In vitro anticancer activity of a pentacyclic triterpenoid via the mitochondrial pathway in bone-invasive oral squamous cell carcinoma

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Abstract

Context: Oral cancer is the most dreadful cancer worldwide with a 5-year survival rate of approximately 50%. Anticancer therapies such as chemotherapy and radiotherapy result in severe side effects.

Aim: We aimed to evaluate the in vitro anticancer activity of Asiatic acid (AA) on bone-invasive oral squamous cell carcinoma (BHY) cell line.

Settings and Design: This was an in vitro laboratory setting.

Materials and Methods: BHY cell lines were used for the experiment. Confocal microscopy was used to observe cellular alterations. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine the IC50 concentration of AA and flow cytometry to analyze the percentage of cells in each phase of the cell cycle post treatment. Immunoblot assays and semiquantitative reverse transcriptase-polymerase chain reaction (rt-PCR) were used to study the expression level of genes involved.

Statistical Analysis Used: Student’s t-test and one-way analysis of variance were used for statistical analysis.

Results: IC50 concentration of AA was 15.6 µM. On flow cytometry analysis, treatment with 15.6 µM and 31.25 µM of AA for 24 h increased the percentage of cells in the G2/M phase to 45.63% and 53.12%, respectively, compared to 9.62% in control group. Immunoblot analysis and semiquantitative rt-PCR demonstrated an upregulation of p53, cyclin-dependent kinase inhibitors (p21 and p27), caspase-3, caspase-9, cytochrome c and Bax in a time-dependent manner and downregulation of cyclins and anti-apoptotic protein Bcl-2 (**P < 0.05, ***P < 0.001 versus control) post AA treatment.

Conclusion: AA induces apoptosis via the mitochondrial-dependent pathway and causes cell cycle arrest at the G2/M phase in BHY cell line.

Keywords: Anticancer, Asiatic acid, BHY cell line

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most dreadful diseases and causes more deaths when compared to any other oral disease. It is the sixth most common cancer worldwide. Despite the recent advances in treatment modalities, the 5-year survival rate over the past 30 years has remained only approximately 50%. The most important causative risk factors for OSCC are the use of betel quid or tobacco and regular consumption of alcohol. Other infectious agents such as human papillomavirus also play a vital role. Recent literature studies have implicated diets low in vegetables and fresh fruits in the etiopathogenesis of OSCC. The incidence and prevalence of OSCC is the highest in the Indian subcontinent due to the very prevalent habits of betel quid or tobacco and areca nut chewing.

Today, OSCC is primarily treated by surgery, chemotherapy and radiation either singularly or in combination. Even though today chemotherapy is the most extensively studied anticancer therapy, its safety and efficacy are factors for consideration. It causes unpleasant side effects such as nausea, vomiting, diarrhea, hepatotoxicity, hematoxicity, cardiotoxicity and immunosuppression, further leading to infections. Apart from these side effects, OSCC is a cancer with frequent recurrence and metastasis. The causation of such side effects has encouraged several researchers to formulate effective treatment regimens by innovative and novel approaches. The discovery of active secondary metabolites from natural sources as medicinal compounds can help provide an alternative treatment choice for several patients.

According to the National Cancer Institute, a diet rich in green leafy vegetables and citrus fruits is rich in vitamin A and C; known to lower cancer risks. Naturally available vegetables contain complementary benefits due to the presence of secondary metabolites such as triterpenoids, polyphenols and flavonoids. These compounds have multiple activities such as antioxidant, antiproliferative, antibacterial, antiviral and antitumorigenic. One such pentacyclic triterpenoid, Asiatic acid (AA) derived from a traditional medicinal plant Centella asiatica Linn. (Apiaceae family), was recognized to inhibit cell proliferation of multiple myeloma cells (RPMI 8226) and exhibited arrest at G2/M phase. It has also been proved to be effective in exhibiting cell growth inhibition of human breast cancer cell lines (MCF-7 and MDA-MB-231) by causing an arrest at S-G2/M phase. It is known to trigger the mitochondrial apoptotic pathway by altering Bax/Bcl-2 ratios, cytochrome c release and caspase-9 inactivation.

Studies have shown that AA demonstrates cytotoxic effects on colon cancer cells (HT-29) by inhibiting their growth via Bcl-xl and Bcl-2 correlative apoptosis and against human liver carcinoma cells (HepG2) via upregulating p53 and calcium release. Considering the effective role of AA in various cancer cell lines, our interest was to evaluate its anticancer potential and mechanism in bone-invasive OSCC cell lines (BHY).

Experimental material and methodology

Reagents

Dulbecco’s Modified Eagle’s medium (DMEM) was purchased from Invitrogen Gibco BRL; dimethyl sulfoxide (DMSO), penicillin, streptomycin, trypsin-ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), AA and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) from Gibco BRL (Gaithersburg, MD), Bradford reagent and protease inhibitors (aprotinin, pepstatin A, phenylmethylsulfonyl fluoride [PMSF] and leupeptin) were obtained from Sigma (St. Louis, MO, USA). Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology. All other chemicals were of reagent or analytical grades which were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and drug preparations

BHY cell line and keratinocytes were procured from National Center for Cell Science, Pune. Monolayer cultures were grown and maintained in DMEM along with heat-inactivated 10% FBS, 2 mM L-glutamine (Sigma Chemical Co.), 100 Units/ml penicillin and 100 µg/ml streptomycin supplementation. The cultures were maintained at 37°C in an atmosphere of 5% CO2 incubator at 95% humidified air. AA stock solution was prepared in DMSO and until use was stored at −20°C. For each experiment, the concentrations used were freshly prepared with a final DMSO concentration of 0.1%. Experiments were performed in triplicates.

Assessment of cell viability

To measure the cell viability, MTT colorimetric assay was performed as described by Mosmann.

Nuclear staining

BHY cells were plated at a density of 5 × 10^4 in 6-well plates and were allowed to grow till 70%–80% confluence following which cells were treated with different
concentrations (selected based on the IC50 concentration) of compounds for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with phosphate-buffered saline (PBS) and subsequently treated with 100 µl of dye mixture (1:1) of ethidium bromide [EtBr] and acridine orange [AO]) and viewed under fluorescence microscope. The percentage of apoptotic cells was determined by:

\[
\text{% of apoptotic cells} = \left[ \frac{\text{total number of apoptotic cells}}{\text{total number of cells counted}} \right] \times 100
\]

Flow cytometric analysis
To investigate the effect of AA on the cell cycle distribution, BHY cells (1 × 10^6 cells/ml) were treated with 15.6 µM and 31.25 µM of AA and cultured for 24 h. The treated cells were harvested, washed with PBS and fixed in 75% ethanol at 4°C overnight. After washing twice with ice-cold PBS, cells were suspended in PBS containing 40 µg/ml propidium iodide (PI) and 0.1 mg/ml RNase A, followed by shaking at 37°C for 30 min. Cells (10,000) were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) on the FL2-A detectors. The data were analyzed consequently using WinMDI 2.9 software (TSRI, La Jolla, CA, USA) to determine the percentage of cells in apoptosis, G0/G1 phase, S phase and G2/M phase.

Western blotting
Western blotting was carried out as described previously by Tu et al. [11] BHY cells (1.5 × 10^5) were seeded onto 100 mm culture dishes in the presence or absence of different concentrations of compound (IC50 concentrations) and were treated for 24 h. The medium was removed and the cells were washed with PBS (0.01M, pH 7.2) and lysed on ice in lysis buffer containing 100 µg/ml PMSF, 50 mM Tris base at pH 8.0, 150 mM NaCl, 0.02% NaN3, 1% NP-40, 10 µM aprotinin, 10 µM pepstatin A and 10 µM leupeptin. The supernatant was collected by centrifugation at 10,000×g for 5 min at 4°C and was used as the cell protein extracts. The harvested protein concentration was measured using a protein assay kit (Bio-Rad). Equal amounts of proteins (50–100 µg) were separated on 7.5%–12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene fluoride membrane. Proteins were blocked overnight with 5% nonfat dried milk in PBS-T at 2°C–8°C. After washing in PBS containing 0.1% Tween 20 for three times, the membrane was incubated with the specific primary antibodies in 5% (w/v) skim milk in PBST. 1X Tris-buffered Saline and 0.1% Tween 20 Detergent (TBST). After reaction with HRP-conjugated goat anti-mouse antibody, the immune complexes were visualized by using the chemiluminescence ECL Plus detection reagents following the manufacturer’s procedure (Amersham Biosciences). The same nitrocellulose membrane was stripped and incubated with β-actin monoclonal antibody (Sigma) at a 1:2000 dilution for 1 h which acted as a control for loading and blotting.

Gene expression studies
Semi-quantitative reverse transcriptase-polymerase chain reaction (rt-PCR) was performed to analyze the expression levels of apoptosis-related genes: Bcl2, p53 and caspase-3. Approximately 5 × 10^5 cells in a total volume of 3 ml were seeded in 6-well plate multidis and incubated in the presence of AA at 37°C for 48 h. Beta-actin (housekeeping gene) was used as a control. Post incubation, cells were rinsed with PBS and trypsinized using trypsin 0.02% EDTA mixture. After centrifugation for 5 min at 500×g 4°C, the supernatant was removed, and the pellet was used for rt-PCR studies. Total ribonucleic acid (RNA) was isolated using Total RNA Isolation System (Promega, France). Complementary deoxyribonucleic acid (cDNA) was generated by Reverse Transcription System (Promega, France). 10 µl of cDNA product was used for PCR reaction as templates. PCR was carried out using the gene-specific upstream and downstream primers [Table 1]. According to the manufacturer’s protocol, initial denaturation at 95°C for 3 min was followed by a PCR cycle of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. PCR products were separated on a 1.5% agarose gel and stained with EtBr. Beta-actin was used as an internal loading control. The intensity of individual bands was semiquantitatively assessed using National Institutes of Health Image.

Statistical analysis
Values represent the mean ± standard deviation of three experiments. Statistical analysis was performed according to the GraphPad Prism 5.0. The significance level was calculated using Student’s t-test and one-way analysis of variance to assess the differences between experimental groups. P <0.05 was considered statistically significant.

| Table 1: Oligonucleotide primers for caspase-3, p53, Bcl-2 and β-actin |
|------------------------|-------------------|-------------------|-----------------------------|
| Genes                  | Accession number  | Forward primer    | Reverse primer              | Product size (BP) |
| Caspase-3              | NM_004346         | TCCTCGCGGATGGGATGCTAT | TCAACGGCTGGGATTTCAAG        | 126              |
| p53                    | NM_000546         | TGCTCAAGACGAGGCGCTGAA | CAATCCAGGGAGACGTCTCA        | 145              |
| Bcl-2                  | NM_000633         | GAACCTGCGGAGGATGGGG | GAGCGAGCATGTTCAGGCAGA       | 311              |
| β-actin                | NM_001101         | GAGCACAGAGCGATCCTCCTT | AGAGCGGTACAGGGGATAGCA       | 494              |
RESULTS

Asiatic acid inhibits proliferation of BHY cells
As demonstrated in Figure 1a, treatment with AA significantly inhibited cell viability and proliferation in a dose-dependent manner. Cisplatin was used as a standard compound. The results were expressed as a percentage relative to the cell number. AA exhibited an IC50 (median growth inhibitory concentration value) of 15.6 µM and 3.9 µM, respectively, after 24 h of treatment. The same was chosen for further experiments [Figure 1b].

Cell and nuclear morphology
To assess cellular apoptosis on drug treatment, BHY cells were treated with IC50 concentration of AA for 24 h. After 24 h, the cells were stained with AO/EtBr and examined by confocal microscopy. The nuclear morphology of cells undergoing apoptosis was evaluated using AO/EtBr staining. Both live and dead cells stain with AO (vital dye/green color), whereas those cells that lose their membrane integrity stain with EtBr (red color). Green stain represents viable cells, early apoptotic cells are represented with yellow stain, whereas late apoptotic cells are represented with reddish or orange stain. In control group, uniform green live cells with normal and large nuclei were observed, whereas yellow-, orange- and red-stained cells were observed in AA-treated group [Figure 2a and b]. The above results establish that AA significantly induces apoptosis in oral cancer cells.

Effect of Asiatic acid on cell cycle distribution
Effective chemopreventive agents induce programmed cell death or growth inhibitory effects in cancer cells via cell cycle arrest. To determine whether the growth inhibitory effect of AA was associated with the induction of cell cycle arrest and/or apoptosis; flow cytometry was used to analyse the distribution of cells in different phases of the cell cycle as shown in Figure 3. In comparison to the control, AA

![Figure 1](image1.png)

Figure 1: Asiatic acid reduces the viability of BHY cells. (a) Graphical representation of cell viability. Asiatic acid reduces the viability of BHY cells. Cells visualized by phase-contrast microscope magnification ×20 (b) Morphological changes in cells induced by Asiatic acid. Control group received 0.1% dimethyl sulfoxide. Scale bar 100 µm

![Figure 2](image2.png)

Figure 2: Acridine orange/ethidium bromide staining of BHY cells to detect apoptosis under confocal microscopy. (a) Control cells presenting normal nuclear morphology; ×20. (b) Apoptotic cells (Asiatic acid treated) presenting morphological changes; chromatin condensation and nuclear fragmentation (white arrows); ×40. Scale bar 20µm
treatment resulted in a dose-dependent increase of BHY cells in the G2/M phase. After treatment with 15.6 µM and 31.25 µM of AA for 24 h, the percentage of cells in the G2/M phase was 45.63% and 53.12%, respectively, when compared to 9.62% of control cells [Figure 3a], thereby inducing G2/M arrest. The compound showed a decrease in the S phase associated with a concomitant increase in G2/M phase when compared to the untreated cells [Figure 3b].

**Effect of Asiatic acid on the expression of cyclin level**

To examine the status of the intracellular signaling molecules responsible for promoting growth inhibitory activity of AA in BHY cell lines, cells at a density of $1 \times 10^6$ were seeded in 100 mm dishes further treated with DMSO (vehicle) and IC50 concentration of AA for 24 h. After 24 h of treatment, protein extracts were prepared from cells for performing Western blot analysis. Cyclin B, E, Cdc-2 and Cdc-25c protein expressions were measured. AA significantly downregulated the expression of cyclin B, E, Cdc-2 and Cdc-25c in a time-dependent fashion [Figure 4a and b] ($**P < 0.05$). These results suggest that AA prevents the cell from progressing into the S phase of the cell cycle and prevents improper DNA synthesis.

**Asiatic acid induces G2/M arrest and apoptosis via p53**

p53, a policing gene, plays a vital role in cell cycle arrest, apoptosis and DNA repair. p21, a cyclin-dependent kinase 2 inhibitor, is known for its ability to be upregulated by p53, both of which are integrated in G1 and G2 arrest machinery in response to DNA damage. The common pathway reports that p53 upregulates p21 resulting in decrease of cyclin B1/Cdk1 complex, which further leads to arrest at G2/M phase. We carried out a study to evaluate whether AA induces apoptosis by G2/M phase arrest via P53-dependent or independent pathway using Western blot and rt-PCR analysis. BHY cells were treated with IC50 concentration of AA. Immunoblot and gene expression analysis demonstrated that AA treatment resulted in an increased expression of p53 protein in a time-dependent manner [Figures 4c and d and 5].

**Effect of Asiatic acid on the expression of Bcl-2 family members**

To examine the status of the intracellular signaling molecules responsible for the growth-inhibiting activity of AA in BHY cells, cells at a density of $1 \times 10^6$ were seeded in 100 mm dishes, further treated with DMSO (vehicle), and IC50 concentration (15.6 µM) of AA for different time intervals. After 24 h of treatment, protein extracts were prepared from cells and utilized for performing Western blot analysis and mRNA expression by rt-PCR. The Bcl-2 protein family contains both pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2 that regulate mitochondrial outer membrane integrity which initiates cytochrome c release in the cytosol which activates pro-caspase into its active form leading to apoptotic cell death. Therefore, we measured the protein and mRNA expression of Bcl-2 family proteins by Western blot analysis. AA significantly increased the expression of pro-apoptotic proteins Bax [Figures 6a and b] and downregulated the expression of anti-apoptotic protein Bcl-2 [Figures 5 and 6a, b] in a time-dependent fashion. These results suggest that Bcl-2 family members play a major role in AA-induced apoptosis in oral cancer cells.

**Effect of Asiatic acid on the expression of cytochrome c and caspases**

To analyze the involvement of mitochondrial release of...
cytochrome c in BHY cell lines and to determine whether caspase-mediated pathway is involved in AA-induced apoptosis, the cells were treated with AA (15.6 µM) for 24 h. We examined the protein expression of caspase-3, caspase-9 and poly adenosine diphosphate-ribose polymerase (PARP) by Western blot and the gene expression of caspase-3 using rt-PCR analysis. Immunoblotting analysis revealed that drug treatment increased caspase-9 and caspase-3 expressions in oral cancer cells in a time-dependent manner which is evident from the increase in its active form as compared to control [Figure 6c and d]. Figure 5 exhibits a significant increase in caspase-3 levels. These results indicate that AA can initiate the release of cytochrome c from the mitochondria into the cytosol post treatment, supporting that AA induces apoptosis in oral cancer cells involving signaling at the mitochondrial level.

DISCUSSION

For maintaining the development and homeostasis of multicellular organisms, programmed cell death/apoptosis is crucial. One of the many hallmarks of tumorigenicity is inefficient apoptosis. Moreover, inducing programmed cell death is a vital target for cancer therapy. Chemoprevention is a promising way to
impede the development of such cancers. Large numbers of minor dietary components have been reported to inhibit various stages of carcinogenesis. In the present study, we investigated the anti-apoptotic effect of AA via the intrinsic pathway. We also evaluated its effect on the different phases of the cell cycle for BHY cell line arrest.

In our study, we observed suppressed cell viability of BHY cells above the IC50 concentration of AA (15.6 µM) when treated for 24 h, compared to a recent study performed by Li et al., who reported suppression of cell viability of tongue cancer cells (Tca8113) when treated with 40 µM of AA for 24 h. From our knowledge, this is the first study to investigate the anticancer properties of AA in BHY oral cancer cell lines, with the second study carried out recently on tongue cancer cell lines (Tca8113) by Li et al.

However, the detailed mode of action of AA in induction of apoptosis has not been well established in oral cancer cell lines. In the present study, we describe a molecular basis for the antiproliferative effects of AA in BHY cells. We observed that AA treatment in a dose-dependent manner inhibited cell viability, with a cell cycle arrest at G2/M phase leading to an increased apoptotic cell death and an increase in the expression levels of p53 protein. On treatment for 24 h, an increase in the cell population at G2/M phase was observed with a concomitant decrease at G1 phase demonstrating a hallmark of apoptotic cell death. Similar results were reported by Lee et al., describing the antiproliferative and inhibitory properties of AA by demonstrating downregulation of Ras/MEK pathway and cell cycle arrest of glioblastoma multiforme (GBM) cells at G1/S and G2/M phase of the cell cycle. Lee et al. reported the anti-apoptotic effect of AA via the release of intracellular calcium and elevated expression of p53 in HepG2 human hepatoma cells. The induction of apoptosis by AA was confirmed by visualization of morphologic changes such as cells rounding, granulation and nuclear condensation by confocal microscopy [Figure 2].

In our study, we observed time- and dose-dependent treatment with AA significantly upregulated pro-apoptotic protein Bax and downregulated anti-apoptotic protein Bcl-2. Similar results were reported by Lee et al. in GBM cell lines.

On fluorescence-activated cell sorting analysis, a noticeable accumulation of BHY cells in G2/M phase was observed prior to the induction of a sub-G1 cell population. The results obtained suggest that G2/M-phase arrest might be an upstream event leading to apoptosis. It has been well established that blockage of cell cycle progression at mitotic phase leads to apoptotic cell death. Several anticancer agents have been associated with the activation of apoptotic pathways. Inactivation and cleavage of several cellular
proteins such as PARP and lamin occur as a result of activation of both the initiator (caspase-9) and executioner caspases (caspase-3), resulting in an apoptotic cell death in many cell types. Our results show that AA promotes cleavage of both caspase-3 and caspase-9, accompanied by PARP cleavage, indicating that the mechanism of AA-induced apoptosis involves a caspase-mediated pathway. Our results were concurrent with a study performed by Wu et al. who reported that AA inhibited cell proliferation and promoted cell death both *in vitro* (human lung cancer cells – A549) and *in vivo* (mouse Lewis lung cancer cells) in a time- and dose-dependent manner by inducing mitochondrial-mediated apoptosis. The antitumor activity of AA was determined by the activation of PARP, caspase-9 and caspase-3 leading to the mitochondrial release of cytochrome c thereby activating the intrinsic pathway of apoptosis. Liu et al. reported significant upregulation of caspase-3, caspase-8, caspase-9 and PARP on treatment with AA in cisplatin-resistant nasopharyngeal carcinoma cell lines (NPC). AA induced apoptosis in cisplatin-resistant NPC cell lines by upregulating pro-apoptotic proteins Bax and Bak. Li et al. reported the antitumor property of AA. AA treatment *in vitro* inhibited tongue cancer cells (Tca8113) via the mitochondrial apoptotic pathway. Western blotting analysis of treated Tca8113 cells revealed that AA treatment decreased Bcl2 levels and increased pro-apoptotic proteins such as Bax and cleaved caspase-3.

AA is a known chemopreventive agent, but the underlying mechanisms for BHY cells are not well understood. In the present study, we have demonstrated that the possible role of AA was (i) to directly inhibit the percentage of cell number (BHY); (ii) induce apoptosis of BHY cells, via intrinsic pathway; and (iii) *in vitro* by interfering/arresting the cells at the G2/M phase, thereby leading to apoptosis. The morphological picture displayed a decrease in the number of BHY cells post treatment with 15.6 µM of AA.

Several researchers have demonstrated inhibition of cell proliferation and induction of apoptosis on treatment with AA in breast cancer, human hepatoma, melanoma, gastrointestinal tumors and glioblastomas. For instance, Hsu et al. in 2005 stated that AA inhibited cell cycle progression at the S-G2/M phase by downregulating the expression of Cdc2, Cdc25C, cyclin B1 and cyclin A and upregulating p21/Cdc2 interaction. Similar results were appreciated in our study; AA acid upregulated cyclin-dependent kinase inhibitors p21 and p27 and downregulated cyclins B, E, Cdc2 and Cdc25c in BHY cells in a time-dependent manner. Apparently, the mechanism of action of AA is not the same in all cell types.

**CONCLUSION**

The results of the present study indicate that AA efficiently induces G2/M cell cycle arrest and mitochondria-dependent apoptosis in human oral cancer cells. On treatment with AA, BHY cells exhibited typical morphological changes associated with apoptosis. In addition, apoptosis was mediated by caspase-3 activation and cytochrome c release. These observations indicate that mitochondria-mediated process may be involved in the induction of apoptosis of BHY cells treated with AA. The growth inhibition is related to the G2/M phase cell cycle arrest associated with the upregulation of tumor suppressor gene p53 and Bax and downregulation of Bel-2, cyclin B, E, Cdc-2 and Cdc-25c. The study results offer a molecular foundation for AA as an antitumor agent in oral cancer therapy. Ability of AA to induce cell cycle arrest at G2/M phase followed by very effective induction of apoptosis in tumor cells at relatively low concentration, gives us an additional argument to consider this compound as a potent and promising therapeutic agent against oral cancer. *In vitro* studies provide factual evidence of the different pathways involved in anti-tumorigenicity and lay a foundation for further *in vivo* research. During *in vitro* studies, the effect of AA on cell lines is tested with the lack of a biological system (ADME properties); therefore, lower concentrations of AA exhibited a significant effect. On the contrary, *in vivo* studies are vital to evaluate the effect of AA as a potent anticancer agent for oral cancer, wherein the dose is calculated based on maximum tolerated dose with single and repeated dose studies and confirmed based on the toxicity and pharmacokinetic model.

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**Conflicts of interest**

There are no conflicts of interest.

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