ANTI-CANCER ACTIVITY OF CASSIA AURICULATA LEAF EXTRACT ON ORAL SQUAMOUS CELL CARCINOMA CELL LINES - AN INVIVO AND IN VITRO STUDY

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ABSTRACT:
Objective: To evaluate the anticancer activity of Cassia auriculata leaf extract on Oral Squamous Cell Carcinoma cell lines.
Material and methods: Biopsy of oral cancer tissues samples were taken, processed and cell lines were developed. Active component of Cassia auriculata was identified and the cell lines were treated with active component at 25µg, 50µg &100µg and Cisplatin at4µg, 6 µg &8 µg at 24,48 and 72 hours and cytotoxicity and apoptotic activity was assessed with Propidium Iodide and Acridine Orange and Ethidium Bromide staining.
Results: The study revealed that the p values obtained were less than 0.05 at concentration 25µg, 50µg &100µg concentration of the test component at 24, 48 and 72 hours and on comparison the promising dose was identified to be 6 µg of Cisplatin and 50 µg/ml of the active component was sufficient to induce cytotoxicity at all 3 time periods. Significant amount of PI positive cells along with significant cells in AO/EB staining was noted. Cassia auriculata's active component decreased the cancer cell viability with increase in concentration in a more efficient manner when compared to Cisplatin. Thus Cassia auriculata can be used as an effective anti cancer drug will lesser side effects.
Key words: Oral cancer, Cassia auriculata, Cisplatin & apoptosis

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INTRODUCTION:

Oral Squamous Cell Carcinoma (OSCC) is the most common type of cancer in the Head and Neck region and it ranks as the 6th most common type of cancer in the world [1]. It poses a major challenge due to high morbidity and mortality rates[2]. The etiological factors of OSCC include habits such as tobacco, alcohol consumption and chronic irritation from ill-fitting dentures, sharp or fractured

teeth[3]. The evolution of OSCC per say is a multi phase process involving the theory of field cancerization in tandem with the combination of individual's genetic predisposition along with the above-mentioned etiological factors and due to this, activation of mutation and oncogenes takes place resulting in cell growth and proliferation[4]. Combination therapies of surgery, chemotherapy and radiotherapy are the standard canonical treatment for cancer[5] but these methods are associated with side effects such as esthetic and functional loss[6]. Although these standard methods are only palliative line of treatment no measures have been made till date to treat cancer in both curative manner at the same time lessening the side effects. Herbal preparations using medicinal plant extracts have been in use for a very long time in India as an acceptable, non-invasive and a cost- effective method[7] Cassia auriculata is one such medicinal plant used in traditional medicine[8.] The natural extracts of this plant is proven to have anti-microbial, anti-inflammatory, antioxidant, anti-hyperlipidemic, antipyretic and anticancer effects[9]. The leaf extract is shown to have cytotoxic effects in the dose relying manner in the human breast adeno carcinoma and human larynx carcinoma[10]. The role of Cassia auriculata on oral cancer is not yet studied.

Hence the present study was done to assess the anti-cancer activity of Cassia auriculata leaf extract on Oral Squamous Cell Carcinoma cell lines.

MATERIALS AND METHOD:

This study was approved by the ethical committee of SRM University, SRMDC/IRB/2020/MDS/NO.604

Materials used:

Source of samples:

OSCC tissue samples were obtained from biopsy.

Plant component:

Identification of CALE (Cassia auriculata Leaf extract) active component was based on the literature. It was identified to be 3-O-Methyl-D- Glucose[11]3-O-Methyl-D- Glucose (sigma Aldrich). To prepare the aqueous extract the powder was dissolved in a suitable amount of distilled water and was used in the concentration of 1mg/ml.

Methods:

Establishment of cell culture:

The tissue samples from biopsy were washed with PBS, Betadine and boric acid then minced washed

and centrifuged. A solution containing mixture of DMEM, collagenase and hyaluronidase was used to separate the ground substance. The cells were then filtered, characterized morphologically and maintained at temperature of 37°C with 5% CO_2 in 95% air humidified incubator. The cells were subculture for multiple passages. The cells were quantified based on the confluence. Those cells that had attained 70-80% of confluence was used for further procedures.

Testfor cytotoxicity induction and IC 50 determination:

Upon reaching the confluence of 80% the cells were seeded on to 96 well plates and incubated. The cells were then subjected to 25µg, 50µg &100µg of 3-O-Methyl-D- Glucose and 4µg, 6 µg &8 µg of Cisplatin as control for 24, 48 and 72 hours. At the end of each time period 20 µl MTT solution was added, incubated for 4 hours followed by addition of 200 µl of DMSO for dissolving the Formazan. The optical density of each well was spectrophotometrically measured at a wavelength of 570 nm by an ELISA microplate reader. Inhibitory dose(IC 50) was measured by standard curve method.

Apoptotic activity assessment of by Acridine orange /Ethidium Bromide dual staining [AO/EB]&Propidium Iodide [PI] staining:

The cells were treated with IC 50 doses of the test and the control extracts then stained with 20µl of AO/EB solution and PI stain for 5 minutes. The stained cells were viewed under a fluorescence microscope (Invitrogen EVOS FL imaging; 40X magnification).

Statistical analysis:

Statistical analysis of the data obtained was performed using SPSS 22 software. The descriptive statistics such as mean and standard deviations were calculated for the individual groups. the confidence interval was set at 95%. Since the data did not follow the normal distribution, Kruskal - Wallis test and t-test was done to analyse the difference between individual groups. Significance was kept at 0.05. Data <0.05 were considered statistically significant.

RESULTS:

Cytotoxicity of 3-O-Methyl-D- Glucose in OSCC cell lines:

The cells were incubated with the concentration of 25µg, 50µg &100µg of 3-O-Methyl-D- Glucose for 24, 48 and 72 hours [Table1]. The mean viability of cells decreased with increasing concentration of 3-O-Methyl-D- Glucose. Maximum reduction was seen at 100 µg at which 11.26% of the cells were viable at 72 hours [Figure 1]. IC treated cells [Figure 3A].

Cytotoxicity of Cisplatin in OSCC cell lines:

The cells were incubated with the concentration of 4µg, 6µg & 8µg of Cisplatin as control for 24, 48 and 72 hours [Table 2]. The mean viability of cells also decreased comparably with increasing

concentrations. Maximum reduction was seen at8µg at which 17% of the cells were viable at 72 hours [Figure 2]. IC treated cells [Figure 3B].

Comparison between concentrations and time periods:

Cell viability decreased with increasing concentration of the test and the control extracts. On statistical analysis significant p value of 0.05 was obtained at concentrations 6 µg and 50 µg/ml in all the three time periods (24, 48 and 72 hours) [Table 3]suggesting a promising dose of 6 µg/ml of Cisplatin and 50 µg/ml of 3-O-Methyl-D- Glucose can induce cytotoxicity in a very effective manner.

Apoptotic activity assessment:

With PI staining increased red fluorescence with PI positive cells [Figures 4A&4B]was seen in 3-Methyl-D- Glucose treated cells suggesting higher cell damage and cell death in comparison with Cisplatin. AO/EB staining [Figures5A&5B] revealed 3- Methyl-D- Glucose treated cancer cells with a light green nucleus and late apoptotic cells showing bright orange sites of condensed chromatin with nucleus that delineate them from necrotic cells with orange-reddish fluorescence. Necrotic cells were fluorescent red. The above changes were more prominent in the 3- Methyl-D- Glucose when compared to Cisplatin.

Table 1: Table depicting the 24 hours, 48 hours and 72 hours alteration in viability of Oral Squamous Cell Carcinoma Cells on exposure to increasing concentration of Cassia auriculata.

24HOURS	CONTROL	25 µg	50 µg	100 µg
	0.731	0.453	0.213	0.13
	0.734	0.429	0.197	0.118
	0.732	0.445	0.21	0.127
MEAN	**0.732**	**0.442**	**0.206**	**0.125**
SD	**0.0015**	**0.0122**	**0.00850**	**0.0624**
48 HOURS	**CONTROL**	**25 µg**	**50 µg**	**100 µg**
	0.693	0.327	0.189	0.214
	0.698	0.322	0.17	0.121
	0.697	0.332	0.14	0.129
MEAN	**0.697**	**0.327**	**0.166**	**0.12**
SD	**0.00265**	**0.00500**	**0.0247**	**0.0040**
72 HOURS	**CONTROL**	**25 µg**	**50 µg**	**100 µg**

	0.632	0.191	0.146	0.072
	0.639	0.194	0.115	0.089
	0.637	0.198	0.11	0.077
MEAN	**0.636**	**0.1943**	**0.123**	**0.08**
SD	**0.00361**	**0.00351**	**0.01950**	**0.00854**

Figure 1: Graphical representation of alteration in viability with increase in concentration of Cassia auriculata over 24, 48and 72 hours.

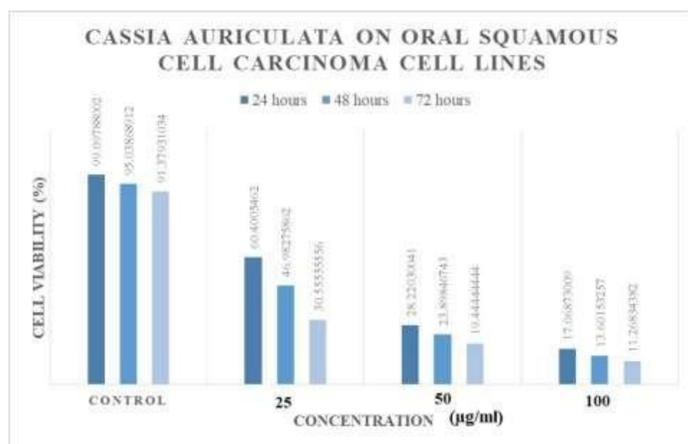

Table 2: Table depicting the 24 hours, 48 hours and 72 hours alteration in viability of Oral Squamous Cell Carcinoma Cells on exposure to increasing concentration of Cisplatin.

24 HOURS	**CONTROL**	**4 µg**	**6 µg**	**8 µg**
	0.731	0.492	0.245	0.159
	0.739	0.497	0.263	0.153
	0.732	0.507	0.256	0.152
MEAN	0.734	0.498	0.254	0.154
SD	0.00436	0.00764	0.00907	0.0037
48 HOURS	**CONTROL**	**4 µg**	**6 µg**	**8 µg**
	0.708	0.39	0.215	0.197
	0.699	0.387	0.233	0.191
	0.689	0.371	0.226	0.176
MEAN	0.7016	0.3826	0.224	0.188
SD	0.0055	0.0102	0.0090	0.018
72 HOURS	**CONTROL**	**4 µg**	**6 µg**	**8 µg**

	0.659	0.281	0.195	0.105
	0.651	0.287	0.193	0.112
	0.648	0.284	0.196	0.116
MEAN	**0.652**	**0.284**	**0.194**	**0.111**
SD	**0.0056**	**0.003**	**0.00153**	**0.0055**

Figure 2: Graphical representation of alteration in viability with increase in concentration of Cisplatin over 24, 48and 72 hours.

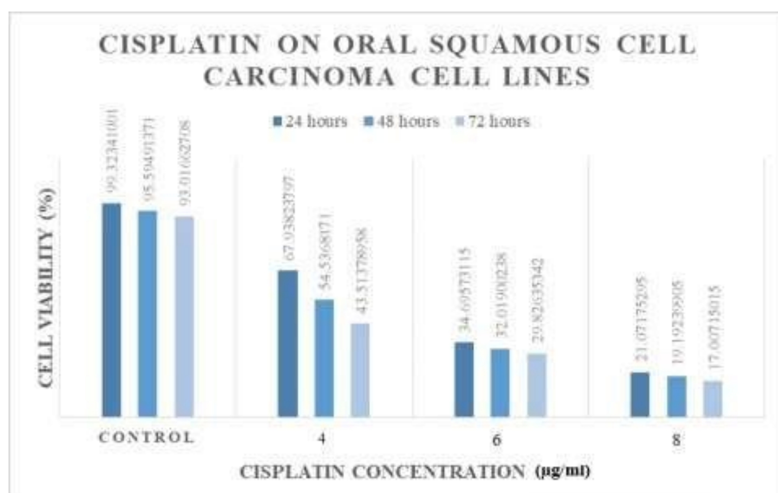

Figure 3: 3A-Microscopic images depicting post treatment of the cells with IC50 concentration of 3-Methyl-D-Glucose , 3B - post treatment of the cells with IC50 concentration of Cisplatin.

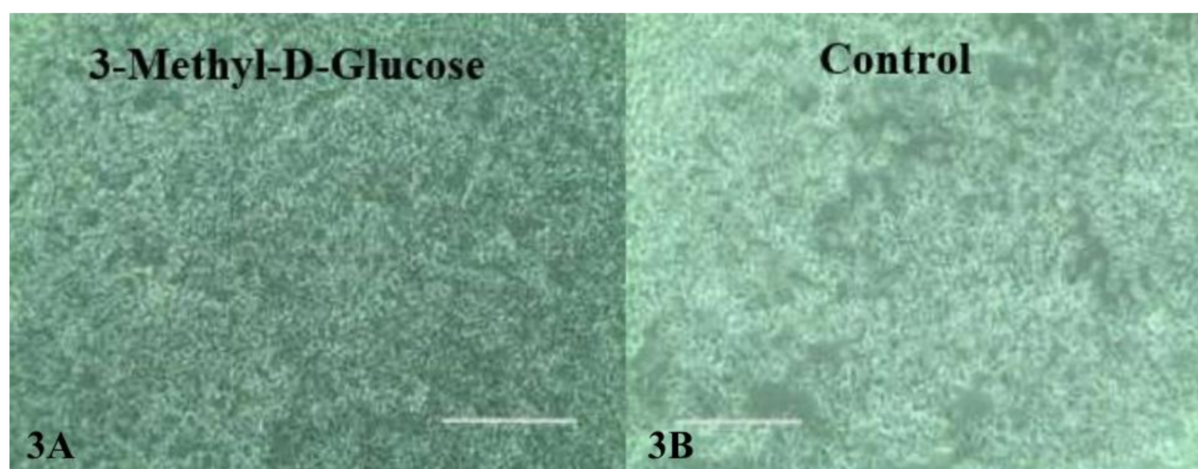

Table 3: Table depicting the comparison between inter groups for dosage and time periods to identify the promising dose of 3-O-Methyl-D- Glucose and Cisplatin

TIME PERIOD	INTERGROUPS	t-VALUE	MEAN DIFFERENCE	P VALUE	SIGNIFICANCE

24 HOURS	CONTROL	0.625	0.00167	0.566	Not significant
	4 µg&25 µg	6.771	0.05633	0.002	Significant
	6 µg& 50 µg	6.685	0.04800	0.003	Significant
	8 µg& 100µg	7.036	0.02967	0.002	Significant
48 HOURS	CONTROL	1.606	0.00567	0.210	Not significant
	4 µg&25 µg	8.478	0.0567	0.004	Significant
	6 µg& 50 µg	3.839	005833	0.18	Significant
	8 µg& 100µg	9.500	0.633	0.001	Significant
72 HOURS	CONTROL	4.287	0.01667	0.01	Significant
	4 µg&25 µg	33.625	0.08967	0.13	Not significant
	6 µg& 50 µg	6.286	0.07100	0.02	Significant
	8 µg& 100µg	5.265	0.03100	0.006	Not significant

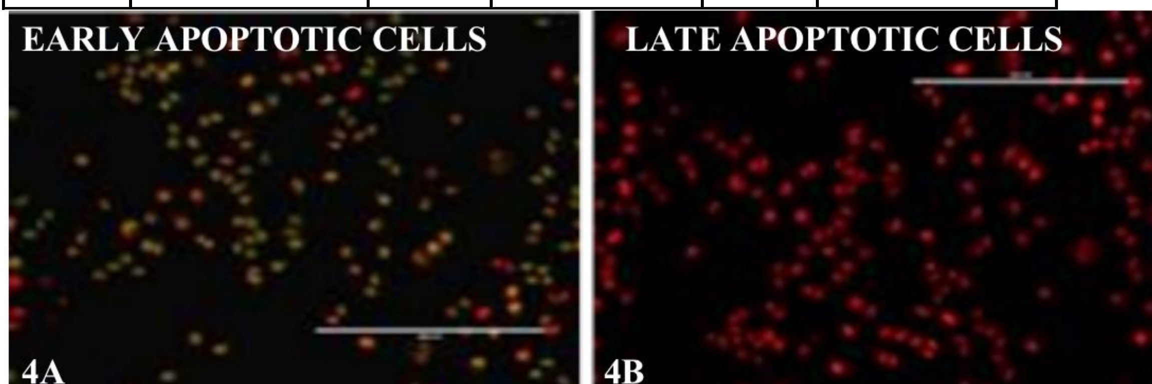

Figure 4: Images depicting AO/EB and PI-stained cells showing 4A-early and 4B-late apoptosis in the 3- Methyl –D-Glucose treated cells.

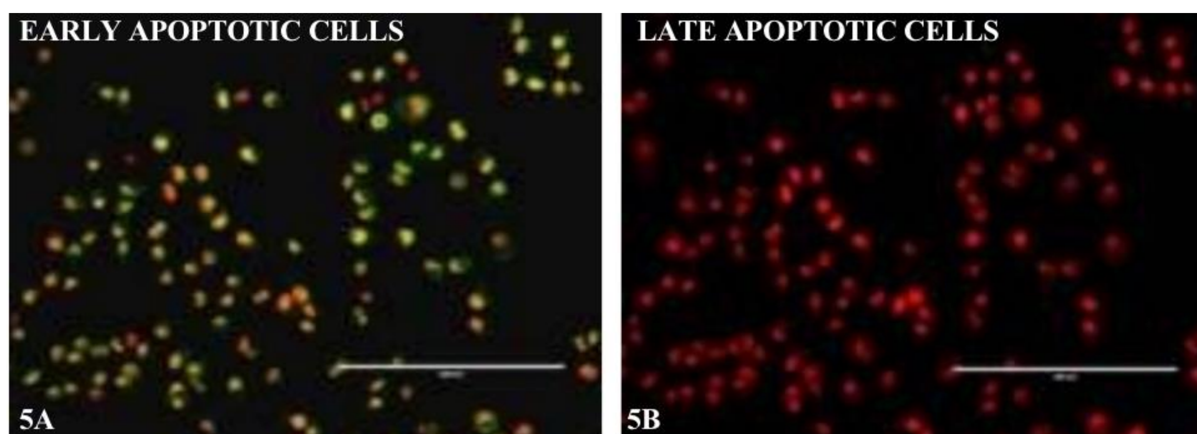

Figure 5: Images depicting AO/EB and PI-stained cells showing 5A-early and 5B-late apoptosis in the Cisplatin treated cells.

DISCUSSION:

Oral cancer is a multifactorial disease that is characterized by abnormal and uncontrolled proliferation of cells. But, most of the patients are unaware to even recognize the early symptoms of this tumor development. Though there are conventional modes of treatments available the side effects posed by these treatments call for alternative adjunctive therapeutics and this is where the traditional ayurvedic plant-based medicines play an important role.

In the present study we have assessed the anti- cancer property of one such medicinal plant i.e Cassia auriculata against the oral cancer cells. We found that the active component of Cassia showed notable cytotoxicity against the cancer cell lines when compared to the control. Despite the fact that there are no similar papers to converse about the cytotoxic effect of 3-Methyl-D-Glucose on Oral Squamous Cell Carcinoma cells, there are limited researches which have assessed the cell viability in different cancer cell lines and with different parts of the Cassia. **R. Prasanna et al**[10]used the ethanolic extract of the 3-Methyl-D- Glucose and assessed the Cytotoxic effect on the MCF -7 and Hep-2 cells with HBL- 100 and Vero cells as standards with the IC $_{50}$ values of 400 and 500 µg/ml. Cytotoxicity was observed in a dose dependent manner which was similar to our study. **V Duraipandian et al** [12]assessed the anticancer activity of Rhein – a component of the Cassia auriculata flower and tested against the human colon adenocarcinoma cell lines and the normal VERO cell lines, and found that Rhein exhibited minimal cytotoxicity towards the normal cell lines and the maximum toward the human colon adenocarcinoma with the IC $_{50}$ Values of 100, 25, 15 and 12.5 µg/ ml for varying time periods. **Esakkirajan et al**[13] assessed the apoptosis mediated anti proliferative effect of compound isolated from Cassia auriculata leaves against human colon cancer cell line and they

found that the isolated leaf component exhibited dose dependent decrease in the cell viability. All these studies prove that Cassia auriculata extracts exhibited significant cytotoxicity against the cancer cell lines in a dose dependent manner.
The promising dose of the active component was observed by comparison between the groups and identifying the levels of significance. There are no relevant previous studies that have assessed the effective dosage of Cassia's active component against the cancer cells and in our study, we have made a novel attempt to identify the promising dosage against the OSCC cell lines, and it was found to be 50 µg/ml of 3-O-Methyl-D- Glucose which was sufficient to induce cell death. These findings mark our study unique from the other studies.

Further more in our study we assessed the apoptotic activity by use of Propidium Iodide together with AO/EB staining for examination of early and late apoptosis. The morphological characters of apoptosis are different from necrosis[14].We found that apoptotic cells had condensed chromatin with fragmented nucleus. A morphologically significant number of PI positive cells, light green colored early apoptotic cells, bright orange sites of condensed chromatin which suggested late apoptosis and cells with orange-reddish fluorescence were seen. All these changes were quantitatively increased in cells treated with 3-O-Methyl-D- Glucose when compared to Cisplatin thus suggesting the significance of Cassia auriculata. Similarly **Esakkirajan et al**[13]in his study examined the anti-proliferative property of the isolated component of the Cassia leaves against the Human Colon Cancer Cell lines (HCT 15). He found that the cells treated with 20 and 100 µg /ml concentration of the isolated component of Cassia exhibited apoptosis and found significant increase in the number of PI positive cells indicating the apoptosis potential and they also performed Lactate dehydrogenase assay where the leak of lactate dehydrogenase through cell membrane due to necrosis or apoptosis was also noted. **R. Prasanna et al** [10] had assessed the anti-cancer activity of CALE against MCF-7 and treated with Propidium Iodide and the apoptosis was assessed along with flow cytometry to investigate the cell cycle regulation and they found out that MCF and Hep – 2 showed increased number in apoptotic cells characterized by nuclear condensation and fragmentation when compared to controls which is similar to our study. Flow cytometry revealed significant reduction in the DNA content in the MCF cells noted at sub-G_0/G_1 or A_0 region that indicated apoptosis with failure at G_1 phase.

LIMITATION:

The limitation of our study is that the findings are based on cytotoxicity, or the cell viability

assays genetic aspects such as the molecular part is not assessed.

CONCLUSION:

Our study was an attempt to evaluate the anti-cancer property of the Active component of Cassia auriculata leaf extract. The results showed a dose dependent increase in the anti-cancer property of Cassia auriculata leaf extract and showed significant apoptotic effect compared to Cisplatin thus proving its efficacy as an emerging anti- cancer drug which can be considered after further validation in the treatment of OSCC.

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