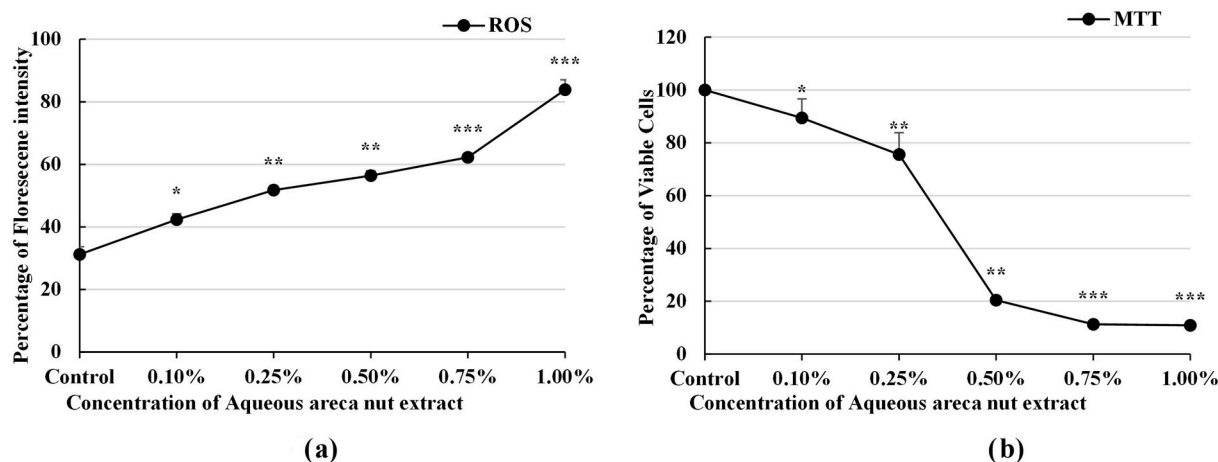
(a)

(b)

Fig. 1(a&b). Generation of ROS and its consequences on viability of KB cells: Results were stated as (a) % fluorescence intensity (ROS) and (b) % of viable cells (MTT) as compared to control (mean ± SD, n = 4). The represented data was statistically significant distinct from control group if *P < 0.05, **P < 0.01 and ***P < 0.001 by performing student's t-test.

treated and untreated control samples were subjected to SDS-PAGE followed by transfer on to PVDF membrane electrophoretically. The blotted membrane was blocked with 5 % Carnation fat-free milk at room temperature for 1 h followed by treatment of primary antibody (PCNA, CDK4, RB, p53, p21, c-Jun or c-Fos) in blocking solution (1:1000) (Neo Biolab, USA) for overnight, then washed in TBST for twice or thrice and later incubated with anti-rabbit/mouse secondary antibody-HRP (1:1000) (Merck-Millipore, India) for 2 h at room temperature. Immunoreactive proteins bands were visualized using Luminata Forte Western HRP substrate as per the requirements provided by the supplier in a Syngene Gel Documentation system (MD, USA). Immunoreactive bands were quantified using image analysis software (ImageJ). GAPDH was used as a positive control and for normalization.

2.8. Statistical analysis

Statistical analysis for cell viability/oxidant and antioxidant assays was performed by Student's t-test. Statistical analysis for RT-PCR and blotting analysis was performed by using one-way ANOVA followed by post hoc Tukey test. Experimental data were represented as mean ± SD from at least three independent experiments. Difference between control and ANE treated cell samples were considered significant if the level was

*P < 0.05, **P < 0.01 and ***P < 0.001 for Student's t-test while *P < 0.05 for one-way ANOVA.

3. Results

3.1. Effect of ANE on cell viability, oxidant, and anti-oxidant levels in buccal KB cells

3.1.1. ANE amplified the liberation of free radicals with reduction in viability of KB cells

Buccal cells ~~on~~ were treated with different concentration of ANE to determine cell viability by MTT assay and ROS generation by DCFDA method. Results of the study showed increase in concentration of ANE increased the generation of free radicals with decreased viability of KB cells when compared to untreated control. Maximum of 2.7-fold increase in generation of ROS was observed at higher concentration of ANE (1%) (Fig. 1a) with 90% reduction in viability of KB cells (Fig. 1b). The 50% inhibitory concentration was recorded at 0.35% ANE in buccal KB cells. Therefore, the cytotoxic nature of areca nut extract resulted in elicitation of free radicals that could be responsible for decreased cell viability of human buccal KB cells.
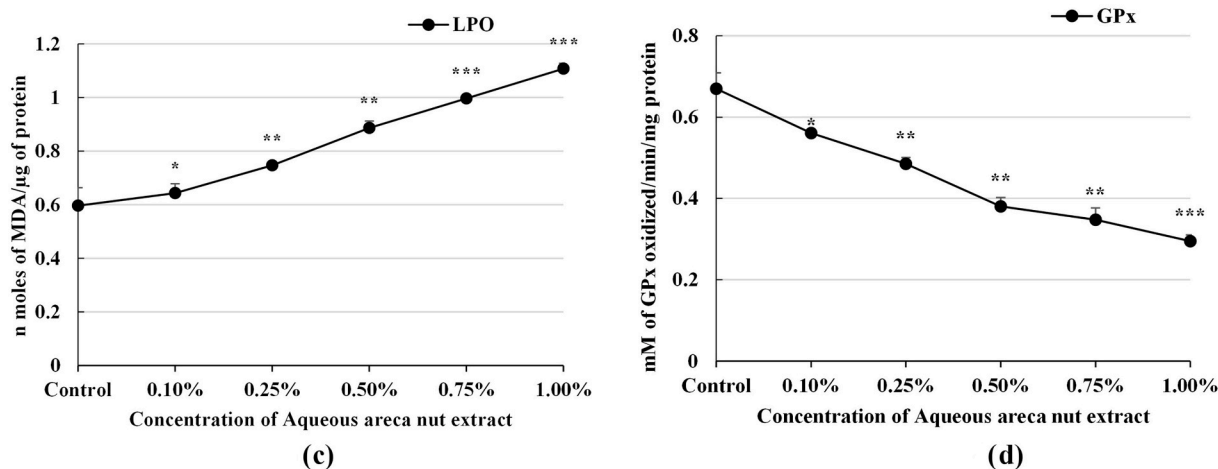

(c)

(d)

Fig. 1(c&d). Depicts the level of lipid peroxidation and the GPx activity in ANE treated KB cells: (c) The levels of lipid peroxidation is expressed as n moles of MDA/μg of protein and (d) GPx enzyme activity is expressed as mM of glutathione peroxidase oxidized/min/mg protein. The OD values were represented as mean ± SD (n = 3). The represented data was statistically significant distinct from control group if *P < 0.05, **P < 0.01 and ***P < 0.001 by performing student's t-test.
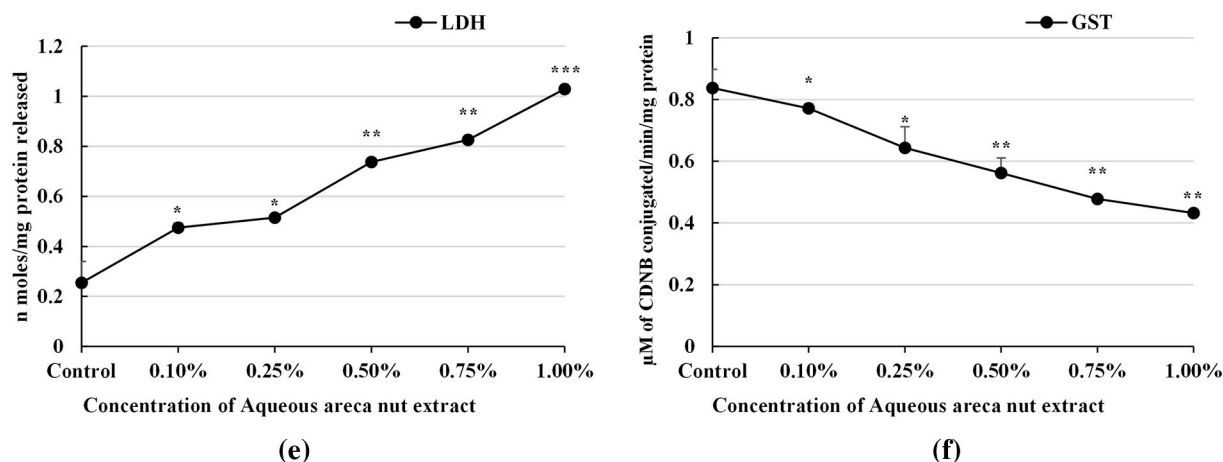
3

(e)

(f)

Fig. 1(e&f). Displays increased leakage of LDH with diminished activity of GST in ANE treated KB cells: Results of (e) LDH is expressed as n moles/mg protein and (f) GST activity as μM of Chloro Dinitro Benzene conjugated/min/mg protein as compared to control. The OD values were represented as mean ± SD (n = 3). The represented data was statistically significant distinct from control group if *P < 0.05, **P < 0.01 and ***P < 0.001 by performing student's t-test.
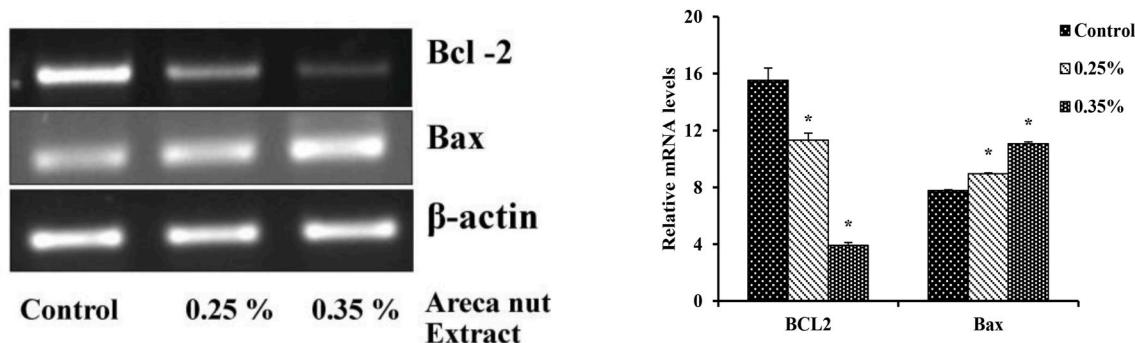

Fig. 2a. Indicates the mRNA expression of apoptotic genes in ANE treated buccal cells (Reverse transcription PCR): Data presented are mean ± SD (n = 3) and changes in mRNA expression of apoptotic genes are statistically significant, if *P < 0.05 when compared with control and was evaluated using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.

3.1.2. Decreased enzymatic functioning of GPx augmented lipid peroxidation on ANE exposure

ANE treated KB cells were subjected to LPO and GPx assay to determine the levels of lipid peroxides and the activity of GPx, respectively. Results showed increased malondialdehyde (MDA) levels along with decreased enzyme activity of glutathione peroxidase with increasing concentration of aqueous areca nut extract. About 1.8-fold rise in formation of MDA was observed at 1% concentration of ANE as compared to control (Fig. 1c). While a maximum of 57% decline in the action of glutathione peroxidase enzyme was recorded at 1 % concentration of ANE when compared to untreated control sample (Fig. 1d). Thus, results of the study confirms the lethal effect of ANE in KB cell with increased generation of free radicals that lead to increased degradation of lipid moieties which could be due to lack of antioxidant defensive action of GPx in detoxifying the liberated free radicals.

3.1.3. Loss of membrane integrity with decreased GST activity in ANE treated buccal cells

ANE exposed buccal KB cells were assessed for loss of membrane integrity and GST activity. Results of the study showed that ANE treated cells showed increased leakage of LDH into culture supernatant with a maximum of 4-fold increase in leakage of LDH was noted at higher concentration of ANE (1%) as compared to control (Fig. 1e). While the enzyme activity of GST was found to be decreased with increasing concentration of areca nut extract. A maximum of 52% decrease in activity of GST was observed at 1% areca nut extract as compared to control (Fig. 1f). Further results of the study depicts the detrimental

effect of ANE on KB cells with increased leakage of LDH that may cause increased membrane damage due to excessive production of free radicals as a result of decreased activity of potent antioxidant (GST) that could possibly reduce the viability of KB cells.

3.2. Impact on mRNA levels of aqueous areca nut extract on apoptosis and cell cycle progression

3.2.1. ANE induced apoptosis by decreased mRNA levels of anti-apoptotic BCL2 in KB cells

The mRNA expression of anti and pro apoptotic genes in ANE treated KB cells were analyzed by RT-PCR studies. Outcome of the study demonstrated that 0.25 and 0.35 % ANE showed decreased mRNA expression of BCL2 by 28 and 75 % respectively. Wherein the pro-apoptotic Bax showed a marginal increase in the level of mRNA expression when compared to control (Fig. 2a). This altered ratio between anti and pro apoptotic proteins BCL2/Bax could be due to the lethal effect of areca nut extract that has driven the cells to undergo the programmed cell death.

3.2.2. Down regulated expression of Cyclin and its dependent kinases in ANE treated KB cells

The mRNA expression of Cyclin E1, Cyclin D1, CDK4, Rb, E2F, p53, p21 and p16 on treatment with ANE was examined by RT-PCR studies. Results of the study showed that aqueous areca nut extract (at concentrations 0.25 and 0.35 %) treated KB cells showed decreased mRNA levels of cyclin E1 by 44 and 54%, cyclin D1 by 72 and 78%, CDK4 by 18
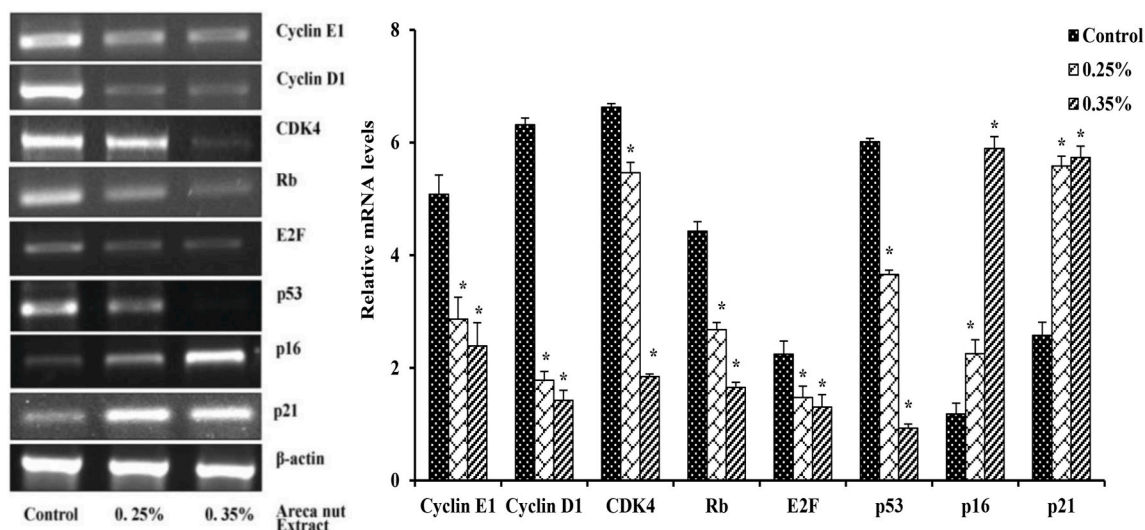
Fig. 2b. Represents the effect of ANE on the expression of cell cycle regulators in KB cells (Reverse transcription PCR): Data shown are mean ± SD (n = 3) and differences in mRNA levels of cell cycle regulators are statistically significant, if *P < 0.05 when compared to control, as analyzed using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.
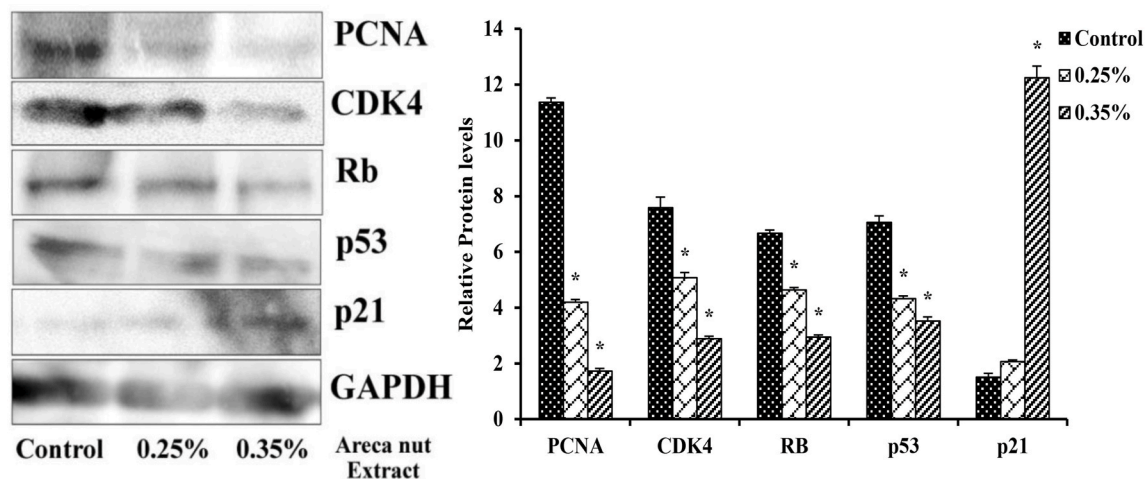

Fig. 2c. Shows the implications of ANE on protein levels of cell cycle regulators in KB cells (Western Blotting): Data shown are mean ± SD (n = 3) and differences in protein levels of cell cycle regulators are statistically significant: if *P < 0.05 compared with control using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.

and 73%, Rb by 40 and 63%, E2F by 35 and 42% and p53 by 40 and 85% respectively when compared to untreated control cell samples. While the mRNA level of p16 (1.9 and 5.0-fold) and p21 (2.1 and 2.2-fold) was found to be significantly upregulated in KB cells on treatment with 0.25 and 0.35 % ANE respectively (Fig. 2b). Thereby results of the study showed the increased expression of CDK inhibitor p16 and p21 which could possibly trigger G1/S cell cycle arrest by downregulating the cell cycle regulators which are involved in transition of G1/S phase in ANE treated KB cells.

3.2.3. Decreased protein levels of major cell cycle regulators on ANE treatment in KB cells

Cells were treated with different concentrations of aqueous areca nut extract for 48 h and were analyzed for protein levels of PCNA, CDK4, RB, p53 and p21 by western blotting. KB cells on treatment with ANE (0.25 and 0.35%) showed decreased protein levels of PCNA by 64 and 85%, CDK4 by 34 and 63%, RB by 31 and 57% and p53 by 39 and 51% respectively in a dose dependent manner as compared to control. While the protein levels of p21 were found to be significantly increased by 2.5-

fold at 0.35 % concentration of aqueous areca nut extract in treated KB cells as compared to control (Fig. 2c). Thereby, the results of mRNA transcripts were in accordance with protein levels which in turn confirms the detrimental effect of aqueous areca nut extract in triggering G1/S phase cell cycle arrest and may drive the cells to undergo apoptosis.

3.3. Effect of areca nut extract on mRNA and protein levels of AP-1 transcription factors

3.3.1. ANE downregulated the mRNA transcripts of Jun/Fos AP-1 factors in KB cells

The mRNA levels Jun and Fos AP-1 subunits in aqueous areca nut extract treated KB cells were analyzed by RT-PCR. Results of the study showed decreased mRNA expression of Jun and Fos family members of AP-1 factors in a dose dependent manner in treated KB cells as compared to control. There was a significant decrease in mRNA levels of c Jun by 33 and 44%, Jun B by 11 and 29%, c Fos by 34 and 62%, Fra 1 by 40 and 67% and Fra 2 by 40 and 75% with (at concentration 0.25 and 0.35 %)
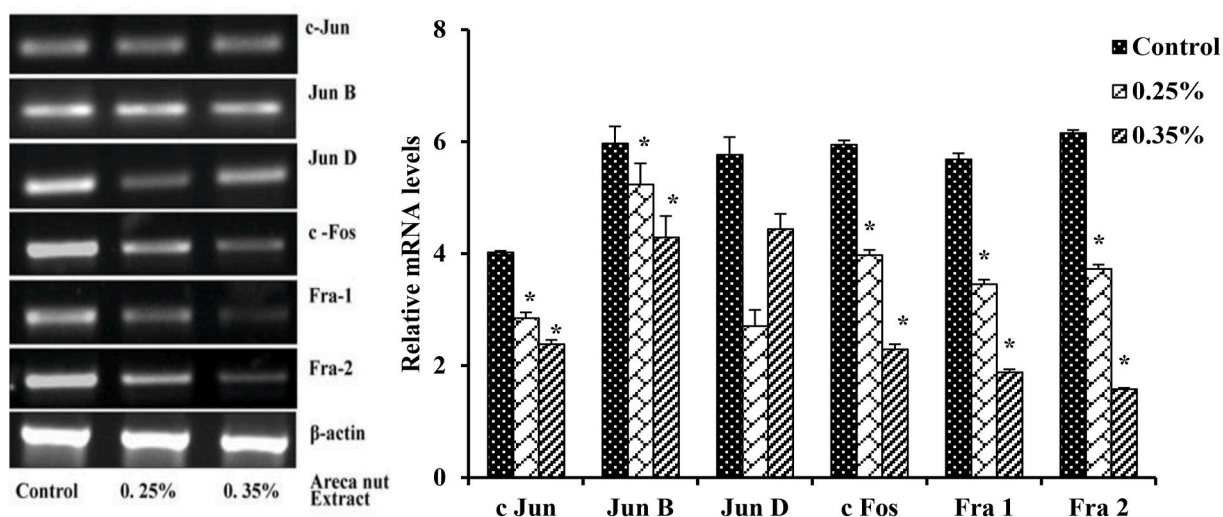
Fig. 3a. Shows the effect of ANE on the mRNA expression of AP-1 factors in KB cells (Reverse transcription PCR): Data shown are mean $\pm$ SD (n = 3) and differences in mRNA levels of AP-1 factors are statistically significant, if $*P < 0.05$ compared with control, as analyzed using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.

Fig. 3b. Blot results shows the effect of ANE on the protein levels of c-Jun and c-Fos AP-1 factors in KB cells (Western Blotting): Data shown are mean $\pm$ SD (n = 3) and differences in protein levels of c- Jun and c- Fos AP-1 factors are statistically significant, if $*P < 0.05$ compared with control, as analyzed using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.
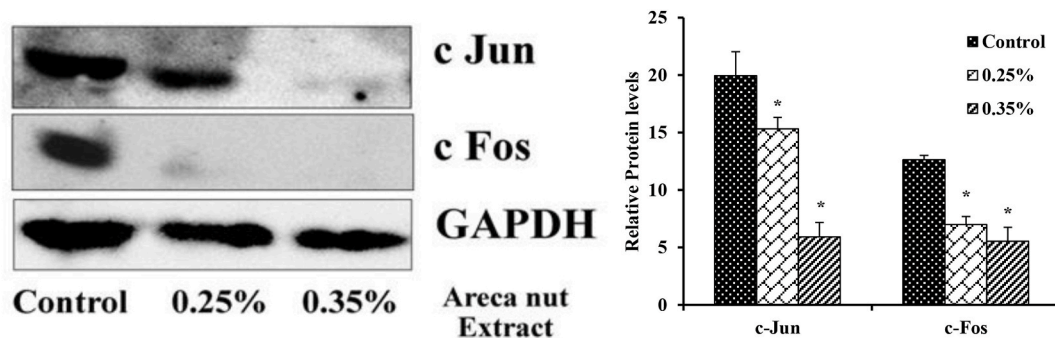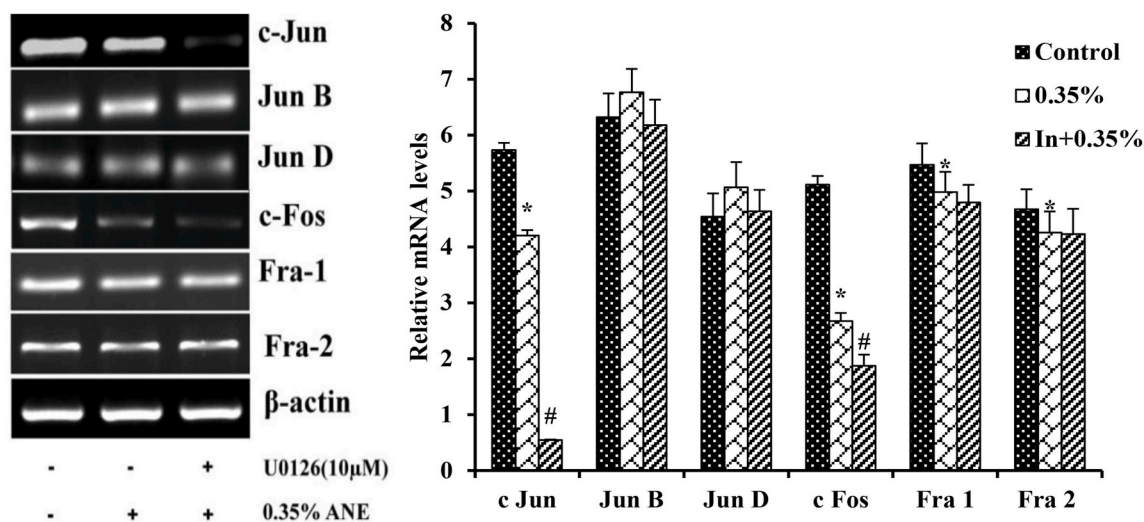

Fig. 4a. Represents the outcome of U0126 on the expression of AP-1 factors in ANE treated KB cells (Reverse transcription PCR): Data shown are mean $\pm$ SD (n = 3) and differences in mRNA levels of AP-1 factors are statistically significant: if $*P < 0.05$ compared with control and $\#P < 0.05$ as compared to areca nut values using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.

Fig. 4b. Effect of SP600125 on the expression of AP-1 factors in aqueous areca nut extract treated KB cells (Reverse transcription PCR): Data shown are mean $\pm$ SD (n = 3) and differences in mRNA levels of AP-1 factors are statistically significant: if *P < 0.05 compared with control and #P < 0.05 as compared to areca nut values using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.
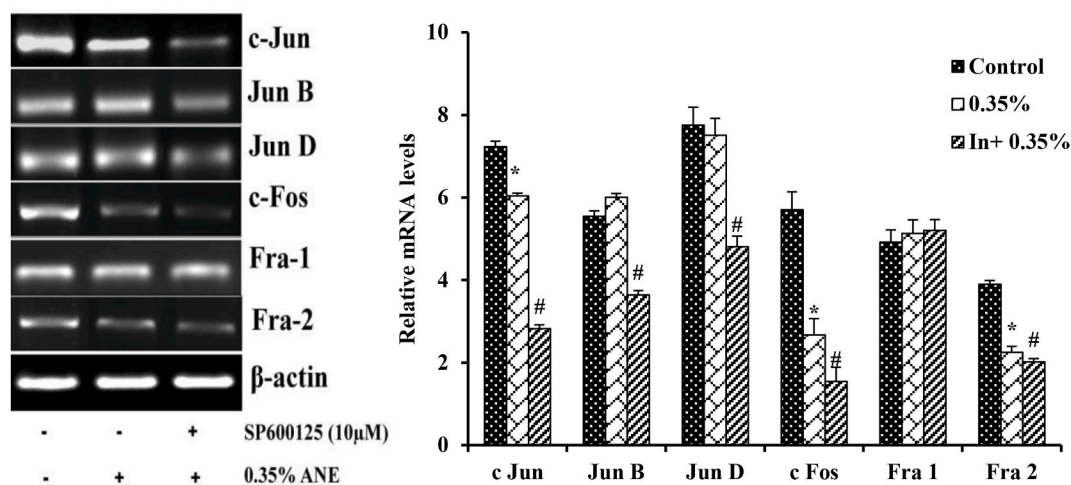
aqueous areca nut extract treated KB cells respectively as compared to control (Fig. 3a). Jun and Fos AP-1 factors are known to be actively involved in regulation of cell growth, proliferation, and death. Thus, RT-PCR studies displayed decreased expression of Jun and Fos AP-1 sub-units that are prime for modulating proliferative status in extract treated cells which exert its lethal effect on KB cells and drive them to undergo apoptosis.

3.3.2. Decreased protein levels of c-Jun and c-Fos AP-1 factors in ANE treated cells

KB cells on treatment with different concentration of ANE was analyzed for protein levels of c-Jun and c-Fos AP-1 subunits by western blotting. Results of the studies showed decreased protein levels of c-Jun and c-Fos in a dose dependent manner on treatment with aqueous areca nut extract in KB cells as compared to control (Fig. 3b). KB cells on treatment with 0.25 and 0.35% aqueous areca nut extract showed downregulated protein levels of c-Jun by 24 and 71 % and c-Fos by 45 and 57% respectively as compared to control. Results of the study are in accordance with that of mRNA levels of AP-1 factor, with decreased protein levels of c-Jun and c-Fos AP-1 members that are engaged in regulation of various biological processes.

3.4. Role of MAPK in activation of AP-1 transcriptional factor

3.4.1. Effect of ERK inhibitor (U0126) on the mRNA expression of AP-1 factors in ANE treated KB cells

KB cells were pretreated with U0126 inhibitor for 1 h before treatment with ANE for 48 h and were compared with cells treated only with areca nut extract to study the role of ERK pathway on downstream expression pattern of AP-1 subunits. Cells on treatment with (0.35 %) areca nut extract showed significant decrease in the mRNA expression of c-Jun by 37 %, c-Fos by 48 % and Fra 1 by 10 % as compared to control. KB cells on treatment in combination with U0126 along with 0.35 % areca nut extract showed a significant decrease in mRNA levels of c-Jun by 87 %, Jun D by 23% and c-Fos by 30 % as compared to areca nut treated KB cells (Fig. 4a)

3.4.2. Effect of JNK inhibitor (SP600125) on the mRNA expression of AP-1 factors in ANE treated KB cells

KB cells were pretreated with SP600125 inhibitor for 1 h before treatment with aqueous areca nut extract for 48 h and compared with cells treated only with areca nut extract to study the role of JNK pathway on downstream expression pattern of AP-1 subunits. Cells treated with
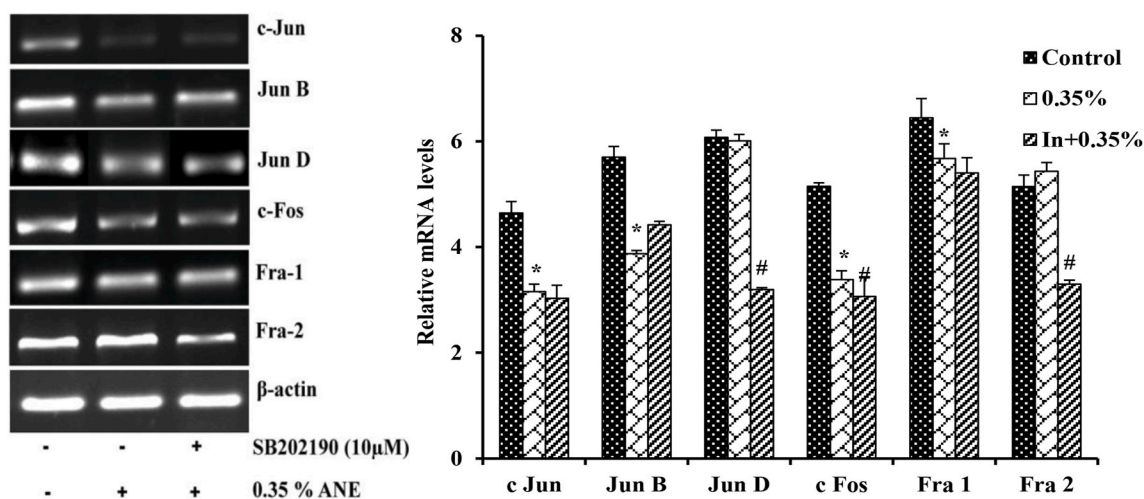

Fig. 4c. Effect of SB202190 on the expression of AP-1 factors in aqueous areca nut extract treated KB cells (Reverse transcription PCR): Data shown are mean $\pm$ SD (n = 3) and differences in mRNA levels of AP-1 factors are statistically significant: if *P < 0.05 compared with control and #P < 0.05 as compared to areca nut values using one-way ANOVA followed by post hoc Tukey test. The bar graph represents their respective densitometric analysis.

0.35 % areca nut extract showed prominent decrease in mRNA expression of c-Jun by 20 %, c-Fos by 74 % and Fra-2 by 43 % subunits of AP-1 as compared control. Cells on treatment in combination with SP600125 along with areca nut extract; showed significant downregulated mRNA levels of c-Jun by 53 %, Jun B and Jun D by 41 %, c-Fos by 47 % and Fra-2 by 11 % as compared to areca nut extract treated cells (Fig. 4b).

3.4.3. Effect of p38 inhibitor (SB202190) on mRNA levels of AP-1 factors in ANE treated KB cells

KB cells were pretreated with SB202190 inhibitor for 1 h before treatment with aqueous areca nut extract for 48 h and compared with cells treated only with areca nut extract to study the role of p38 pathway on downstream expression pattern of AP-1 subunits. Cells treated with 0.35 % areca nut extract showed significant decrease in the mRNA expression of c Jun by 37 %, Jun B by 27 %, and c Fos by 41 % as compared control. Cells on treatment in combination with SB202190 along with areca nut extract showed significant decrease in mRNA levels of Jun D by 51 %, c-Fos by 11 %, and Fra 2 by 42 % as compared to areca nut extract treated KB cells (Fig. 4c).

4. Discussion

Areca catechu, a popularly known masticator and a psychoactive substance [29] often used in numerous geographically locations in and around the world specially in Asian subcontinent from centuries [30]. It is reported to possess several medicinal properties and has been used in traditional Indian medicine for managing several ailments [31]. Besides habitual chewing of betel nut it is also reported to be connected with the development of several pathologies associated with the oral cavity including the development of malignant conditions [16,17]. The International agency for Research on Cancer (IARC) has categorized areca nut as a Group I carcinogen [32] as areca nut chewing along with tobacco, piper betel leaf along with lime or alone has recognized areca nut as a major etiological factor in advancement of oral carcinogenesis [33]. Although several studies report the beneficial/detrimental effects of areca nut, studies pertaining to its toxicity and its detailed mechanistic act are scarce. Most of the reports on areca nut indicate the lethal effect on prolonged exposure of the nut at low to moderate concentration. Present study efforts to examine the toxicological implications of areca nut especially on exposure of small to high-level dose for a shorter incubation time has not clearly understood. Hence, the current study is an attempt to understand the effects of short-term exposure to a high-level dose of ANE on appropriate cell culture model. Human buccal KB cells were used as model system to inspect the toxicological effects of ANE and their implications on biochemical status which would further bring about alterations in various biological processes such as cell cycle control and apoptosis.

Results of cell viability and ROS assay displayed decreased viability of KB cells with induced production of reactive oxygen species thereby confirms the lethal effect of ANE with that of untreated control group. Similar annotations were observed in many experimental models that exhibited increased generation of ROS [34] with induced cytotoxicity and genotoxicity on ANE exposure [35,36]. Excessive production of free radicals was responsible for increased lipid peroxidation with high levels of acid reactive malondialdehyde levels which could be a consequence of decreased activity of detoxifying antioxidant GPx in ANE treated KB cells. These findings were in accordance with previous tryouts that showed enhanced liberation of acid-reactive MDA in human oral keratinocytes with amplified ROS on exposure of gutkha and pan masala extracts [37]. Lipid degradation led to membrane destruction with consequential seepage of lactate dehydrogenase to culture supernatant along with decreased enzyme activity of GST which further ensued in reduced viability of buccal KB cells on treatment of areca nut extract. Earlier studies of Gurunathan et al. (2013) and Kumari and Dutta (2013) marked the increased generation of free radicals with loss of membrane integrity due to persistent exposure of gutkha and pan masala in oral

keratinocytes. Similarly, oral cancer patients who were frequently subjected to betel nut chewing showed deteriorated readings of the antioxidant enzymes [38]. Aqueous areca nut extract exhibited altered balance between production and elimination of liberated free radicals that prompted redox status in buccal KB cells which could affect both at transcriptional as well as translational levels and may be responsible for modulation of several cellular targets which actively participate in regulation of various biological functions like proliferation, growth, transformation, apoptosis and so on.

The consequence caused on treatment of aqueous areca nut extract in KB cells demonstrated decreased mRNA levels of anti-apoptotic BCL2 with increased expression of pro-apoptotic Bax as compared to control. Therefore, altered the ratio of Bax/BCL2 triggered the cells to undergo apoptosis. Earlier studies carried out in human oral and tongue squamous cell carcinoma (SCC), HSC-2 and HSC-3 and HaCaT and Hel (human erythroleukemia) cells exhibited the induction of apoptosis on treatment with areca nut extract [39] with altered Bax/Bcl$_2$ ratio. Apoptotic events could be due to an irreversible arrest in cell cycle progression. Similarly, ANE treated buccal epithelial cells underwent cell cycle arrest at G1/S phase with decreased mRNA expression of Cyclin D1, Cyclin E1, CDK4, RB, E2F and p53 with increased expression of p16 and p21 which take part in transition of cells from G1 to S phase of the cell cycle. Additionally, translational levels of PCNA, CDK4, RB and p53 were downregulated along with increased protein expression of p21 in ANE treated KB cells. Zhou et al. [40], reported HaCaT cells indicated lowered mRNA and blot readings of Cyclin D1, CDK4 and E2F the major cell cycle regulators that initiate cell cycle arrest at G1/S phase on areca alkaloid arecoline exposure. Parallel results were observed in oral carcinoma 3 cells upon areca nut extract exposure for 24 h which displayed G1/S phase of cell cycle arrest [41]. The results of the study reveal that the G1/S phase arrest of cell cycle could be as a due to upregulated levels of p21 and p16 CDK inhibitor. The upstream activation of p21 may be independent of p53 in buccal KB cells on ANE treatment. Comparable findings were noticed in A549 lung cells on treatment with ANE exhibited cell cycle arrest at G1/S phase with increased p21 levels with decreased functioning of p53 [19].

The regulation of downstream apoptotic and cell cycle regulators is modulated by Activator Protein-1 transcription factor that are responsible for carrying out various cellular functions. Activator Protein-1 factor encode genes that behave as "immediate or early" genes and are quickly transcribed in response to various extracellular stimuli, such as growth factor [42,43] and internal and external stressors [44,45]. However, the present study was interested to record the results at 48 h time interval and with IC50 and a dose less than IC50 value of areca nut extract exposure to study the responsiveness of the cell system in terms of induction or repression of AP-1 activity. The research showed reduced expression of AP-1 transcripts along with reduced translational levels of c-Jun and c-Fos on exposure of ANE in buccal KB cells. Thereby, the reduced activity of c-Jun AP-1 inhibited the mRNA transcripts of cyclin D1, tumor suppressor p53 with increased levels of CDK inhibitor p21 which play a critical role in arresting buccal cells at G1/S phase of cell cycle on treatment of ANE.

Additionally, these AP-1 factors are activated and regulated by upstream MAPKs. To explicate the activation of AP-1 was through MAPKs specific inhibitors of ERK, JNK and p38 MAPKs were used along with aqueous areca nut extract to the study mRNA expression pattern of Jun and Fos AP-1 subunits. The results of the study showed reduced mRNA levels of almost all the AP-1 subunits on treatment of JNK inhibitor alone and in combination with aqueous areca nut extract in KB cells when compared to cells on treatment with ERK and p38 MAPK and areca nut extract. Areca nut extract exhibited pleiotropic effects on many biological systems. Reports have confirmed the activation of JNK by induced ROS [46]. Pant et al. reported the activation TGF-β pathway in oral submucous fibrosis an areca related oral disorder is induced through JNK/ATF2/Jun axis [47]. Further, areca nut extract treatment SAS and OC3 cells showed low and transient JNK activation [41]. Thereby, ROS
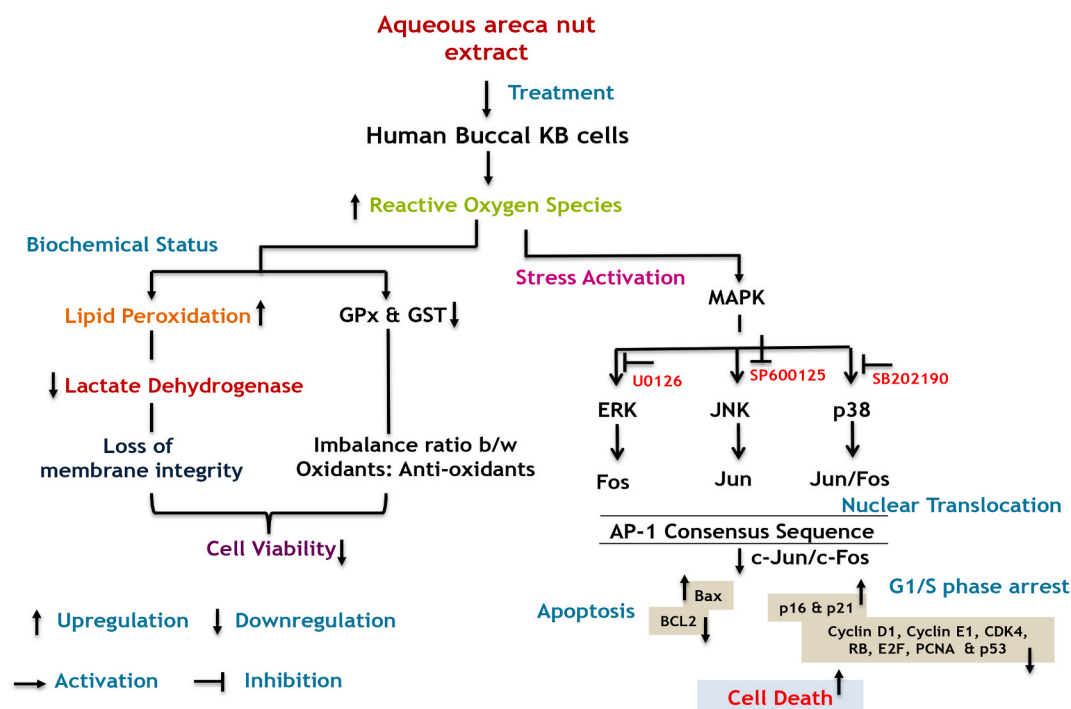
Fig. 5. Proposed mechanistic act of aqueous areca nut extract on human buccal KB cells.

act as a potent signaling molecule which aid in activation of numerous signaling cascade which could act as a double-edged sword that demonstrated both beneficial and harmful effects. Additionally, JNK and p38 MAPKs and their targets when deregulated plays a crucial role in malignant transformation which could be the possible trigger in the present study and numerous reports are in correspondence with the above results [48]. Further, these oncogenic players which are downstream targets of JNK MAPKs could possibly be the therapeutic targets for cure of various cancer forms in the near future [49].

5. Conclusion

The research investigation illuminates the act of aqueous areca nut extract on buccal KB cells. The study provides inference on toxic effect of aqueous areca nut extract on biochemical parameters that could influence various transcriptional factors which regulate their respective target genes which works in a coordinated manner to perform various cellular functions and thereby decides the fate of the cell (Fig. 5). Further, the experimental analysis aids in understanding the pathological state of various oral disorders caused due to constant usage of areca nut extract and helps in designing new therapeutic modalities that specifically target those marker genes thereby facilitates in better disease management.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101090.

References

[1] W. Peti, R. Page, Molecular basis of MAP kinase regulation, Protein Sci. 22 (2013) 1698–1710, https://doi.org/10.1002/pro.2374.
[2] M. Soares-Silva, F.F. Diniz, G.N. Gomes, D. Bahia, The mitogen-activated protein kinase (MAPK) pathway: role in immune evasion by trypanosomatids, Front. Microbiol. 7 (2016), https://doi.org/10.3389/fmicb.2016.00183.
[3] J.S.C. Arthur, S.C. Ley, Mitogen-activated protein kinases in innate immunity, Nat. Rev. Immunol. 13 (2013) 679–692, https://doi.org/10.1038/nri3495.
[4] D.M. Owens, S.M. Keyse, Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases, Oncogene 26 (2007) 3203–3213, https://doi.org/10.1038/sj.onc.1210412.
[5] J.M. Kyriakis, J. Avruch, Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update, Physiol. Rev. 92 (2012) 689–737, https://doi.org/10.1152/physrev.00028.2011.
[6] M. Karin, Z. Liu, E. Zandi, AP-1 function and regulation, Curr. Opin. Cell Biol. 9 (1997) 240–246, https://doi.org/10.1016/s0955-0674(97)80068-3.
[7] Wagner, AP-1 reviews, Oncogene 20 (2001), https://doi.org/10.1038/sj.onc.1204415, 2333-2333.
[8] A.G. Turjanski, J.P. Vaqué, J.S. Gutkind, MAP kinases and the control of nuclear events, Oncogene 26 (2007) 3240–3253, https://doi.org/10.1038/sj.onc.1210415.
[9] P. Monje, M.J. Marinissen, J.S. Gutkind, Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor, Mol. Cell Biol. 23 (2003) 7030–7043, https://doi.org/10.1128/mcb.23.19.7030-7043.2003.
[10] B.J. Pulverer, J.M. Kyriakis, J. Avruch, E. Nikolakaki, J.R. Woodgett, Phosphorylation of c-jun mediated by MAP kinases, Nature 353 (1991) 670–674, https://doi.org/10.1038/353670a0.
[11] M.J. Marinissen, M. Chiariello, M. Pallante, J.S. Gutkind, A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5, Mol. Cell Biol. 19 (1999) 4289–4301, https://doi.org/10.1128/mcb.19.6.4289.

[12] F. Miralles, G. Posern, A.I. Zaromytidou, R. Treisman, Actin dynamics control SRF activity by regulation of its coactivator MAL, Cell 113 (2003) 329–342, https://doi.org/10.1016/s0092-8674(03)00278-2.

[13] IARC, Betel-quid and areca-nut chewing, IARC Monogr. Eval. Carcinog. Risk Chem. Humans 37 (1985) 137–202.

[14] R. Maher, A.J. Lee, K.A. Warnakulasuriya, J.A. Lewis, N.W. Johnson, Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan, J. Oral Pathol. Med. : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 23 (1994) 65–69.

[15] P.R. Murti, R.B. Bhonsle, P.C. Gupta, D.K. Daftary, J.J. Pindborg, F.S. Mehta, Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing, J. Oral Pathol. Med. : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 24 (1995) 145–152.

[16] R.V. Prabhu, V. Prabhu, L. Chatra, P. Shenai, N. Suvarna, S. Dandekeri, Areca nut and its role in oral submucous fibrosis, Journal of clinical and experimental dentistry 6 (2014) e569–575, https://doi.org/10.4317/jced.51318.

[17] M. Hosein, S. Mohiuddin, N. Fatima, Association between grading of oral submucous fibrosis with frequency and consumption of areca nut and its derivatives in a wide age group: a multi-centric cross sectional study from karachi, Pakistan, Journal of cancer prevention 20 (2015) 216–222, https://doi.org/10.15430/jcp.2015.20.3.216.

[18] K.K. Wary, R.N. Sharan, Cytotoxic and cytostatic effects of arecoline and sodium nitrite on human cells in vitro, Int. J. Canc. 47 (1991) 396–400, https://doi.org/10.1002/ijc.2910470315.

[19] R. Nagesh, K.M. Kiran Kumar, M. Naveen Kumar, R.H. Patil, K. Kavya, R.L. Babu, G.T. Ramesh, S. Chidananda Sharma, Aqueous areca nut extract induces oxidative stress in human lung epithelial A549 cells: probable role of p21 in inducing cell death, Gene Reports 6 (2017) 103–111, https://doi.org/10.1016/j.genrep.2016.12.008.

[20] R. Nagesh, K.M. Kiran Kumar, M. Naveen Kumar, R.H. Patil, S.C. Sharma, Stress activated p38 MAPK regulates cell cycle via AP-1 factors in areca extract exposed human lung epithelial cells, Cytotechnology 71 (2019) 507–520, https://doi.org/10.1007/s10616-019-00297-3.

[21] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[22] K.M. Kiran Kumar, M. Naveen Kumar, R.H. Patil, R. Nagesh, S.M. Hegde, K. Kavya, R.L. Babu, G.T. Ramesh, S.C. Sharma, Cadmium induces oxidative stress and apoptosis in lung epithelial cells, Toxicol. Mech. Methods 26 (2016) 658–666, https://doi.org/10.1080/15376516.2016.1223240.

[23] L. Flohé, W.A. Günzler, [12] Assays of glutathione peroxidase, Methods in Enzymology, Academic Press1984, pp. 114-120.

[24] J. King, A routine method for the estimation of lactic dehydrogenase activity, J. Med. Lab. Technol. 16 (1959) 265–272.

[25] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases, The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.

[26] S.C. Sharma, J.W. Clemens, M.D. Pisarska, J.S. Richards, Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin, Endocrinology 140 (1999) 4320–4334, https://doi.org/10.1210/endo.140.9.6965.

[27] M. Naveen Kumar, R.L. Babu, R.H. Patil, K.M. Kiran Kumar, S.M. Hegde, R. Nagesh, K. Kavya, G.T. Ramesh, S.C. Sharma, C. Srinivas, Protein kinases orchestrate cell cycle regulators in differentiating BeWo choriocarcinoma cells, Mol. Cell. Biochem. 452 (2019) 1–15, https://doi.org/10.1007/s11010-018-3407-8.

[28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[29] R.J. Sullivan, E.H. Hagen, Psychotropic substance-seeking: evolutionary pathology or adaptation? Addiction 97 (2002) 389–400.

[30] IARC, Tobacco habits other than smoking; betel-quid and areca-nut chewing; and some related nitrosamines, IARC Monogr. Eval. Carcinog. Risk Chem. Humans 37 (1985) 1–268. IARC Working Group. Lyon, 23-30 October 1984.

[31] W. Peng, Y.J. Liu, N. Wu, T. Sun, X.Y. He, Y.X. Gao, C.J. Wu, Areca catechu L. (Arecaceae): a review of its traditional uses, botany, phytochemistry, pharmacology and toxicology, J. Ethnopharmacol. 164 (2015) 340–356, https://doi.org/10.1016/j.jep.2015.02.010.

[32] IARC, Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines, IARC Monogr. Eval. Carcinog. Risks Hum. 85 (2004) 1–334.

[33] C.Y. Chou, S.Y. Cheng, J.H. Liu, W.C. Cheng, I.M. Kang, Y.H. Tseng, C.M. Shih, W. Chen, Association between betel-nut chewing and chronic kidney disease in men, Publ. Health Nutr. 12 (2009) 723–727, https://doi.org/10.1017/S1368980008003339.

[34] W.T. Ji, S.R. Yang, J.Y. Chen, Y.P. Cheng, Y.R. Lee, M.K. Chiang, H.R. Chen, Arecoline downregulates levels of p21 and p27 through the reactive oxygen species/mTOR complex 1 pathway and may contribute to oral squamous cell carcinoma, Canc. Sci. 103 (2012) 1221–1229, https://doi.org/10.1111/j.1349-7006.2012.02294.x.

[35] S.L. Chiang, S.S. Jiang, Y.J. Wang, H.C. Chiang, P.H. Chen, H.P. Tu, K.Y. Ho, Y.S. Tsai, I.S. Chang, Y.C. Ko, Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling, Toxicol. Sci. : an official journal of the Society of Toxicology 100 (2007) 66–74, https://doi.org/10.1093/toxsci/kfm201.

[36] C.C. Lin, M.C. Chang, H.H. Chang, T.M. Wang, W.Y. Tseng, T.F. Tai, H.W. Yeh, T.T. Yang, L.J. Hahn, J.H. Jeng, Areca nut-induced micronuclei and cytokinesis failure in Chinese hamster ovary cells is related to reactive oxygen species production and actin filament deregulation, Environ. Mol. Mutagen. 50 (2009) 367–374, https://doi.org/10.1002/em.20463.

[37] M. Bagchi, J. Balmoori, D. Bagchi, S.J. Stohs, J. Chakrabarti, D.K. Das, Role of reactive oxygen species in the development of cytotoxicity with various forms of chewing tobacco and pan masala, Toxicology 179 (2002) 247–255, https://doi.org/10.1016/S0300-483x(02)00357-8. Pii S0300-483x(02)00357-8.

[38] S. Gurudath, K. Ganapathy, S. D, A. Pai, S. Ballal, A. Ml, Estimation of superoxide dismutase and glutathione peroxidase in oral submucous fibrosis, oral leukoplakia and oral cancer–a comparative study, Asian Pac. J. Cancer Prev. APJCP : Asian Pac. J. Cancer Prev. APJCP 13 (2012) 4409–4412.

[39] L. Sari, G. Subita, E. Auerkari, Areca Nut Extract Demonstrated Apoptosis-Inducing Mechanism by Increased Caspase-3 Activities on Oral Squamous Cell Carcinoma [version 1; Referees: 2 Approved with Reservations, 2018, https://doi.org/10.12688/f1000research.14856.1. F1000Research 7.

[40] Z.S. Zhou, M. Li, F. Gao, J.Y. Peng, H.B. Xiao, L.X. Dai, S.R. Lin, R. Zhang, L.Y. Jin, Arecoline suppresses HaCaT cell proliferation through cell cycle regulatory molecules, Oncol. Rep. 29 (2013) 2438–2444, https://doi.org/10.3892/or.2013.2360.

[41] H.H. Lu, S.Y. Kao, T.Y. Liu, S.T. Liu, W.P. Huang, K.W. Chang, S.C. Lin, Areca nut extract induced oxidative stress and upregulated hypoxia inducing factor leading to autophagy in oral cancer cells, Autophagy 6 (2010) 725–737, https://doi.org/10.4161/auto.6.6.12423.

[42] M. Karin, Z.-g. Liu, E. Zandi, AP-1 function and regulation, Curr. Opin. Cell Biol. 9 (1997) 240–246, https://doi.org/10.1016/S0955-0674(97)80068-3.

[43] K. Ryder, D. Nathans, Induction of protooncogene c-jun by serum growth factors, Proc. Natl. Acad. Sci. Unit. States Am. 85 (1988) 8464, https://doi.org/10.1073/pnas.85.22.8464.

[44] P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, Biochim. Biophys. Acta Rev. Canc 1072 (1991) 129–157, https://doi.org/10.1016/0304-419X(91)90011-9.

[45] H. Zhou, J. Gao, Z.Y. Lu, L. Lu, W. Dai, M. Xu, Role of c-Fos/JunD in protecting stress-induced cell death, Cell Prolif 40 (2007) 431–444, https://doi.org/10.1111/j.1365-2184.2007.00444.x.

[46] Y. Son, Y.-K. Cheong, N.-H. Kim, H.-T. Chung, D.G. Kang, H.-O. Pae, Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? J Signal Transduct 2011 (2011) https://doi.org/10.1155/2011/792639, 792639-792639.

[47] I. Pant, S.G. Rao, P. Kondaiah, Role of areca nut induced JNK/ATF2/Jun axis in the activation of TGF-β pathway in precancerous Oral Submucous Fibrosis, Sci. Rep. 6 (2016) 34314, https://doi.org/10.1038/srep34314.

[48] E.F. Wagner, Á.R. Nebreda, Signal integration by JNK and p38 MAPK pathways in cancer development, Nat. Rev. Canc. 9 (2009) 537–549, https://doi.org/10.1038/nrc2694.

[49] C. Bubici, S. Papa, JNK signalling in cancer: in need of new, smarter therapeutic targets, Br. J. Pharmacol. 171 (2014) 24–37, https://doi.org/10.1111/bph.12432.

[50] Hiroshi Ohkawa, et al., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (2) (1979) 351–358. In press, https://doi.org/10.1016/0003-2697(79)90738-3.