EVALUATION OF ANTI CARCINOGENIC ACTIVITY OF *TRIFOLIUM PRATENSE* ON ORAL CANCER CELL- AN IN VITRO STUDY

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TYPE OF STUDY: Original research

Running title : Evaluation of anti carcinogenic activity of *trifolium pratense* on oral cancer cell

ABSTRACT :

Introduction:

Trifolium pratense also known as the red clover is widely distributed in the tropics and in the subtropical regions. It is generally consumed in the form of tea by the northern states of India and some tribal people of Nepal and Bhutan. Studies reveal that it is rich in antioxidant and anti-inflammatory activity. It is due to the presence of unique isoflavones found in Trifolium pratense are Biohanin A and formononetin.

Aim :

The main aim of the study is to find out whether Trifolium pratense extract has antiproliferative activity against oral squamous carcinoma cells .

Materials and methods :

The dried buds of Trifolium pratense flowers were purchased commercially and then powdered Then MTT assays was carried out to find out it's inhibitory activity against oral carcinoma cells

Results and discussion:

From the assay it is evident that it shows a potent inhibitory activity against oral squamous carcinoma cells . Linear regression analysis revealed that the IC50 was found to be at 53.13µg/ml which is higher than that of other species of this family.

Conclusion :

From the above study it is evident that Trifolium pratense has a very good inhibitory activity and hence can be used in the treatment of oral cancer.

Keywords : Trifolium pratense, oral squamous cell carcinoma, Anticarcinogenic activity,BiohaninA, MTT assay, innovative technique.

INTRODUCTION

Phytochemicals are a special type of organic compounds that are naturally present in plants.[1,2] **Nearly 1,000 phytochemical compounds with anti carcinogenic properties have been identified but only few are clinically used.**[3] **The use of synthetic chemotherapeutic ways in treatment of cancer patients leads to side effects such as nausea,vomiting, diarrhea, alopecia,bleeding, bone marrow destruction etc.**[4,5]**Drugs such as Methotrexate,Mechlorathamine have a strong effect on the patients immune system by destroying the leukocytes and making them immune compromised . Also the**

use of these drugs may also lead to systemic toxicity which may sometimes become unnoticed and make the patients life under risk.[6,7] **Hence the use of plant extract is essential as they have less toxicity or non toxic and less side effects when compared to chemically synthesised drugs.**[8]

The plant Trifolium pratense also known as the red clover is a short perennial flowering herb generally found in the tropics and in the subtropical regions.[9]**The unique isoflavones found in Trifolium pratense are Biohanin A and formononetin. Other isoflavones are found in leaves include daidzein , genistein, pratensein, prunetin, irilin B, calycosin, methylorobol, afrormosin, texasin, pseudobaptigenin irilone and flavonoids (for example, quercetin and kaempferol). Also the plant contains other phenolic substances such as phenolic acids (caffeic, rosmarinic and chlorogenic acid)**[10]**.**[11] **Other members of the Trifolium species exhibited biologically active activities including antioxidant activity, anticestodal activity, cytostatic activity,anti-inflammatory activity, cytotoxic activity and estrogenic activity. These plant species extracts are used as a chemoprotective agent against some cancers and cardiovascular diseases in some Ayurvedic medicines.**[9][12]**The plant extract is a primary drug of choice for the treatment of menopausal symptoms. The presence of phytoestrogens also acts as an effective antioxidant as it has tyrosine kinase inhibitory activity. Research proves that these phytochemicals have the capacity to correct the damage caused by UV-radiation-induced oxidative damage to DNA.**[9,13–15]**. Our team has extensive knowledge and research experience that has translate into high quality publications**[16–27]**.**[28–32]

Hence the main aim of the study is to evaluate the anti carcinogenic effect of Trifolium pratense on oral cancer cell-an invitro study

MATERIALS AND METHODS

The dried form of red clover flower (Trifolium pratense) is purchased commercially.

Cell preparation and culturing:

The SCC – 25 oral squamous carcinoma cell lines was procured from ATCC with the passage number of 26. Cells were maintained in Dulbecco's Minimum Essential Media (DMEM) and Ham's F – 12 (1:1 ratio) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/mL penicillin and 100µg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO2 at 37 °C. Cells were grown in 75cm 2 culture flasks and after a few passages, cells were seeded for experiments. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using 0.25% Trypsin-EDTA solution.

Cell proliferation assay or MTT assay:

Proliferation of oral squamous carcinoma cells was assessed by MTT assay (Macedo et al., 2019). The proliferation test is based on the colour reaction of mitochondrial dehydrogenase in living cells by MTT. Cells were plated in 96-well plates at a concentration of 5 × 104 cells/well 24 h after plating. After 24h of cell incubation, the medium was replaced with a 100µl medium containing Trifolium procumbens extracted at different concentrations (0.1 – 1000µg/ well) and incubated for 24h. Untreated cells served as control and received only 0.1% DMSO in which the extract was prepared. At the end of treatment period, media from control, Trifolium extract-treated cells was discarded and 50µl of MTT (5mg/ml PBS) was added to each well. Cells were then incubated for 4h at 37°C in the CO2 incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 150µl of DMSO and mixed effectively by pipetting up and down. Spectrophotometric absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570nm . Optical density of each sample was compared with control optical density and graphs were plotted.

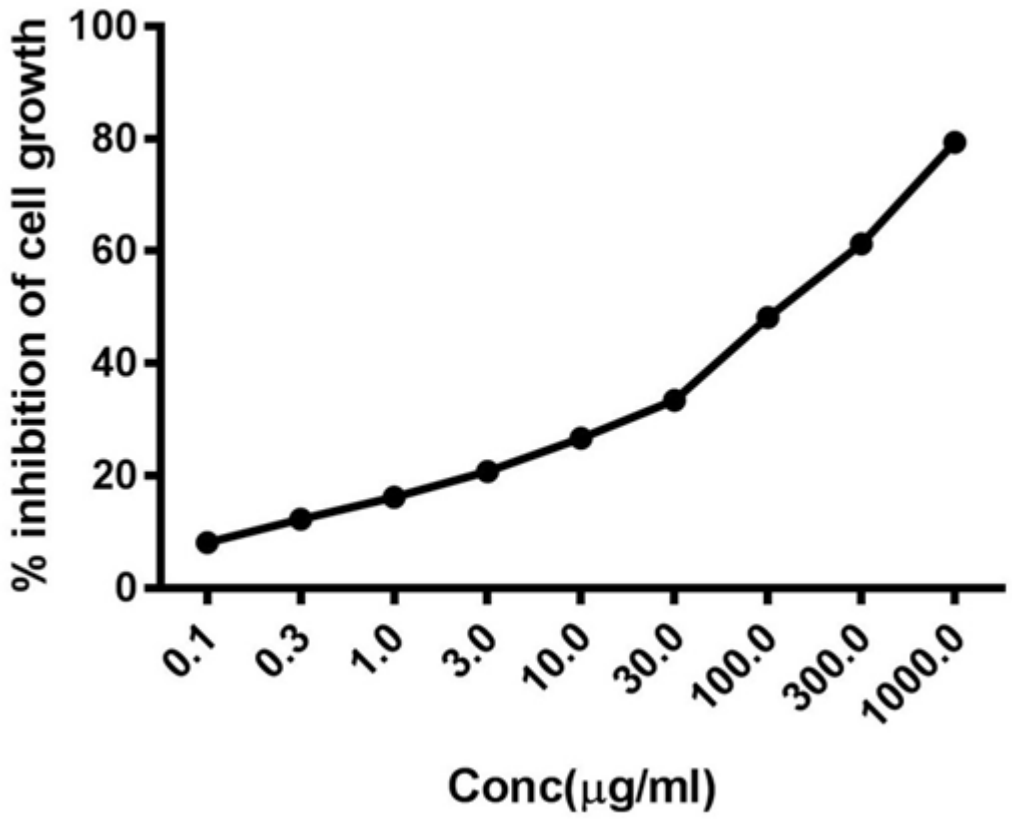
STATISTICAL ANALYSIS:

Data were expressed as mean ± S.E.M and analysed by Tukey's test to determine the significance of differences between groups.

RESULTS :

Inhibitory effect of Trifolium pratense extract against human SCC - 25 oral carcinoma cells

The inhibitory effect of Trifolium procumbens extract was evaluated in SCC - 25 cell lines at a concentration range of 0.1 - 1000 µg/ml. The results demonstrated that Trifolium pratense extract was able to inhibit the proliferation of oral squamous carcinoma cells. Maximum inhibition was found to be 79.37% at a concentration of 1000µg/ml. The IC50 was calculated by linear regression analysis and was found to be 53.13µg/ml. The p value was found to be 0.007 which is statistically significant.



Graph 1: Inhibitory effect of Trifolium procumbens extract against human SCC - 25 oral carcinoma cells

Concentration (µg/ml)	% inhibition of cell growth
0.1	8.15±0.03
0.3	12.26±0.06

1	**16.24±0.29**
3	**20.76±0.06**
10	**26.74±0.48**
30	**33.43±0.38**
100	**48.20±0.13**
300	**61.25±0.22**
1000	**79.37±0.38**

Table 1: Inhibitory effect of Trifolium procumbens extract against human SCC - 25 oral carcinoma cell

Discussion :

In the present study, we illustrated that the extract of Trifolium pratense acts as a good inhibitor of the proliferation of the oral squamous cell carcinoma. Previous enzyme kinetic analysis reveals that inhibition of proliferation involves both competitive and non-competitive inhibitions[33]**.The various phytochemicals present in the plant have good antioxidant and antidiabetic activity and can be used as a potent therapeutic drug. The chief phytochemical Biochanin A suppresses testosterone-induced MCF-7aro cell proliferation, which was attributed to the reduced aromatase activity. Study done by Ramos et al 2012 shows that at the transcriptional level, the phytocompounds reduced the aromatase mRNA abundance in the breast cancer cell line SK-BR-3**[34]**. The study on oral cancer cell lines reveals that the IC50 was found at 53.13 µg/ml. In comparison with nutmeg the IC50 of Trifolium pra tense is more proving that it can be potentially used as an effective drug in the management of oral cancer**[35]**.**

The work done shows that the phytocompound biochanin A at 100 nm and 10 µm was found to be ineffective in inhibiting CYP19 at the enzyme and expression levels in human granulosa-lutein cells. In comparison with genistein, on the other hand, displays a similar suppressive effect on CYP19 in the former study. Similar study done by Ferris et al 2020 on extract of red wine proves that the flavonoids present in red wine inhibits aromatase activity, and reduces mammary hyperplasia in transgenic mice over-expressing CYP19[36]**). The active phytochemical compounds may be procyanidin B dimers and resveratrol. In the present study biochanin A was the only isoflavone demonstrated to inhibit the enzyme activity. Many studies have been reported on biochanin A's chemopreventive effect on breast cancer cell lines Sosnowoki et al**[37]**. This isoflavone shows that it can protect against NDEA induced hepatocellular carcinoma in rats, and mammary tumour virus-induced spontaneous breast cancer in mice. From all these studies it is evident that Trifolium species are a potent natural source of iso- flavonoids and can be used in the treatment of various diseases**[38]**. They have been used as a traditional medicine to treat a variety of disorders. Traditional application of this plant species yields a better effect on humans. However combining the extract with other inorganic chemicals like methanol or ethanol or even with metallic nanoparticles may yield a better result .**

The concentration of soyasapogenol glycosides in the seeds of Trifolium species is similar to the concentration in other leguminous plants. The high concentration of quercetin and the presence of soyasapogenol B glycosides make the seeds of some Trifolium species a promising plant material to be used in human nutrition as nutraceuticals or food additives some of the Trifolium species exhibited other biological activities such as antiinflammatory activity, antioxidant activity, anticestodal activity, cytostatic activity, cytotoxic activity and estrogenic activity ([39]

Phytochemical analysis reveals that the plant T. pratense is one of the most important sources of phytoestrogens in nature. It is mainly due to the isoflavones and coumestans . However, further research must be carried out by obtaining extract from different parts of the plant and whether the combination of Trifolium pratense extract along with nano particles have an increased effect or not. This can also be studied on other cancer cell lines and thus can be used as an alternative drug rather than commercially used synthetic chemotherapeutic drugs.

Limitations: As the study was done in an in vitro manner it must be carried on ex-vivo also . Further its biocompatibility to normal human cells must also be assessed before formulating thia extract into a conventional medicine .

Conclusion:

From the above results it is evident that as the concentration of the extract increases, the inhibitory activity of the extract also increases and the inhibitory concentration 50 was found to be at 53.13µg/ml which is comparatively better than that of the other plants of the same species thus proving that the extract of Trifolium pratense shows a very good inhibitory activity against oral squamous cell carcinoma cells and thus it can be formulated and used in the treatment of oral cancer .

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Conflict of Interest : Nil

Author contributions:

Author Name

1 . V.A.Muralidharan -Study design, data collection, drafting manuscript

2 . Dr. R.V.Geetha -Revising manuscript, final approval of manuscript

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