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Anti-Proliferative Activity of Aescin on Human Oral Carcinoma (KB) Cells and Human Laryngeal Carcinoma (Hep-2) Cells- Via Cell Sensitivity Assays

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Abstract

The incidence of Head and Neck malignancy is expanding around the world. The critical risk factor for these tumors incorporates smoking, liquor, tobacco intake, and viral infection. Natural agents have frequently shown restorative properties with helpfulness in treating different human degenerative diseases. Aescin is the most prominent constituent of *Aesculus hippocastanum* L. *(Hippocastanaceae)* seeds has been conventionally utilized as a therapeutic herb. We determine the anticancer effects of aescin on human oral carcinoma (KB) cells and human laryngeal carcinoma (HEp-2) cells by cell sensitivity assays. To vindicate the impact of aescin compared with positive control cisplatin on anti-proliferative activity in human oral carcinoma and human laryngeal carcinoma cells were investigated by cell sensitivity assays (MTT assay, Tryphan blue dye exclusion method, Alamar Blue assay, and Hexosaminidase assay). The 24-hour treatment of KB cells and HEp-2 cells with different concentrations of aescin suppressed proliferation and induced morphological changes consistent in these cells. Exposure of both cell lines to aescin resulted in a marked increase in loss of cell proliferation and induces cytotoxicity in KB and HEp-2 cells. Taken together, our results demonstrate that aescin modulate cell growth and differentiation in KB and HEp-2 cells by initiate cytotoxicity implying that aescin might be developed as a potential chemotherapeutic agent for the treatment of head and neck cancer.

Keywords: Oral carcinoma, Aescin, cisplatin, cytotoxicity, anti-proliferative.

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INTRODUCTION

Head and neck cancer

Around the world, Head and neck malignancy (HNC) which incorporates tumors of the oral cavity, oropharynx, and larynx/hypopharynx is the 6th most regular disease and positioned as the second most normal disease in India (Globocan 2018) and the reason for oral malignant growth endurance rate for a very long time is 63-64% (Debolina *et al.,* 2019). According to Globacon, lip and oral cancer are responsible for over 354,864 new cases and an estimated 177,384 deaths in 2018 (Bray *et al.,* 2018). Tobacco use, as well as alcohol, are the most common etiological factors associated with the development of oral cancer. Some other risk factors for oral cancer, which is HPV infection, have also been documented (Debolina *et al.,* 2019 and Mummudi *et al.,* 2019).

Aescin

Aescin (polyhydroxyolean-12-ene 3-O-monodesmosides), a pentacyclic triterpenoid saponin blend (Dorothy *et al.,* 2018). It's an active component of the horse chestnut, Aesculus hippocastanum L, which has been utilized for quite a long time as regular home grown medication in China and is presently used to treat certain issues, including hemorrhoids, varicose veins, hematoma, and the chronic venous deficiency (Jiang *et al.,* 2011) & (Huang *et al.,* 2014). Aescin exists pharmacodynamic properties, anti- oedematous, anti-inflammatory, and vasoprotective. The hypoglycaemic and anti-obesity effects also have been reported (Cesare *et al.,* 2001) & (Luca *et al.,* 2019). The most recent investigation exhibits the anticancer impact of aescin in various cancer cell line models, like lung cancer (A549), renal cancer (786-O and Caki-1), breast cancer, human acute leukemia, cervical cancer (HeLa), pancreatic cancer (Panc-1, COLO 357, and MIA-Paca) (Wang *et al.,* 2012), human osteosarcoma (U2OS) (Liu *et al.,* 2017), glioma (C6, and A549) (Gülşen *et al.,* 2015), and bladder cancer (T24) Chen *et al.,* 2018).

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Cell sensitivity assays

Cell proliferation assays are imperative to gain an understanding of the molecular mechanisms that modulate cell growth and differentiation. Various bioassay frameworks have been created for the quantitation of cell number, cell endurance, or cell multiplication (Huang *et al.,* 2016). Cytotoxicity is one of the most prominent pointer for biological assessment studies for in vitro. In vitro, drugs have different cytotoxicity mechanisms such as destruction of cell membranes, irreversible binding to receptors, prevention of protein synthesis, etc (Niraj *et al.,* 2017). To assess the cell death induced by these damages, there is a requirement for inexpensively, and reproducible transient cytotoxicity and cell feasibility measures depends on different cell capacities. An expansive range of cytotoxicity examines is presently utilized in the fields of toxicology and pharmacology (Voytik-Harbin *et al.,* 1998). There are various orders for these examines: (i) color rejection tests; (ii) colorimetric measures such as MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-phenylamino-carbonyl)2H-tetrazoliumhydroxide) and exclusion/inclusion of dyes such as neutral red, crystal violet, tryphan blue & methylene blue; (iii) fluorometric measures; and (iv) luminometric tests (Flick and Grifford, 1984) & Gillies *et al.,* 1986). Every one of these bioassay frameworks has its points of interest and restrictions (Scudiero A *et al.,* 1988).). Picking the fitting technique among these examines is significant for getting exact and dependable outcomes. While choosing the cytotoxicity and cell viability examines to be utilized in the investigation, various boundaries must be viewed as, for example, the accessibility in the research facility where the examination is to be performed, test mixes, recognition instrument, explicitness, and affectability (Givens *et al.,* 1990) & (Paul *et al.,* 2019). In this part, the data will be given about in vitro cytotoxicity and feasibility examines, these tests will be characterized and their preferences and inconveniences will be underlined (Voytik-Harbin *et al.,* 1998). This part plans to control the specialist keen regarding this matter to choose the proper examine for their examination.

The intent of the MTT assay

Different measure boundaries were improved for a 96-well configuration to accomplish a perceptible scope of cancer cell numbers indeed, which is like that got with

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conventional (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) and [3H]thymidine examine methods. The MTT assay is used as an indicator of cell viability, proliferation, and cytotoxicity to assess cellular metabolic activity (Alley C *et al.,* 1988). This assay based on mitochondrial activity is constant for most viable cells and that an increase or decrease in the number of viable cells (Gertjan *et al.*, 2011). This colorimetric assay is based on the reduction by of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals of metabolically active cells. The viable cells contain NAD (P)H-dependent enzymes of oxidoreductase which reduce the formazan MTT (Mosmann, 1983, Scudiero *et al.*, 1988). Using a solvent DMSO, the insoluble formazan crystals are dissolved and the resulting purple-colored solution is quantified using a multi-well spectrophotometer by measuring absorbance at 540 – 720 nm. For the identification in bioassays of lymphotoxin, growth factors, and interleukin, the MTT approach was significant (Scudiero *et al.,* 1988). However, to address the technical limitations of less than optimum sensitivity, variable context due to protein precipitation, the low solubility of the formazan product, and insufficient reproducibility, modifications to the original procedure are required.

The role of Trypan blue exclusion

Trypan blue is a dye used to measure live cells by labeling dead cells only. It is used to exclusively mark dead cells to measure live cells. Trypan blue is unable to penetrate the cell membrane of live cells and reach the cytoplasm because live cells have an intact cell membrane (Jayashree *et al.,* 2018). In a dead cell, trypan blue goes through the permeable cell membrane and enters the cytoplasm. Under light microscopy, just dead cells have a blue tone. It is additionally used to wipe out bogus positives from happening during cell tallying by stream cytometry and one of the most punctual and easiest feasibility tests (Paul *et al.,* 2019).

The purpose of the Alamar Blue assay

As of late, another and adaptable metabolic-dye Alamar Blue has gotten economically accessible, depends on the reducing reactions intrinsic to viable cell metabolism, and is generally utilized as a screening strategy to decide the cytotoxic

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capability of medications against disease cells. Alamar Blue examines utilize the indicator Resazurin to demonstrate cell viability by estimating the metabolic limit of cells (Sephra *et al.*, 2012). The blue-colored resazurin is nonfluorescent, while it is pink and strongly red fluorescent in its reduced form, called resorufin (7-hydroxy-3H-phenoxazin-3-one) (Voytik-Harbin *et al.*, 1998). Metabolically active cells hold the capacity to reduce Resazurin into Resorufin, which gives a fluorescent sign, indicates the cell viability (Niraj *et al.*, 2017). Alamar Blue has a few qualities that make this color an alluring contender for use as a quantitative examination of cancer cell proliferation (Sephra *et al.*, 2012). This test offers the upsides of specialized effortlessness, independence from radioisotopes, adaptability in location, no extraction, and fantastic reproducibility and affectability (Bonnier *et al.*, 2015). We envision that this straight forward and flexible Alamar Blue examination, when utilized alone or related to different bioassays, will be a helpful apparatus for researching the intricate instruments of cell expansion.

The motivation behind hexosaminidase assay:

β-hexosaminidase, a bio-active material acts as a potent inflammatory mediator stored in mast cells and is frequently released from activated mast cells in parallel with histamine (Thana *et al.*, 2014). As a synthetic substrate for the ubiquitous cytoplasmic enzyme, hexosaminidase, uses p-nitrophenol-N-acetyl-/3-D-glucosaminide (Huang *et al.*, 2016). The accuracy of this assay with different cell types, including murine and human lymphocytes, macrophages, Chinese hamster ovary, and cancer cell lines, has been tested; the above technique could provide less time consuming, effort, and expense over traditional assays such as Coulter counting, particularly in drug studies and aim at screening multiple compounds for anti-proliferative properties (Givens *et al.*, 1990).

To our knowledge, these colorimetric techniques have not been examined in proliferation assays using human oral carcinoma (KB) cells and human laryngeal carcinoma (HEp-2) cells treated with aescin. Therefore, we investigated the feasibility of these techniques as simple alternatives to electronic cell counting, in drug-induced cytotoxicity assays. In this article, we explore the application of Alamar Blue and β-hexosaminidase, a new and versatile metabolic dye and inflammatory mediator for the

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detection and/or survival of aescin-influenced human oral carcinoma (KB) cells and human laryngeal carcinoma (HEp-2) cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagles Medium (DMEM), Phosphate Buffered Saline (PBS), fetal bovine serum (FBS), 0.25% trypsin EDTA, Antibiotics (penicillin, Streptomycin), Dimethyl sulfoxide (DMSO), 3 – (4,5 – dimethyltiozol – 2 – yl) -2,5 diphenyl tetrazolium bromide (MTT), 7- hydroxyl- 3H – Phenoxazain – 3 – One – 10 Oxide Sodium salt (Resazurin sodium), Trypan blue, Glycine, Triton X- 100, sodium citrate were be obtained from Hi-media lab Ltd, Mumbai, India. Naphtol As-BI AN-acetyl-B-D-glucosaminide (β-hexosaminidase substrate), bovine serum albumin (BSA), and Escin ≥ 95% by TLC (CAS NO. 6805 -41 -0) were being purchased from Sigma – Aldrich and Cis-Diammine Platinum (II) Dichloride (Cisplatin):99.9% was be purchased from Sisco Chem, PVT.LTD. India.

Cell Culture

From NCCS, Pune, India, Human Laryngeal carcinoma [Human epithelial type 2 cells] (HEp-2) and KERATIN-forming tumor cell line Hela (KB) were acquired. In the Dulbecco Modified Eagles Medium (DMEM), cells were cultured and maintained at an appropriate incubation environment. Before drug treatment, Aescin was freshly dissolved in 1XPBS/ethanol.

Cell proliferation assays

The effect of aescin compared with cisplatin on the cell proliferation of HEp -2 & KB cells was determined by MTT, Trypan blue dye exclusion method, Alamar Blue, and hexosaminidase assay as per the experimental protocol shown in Figure.1.

Measurement of Cell proliferation Inhibitory Activity

The influence of aescin on the cell proliferation of HEp-2 and KB cells dependent on the detection of mitochondrial dehydrogenase activity in viable cells was dictated by MTT screening. In 96 well plates with a concentration of 5 X 103 cells/well in a final volume of 100µl with DMEM incubated up to 24h, HEp-2 and KB cells were cultivated.

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The exposure to different concentrations of aescin and positive cisplatin control. After 24h, the cells were incubated for 2h at 37^{o}C with 100µl of MTT solution (1 mg/ml). To break up the formazan crystals, the MTT solution was eliminated and added 100µl of DMSO (Seweryn *et al.,* 2014). In a Read well touch, ELISA plate reader, the plate was read at 570nm (Robonic, India)

Determination of reducing reactions.

The effect of aescin on cell proliferation was determined by Alamar Blue assay based on the detection of redox reaction by mitochondrial enzyme activity in healthy cells. HEp -2 & KB cells were seeded in 96 – well plates at a density of 5 X 10^3 cells / well in a final volume of 100µl with DMEM incubated up to 24h The cell were treated with different concentrations of aescin and positive control cisplatin. After 24h, the cells were incubated with 10µl of Alamar Blue reagent (10%) for 3h at 37 ^{o}c. Following incubation, the cell plates were placed in a darkened environment for 30 minutes at room temperature (Fields & Lancaster 1993). The plate was read at 570nm in a Read well touch, ELISA plate reader (Robonic, India)

Measurement of β-hexosaminidase release activity by cell Adhesion Assay

The cell adhesion assay was performed by the release of β-hexosaminidase correlates well with spectrophotometric estimations of 4-nitrophenol synthesis for the enzyme-substrate response. The effect of aescin on the β-hexosaminidase of HEp -2 & KB cells were determined by Hexosaminidase assay based on the detection of hydrolysis of the NAG substrate (4 – Nitrophenyl N- acetyl – β- D- glucosaminide) by the enzyme. 96-well plates were coated with aescin and positive control cisplatin at various concentrations in PBS (1mg/1ml). After overnight incubation at 4^{o}C were rinsed with PBS and uncoated surfaces were blocked with 2% BSA. After one hour 0.1ml of cell suspension (Cells were suspended in the medium at a density of 3 X 10^5 cells/ml) were added to each well of the coated plates. After incubation of 6hrs 37^{o}C in 5mM citrate buffer (P^H 5.0) containing 3.75mM hexosaminidase substrate (p-nitrephenyl – N – acetyl – β – D – glucosaminide) and 0.25% Triton X – 100. Enzyme activity was blocked by adding 50mM glycine buffer P^H 10.4 containing 5mM EDTA (Landegren *et al.,* 1984).

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The plate was being read at 570nm in a Read well touch, ELISA plate reader (Robonic, India).

Trypan Blue dye exclusion assay

Cells count was determined by trypan blue dye exclusion assay based on the detection of live cells passed intact membranes that exclude certain dyes. HEp – 2 & KB cells were seeded with DMEM. After 24h treatment with aescin and positive control cisplatin the cell was trypsinized, the adherent cells, and were transferred into a centrifuge tube and it was centrifuged at 1500rpm for 5minutes. The pellet was being dissolved in a 1ml medium. After 900 µl of medium and 100 µl of cells were be added in an Eppendorf tube (total 1 ml). The 20 µl of cells from 1 ml stock were mixed with 20 µl trypan blue dye (trypan blue dye diluted with PBS at 1:1 concentration) gently. After 10µl of mixed constituents were counted by the use of a hemocytometer counting chamber (Sanjay *et al.,* 2009).

Statistical analysis.

The quantitative data's are value represents mean±SD (n=3) from experiments and is expressed as a relative percentage of control, statistical data differences were performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) using SPSS version 17.0 for windows. The Statistical significance was identified as $p < 0.05$ values.

RESULTS

Aescin induced morphological changes in KB and HEp-2 cell lines

The morphological change of live cells and cytotoxic cells of KB and HEp-2 cell lines treated with various concentrations of Aescin & cisplatin (5-45 µM) were shown in Figure 2. A & B. KB and HEp-2 cells show significant morphological change as compared to untreated control cells. Taken together, these results suggest that aescin induces cell death or apoptosis by induction of morphological changes.

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Aescin exhibits cytotoxic effects on KB and HEp-2 cell lines.

The level of cell reduction of tetrazolium salt, 3-(4.5-dimethylthiazol-2-yl)- 2.5-diphenyltetrazolium bromide (MTT) was measured to determine the cytotoxicity level. Cells were treated with various concentrations of aescin and cisplatin (5-45µM) for 24 hr in the appropriate environment, which shows the dose depended on the manner, aescin and cisplatin showed maximum cell death at 45µM concentrations (Figure 3. A & B.). Hence, the half inhibitory concentration (IC50) of aescin for KB cell line was 25µM±1.89 and for HEp-2 cells line was 22µM±1.64 as compared with cisplatin IC50 for KB cell line was 26µM±1.98 and for HEp-2 cells line was 23.5µM ±1.78 as determined from the growth inhibition curve.

Aescin dysregulates redox reaction in KB and HEp-2 cell lines.

To determine the redox reaction of aescin in KB and HEp-2 cell lines cells were treated with several concentrations of aescin (5-45 µM) compared with cisplatin (5-45 µM) for 24 hr. Figure 4. A & B. shows the concentration-response curves obtained for the Aescin and cisplatin in KB And HEp-2 cell line with the Alamar Blue. Aescin and cisplatin showed dysregulation of a redox reaction to cause cell death maximum at 45 µM. Hence, the half inhibitory concentration IC50 aescin of Alamar Blue redox reaction for KB cell line was 19.9µM±1.52 and for HEp-2 cells line was 18.58µM ±1.41, and for cisplatin 22.5µM±1.71 was KB cells and HEp-2 cells line was 21.5 µM±1.64 as determined from redox reaction inhibition curve.

Aescin inhibits β-hexosaminidase release activity in KB and HEp-2 cell lines.

To assess, whether aescin could have **β-hexosaminidase release activity** was performed in KB and HEp-2 cell line (Figure 5. A & B) compared with cisplatin-treated as a positive control. Cells were treated with various concentrations of aescin and cisplatin (5-45µM) for 24 hr in the appropriate environment, which shows the dose-dependent manner, aescin showed minimum cell adhesion at 45 µM concentration. Hence, the IC50 of aescin for KB and HEp-2 cells were 27.55µM±2.09 and 24.34µM±1.86; and for cisplatin (IC50) KB and HEp-2 cells were 29.2µM±2.22 and 25.91µM±1.86 as determined from the adhesion inhibition curve. The absorbency of the

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β-hexosaminidase reaction product is directly proportional to the number of cells and the time allowed for the reaction.

Aescin induced cell death on KB and HEp-2 cell lines.

The data generated using the Trypan Blue dye exclusion assay described in this report was presented as relative changes in cell death. To present data in a manner relative to an untreated with the treated group, we have been chosen 15, 25 & 35 μM (KB cells) and 12, 22 & 32 μM (HEp-2) of aescin and cisplatin; by the correlation of Alamar Blue based fluorescence and β-hexosaminidase with cell viability (MTT). Aescin and cisplatin-treated cells show cell death generated in both KB and HEp-2 cells in a dose-dependent manner (Figure 6. A & B).

DISCUSSION

Initially, most existing anti-cancer drugs go through cell viability tests or proliferation assays. The decision to continue research on these agents is also focused on their effect on the viability or proliferation of cells (Niraj *et al.,* 2017). The proliferation of cells is a complicated process involving the concerted operation of several factors. Most of the current understanding of the basic mechanisms involved in cell proliferation, in particular cancer cell lines, has come from the use of cultured cells (Voytik-Harbin *et al.*, 1998). Cytotoxicity and apoptosis are the main effects of drug-induced cell death. Cultural cancer testing strategies often vary in terms of whether they are meant to assess cytotoxicity or viability, and the basis on which they are identified. In addition to extreme changes in cell morphology, is specific for apoptosis and one of the easiest ways to distinguish cell death (Givens *et al.,* 1990). In different settings, techniques may be considered most appropriate, and the use of several complementary techniques could be the most illuminating. Different previous studies with different cell lines have obtained results supporting our findings.

Aescin has been shown to have antitumor effects in vitro in various cancer cells includes pancreatic cancer, colon cancer, gastric adenocarcinoma, human acute leukemia, chronic myeloid leukemia, and; cholangiocarcinoma, hepatoma, and prostate cancer cells. Although there is emerging evidence that aescin has antitumor properties, the

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effects of aescin on KB and HEp-2 cells remain elusive (Jiang *et al.*, 2011, Piao *et al.*, 2014, and Huang *et al.*, 2014). This study is the first to demonstrate the cytotoxic effect of aescin on KB and HEp-2 through the induction of apoptotic cell death which was the common molecular mechanism of anticancer agents (Yuan *et al.*, 2017). We report that treatment with aescin compared with cisplatin induces morphological changes, cytotoxic and apoptotic effects on KB and HEp-2 cells (Fig.1). Our results showed that aescin was cytotoxic to human oral and laryngeal cancer cells (KB and HEp-2) after a 24-h treatment with IC50 values of 25.6±1.90 and 35.0±1.90 μM, respectively by the correlation of Alamar Blue based fluorescence (Fig.4.A&B) and β-hexosaminidase (Fig.5. A&B) with cell viability (MTT) (Fig.3. A&B) and trypan blue dye exclusion method (Fig. A&B). Thus, in standard curves, the MTT assay and trypan blue assay was less sensitive then Alamar blue redox reaction and hexosaminidase assay. The major finding of this study is that Alamar blue, and β-hexosaminidase a colorimetric and fluorometric redox indicator and inflammatory mediator, provides a rapid, quantitative index of cell viability in a culture that can be used to detect cellular damage in KB and HEp-2 cells.

CONCLUSION

This suggests that aescin-treated KB and HEp-2 cells have relatively low mitochondrial enzyme levels or activity in vitro, despite their ability to proliferate rapidly. In conclusion, aescin has potent anti-proliferative effects against KB and HEp-2 cells through morphological changes and mitochondrial-mediated apoptosis. These effects are dose and time-dependent. Also, aescin caused apoptotic effects which were slight intensely observed in HEp-2 cells compared to KB cells. Overall the findings of these studies suggest that aescin may be a suitable chemotherapeutic agent for Head and neck cancer as well as other cancer also.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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