RESEARCH ARTICLE

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Antifibrotic Effect of Baicalin on Arecoline Induced Human Oral Fibroblast: An *In-Vitro* Study

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Abstract

Background: *Baicalin* is a flavonoid obtained from the Chinese herb *Scutellaria baicalensis*, which has a wide varieties of health benefits and scope to be studied for its therapeutic potential in oral fibrosis. **Aim:** The aim of the study was to investigate the antifibrotic effect of a *Baicalin* in arecoline induced human oral fibroblast in vitro setting. **Material and Methods:** Arecoline and ethanolic extracts of *Baicalin* were commercially purchased from Sigma-Aldrich. Human oral fibroblasts were cultured and characterized with specific fibroblast markers, and cells were stimulated with arecoline. An MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was executed to determine the half-maximal inhibitory concentration of arecoline and *Baicalin*. Arecoline-induced cells (25µg/ml) were treated with a non-toxic dose of *Baicalin* (proliferative dose of 25µg/ml). Cytokine (CCL2, CXCL-8, IL17, IL-beta, and IL-6) and fibrotic marker genes were studied by reverse transcription-polymerase chain reaction (RT-PCR). The inhibitory effect of *Baicalin* was studied to prove its antifibrotic properties. **Results:** Arecoline significantly upregulated all inflammatory and fibrotic markers. On treatment with 25µg/ml of *Baicalin*, all inflammatory and fibrotic markers were inhibited. Arecoline affects fibroblast morphology, supporting the fact that arecoline is cytotoxic to cells. **Conclusion:** *Baicalin* can be used as an antifibrotic herb to treat OSMF.

Keywords: *Baicalin*- Antifibrotic- Arecoline- Oral submucous fibrosis (OSMF)- Herbs- Alternative medicine

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Introduction

Oral Submucous Fibrosis (OSMF) is one such disease, which is an insidious, chronic, complex, crippling, debilitating, irreversible, persistent, progressive, scarring, potentially malignant, and collagen metabolic disorder; primarily affecting the oral cavity and occasionally the pharynx and esophagus; wherein the oral mucosa undergoes various changes like inflammation, ulcerations, pigmentation, loss of flexibility, and elasticity, leading to a significant blanched and fibrosed appearance, leading to inflexibility and progressive inability to open the mouth [1].

Tissue Fibrosis and hyalinization are the most fundamental clinic pathological features of OSMF, which prominently affect the patients' quality of life. Myofibroblast and the regular expression of α-smooth muscle actin (α-SMA) are reflected as signs of advanced fibrosis and are said to cause the alteration of OSMF microenvironment, leading to tumorigenesis [2]. Potentially malignant diseases are the term used to describe these warning lesions (PMD). One of the most common diseases (PMD) detected in the oral mucosa is oral submucous fibrosis (OSF) [3].

Arecoline is the most abundant of all areca alkaloids and has been studied and researched as a possible carcinogen. Cytotoxicity and genotoxicity of areca nut to oral fibroblasts and keratinocytes lead to inhibition of growth, attachment, and matrix protein synthesis in cultured human gingival fibroblasts. Arecoline is proven to be mutagenic and can cause sister chromatid exchanges, chromosomal aberrations, and micronuclei in various types of cells. Such arecoline induced effects could somewhat explain the mechanism of malignant transformation in OSMF [4].

Myofibroblast are a unique population of smooth muscle-like fibroblasts, occasionally called stellate cells due to their shape. These cells play an important role in growth factor secretion, the deposition of matrix, and its degeneration. It's alleged that fibrosis in OSMF is due to the pulling of myofibroblast from surrounding tissue in the wound area [5], anticipated human xylosyl transferase-I activity along with α SMA expression as a novel biomarker for myofibroblast [6]. The trans differentiation of fibroblasts to myofibroblast, mediated by growth factors and cytokines expressed by tumor cells, is believed to be an early and key event in carcinogenesis. Two important events, stromal deviations driving invasion and metastasis,

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are hallmarks of malignancy [7].

Several treatment options available, such as intralesional injections of steroids, hyaluronidase, human placental extracts, collagenase, pentoxifylline, iron, and multivitamin supplements, along with surgical approaches (removal of fibrotic bands) and laser ablation, have been tried time and again for improving the mouth opening, but have proven ineffective, as the fibrotic bands recur. Repetitive submucosal injections and multiple needle pricks lead to trismus, which leads to more fibrosis and increases morbidity after a certain period. Western medicines (pharmaceutical preparations) are loaded with chemicals; they cause unbearable short- and long-term side effects. Due to the dearth of antifibrotic medications in Modern medicine, it would be advantageous to develop a natural form of treatment, such as herbal medicine or herbal therapy. There is evidence to support the effectiveness, curativeness, and economic viability of using traditional medicinal herbs and active secondary metabolites (Ayurveda, Siddha, Unani, and Chinese) [8].

Georgi-*Baicalin* is an active flavonoid from the herb *Scutellaria baicalensis*, which has been shown in studies to have anti-inflammatory and antioxidant activities in liver problems. It has been shown that *Baicalin* reduces liver fibrosis, which is thought to be caused by the suppression of TGF-β1, α-SMA and COL1A2 [9]. So, we hypothesized that *Baicalin* would have similar antifibrotic properties in Oral fibrosis.

Materials and Methods

This study was approved by the Institutional Ethical Committee (IEC) in DYPV/252/2019. The study was carried out at the Regenerative Medicine Laboratory, part of the Central Facility of Dr. D.Y. Patil Vidyapeeth, Pune, under all standard guidelines and protocols. Arecoline and ethanolic extracts of *Baicalin* (Sigma-Aldrich 572667) were commercially purchased from Sigma Aldrich, Bangalore, and Karnataka, India.

Primary cell culture

The isolation of gingival tissue was carried out by informed consent from patients. Tissue samples were collected from patients who underwent third molar impaction surgery or any other minor surgical procedure at the Department of Oral and Maxillofacial Surgery. Gingival tissue was washed with PBS (containing antibiotics and antimycotic agents) to remove the blood and impurities. Cell isolation was carried out by using the Expand Cell Culture method described by Chiu et al. [10]. The cell outgrowth was monitored under a microscope after passage 4, cells were used for fibroblastic characterization by using surface marker analysis.

Cytotoxic assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)

The monolayer cell culture was trypsinized, and the cell count was adjusted to 1 x 105 cells/ml using medium containing 10% FBS (ThermoFisher-Catalog Nos-26140). To each well of the 96 well microtiter plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added for 24hrs at 37°C and 5% CO_2. After 24 hours, cells were tittered with different concentrations of Arecoline and *Baicalin*, and the plates were incubated for 48 hours in a CO2 incubator. After 48 hours, the cells were treated with MTT, and the plate was incubated for 3 hours in a CO2 incubator. DMSO (ThermoFisher-Catalog Nos- D12345) was used as a soluble agent for the MTT assay [11]. The absorbance was measured using a microplate reader at a wavelength of 560 nm to determine the cytotoxicity. The IC_{50} values were calculated using Graph Pad Prism 4 software.

Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

To evaluate the expression of cytokines, trizol reagent was added to the culture plate of the treated cell palate collected for gene expression analysis. The RNA expression was carried out as described previously [12]. cDNA synthesis for RTPCR was performed using Verso thermo scientific DNA synthesis kit (ThermoFisher-Catalog Nos-AB1453A).

Statistical analysis

Statistical analysis for the study was done using Statistical Package for Social Sciences (IBM SPSS Statistic for window, version 21.0. Armonk, NY: IBM Corp.). The analysis was done using Kruskal Wallis test at 95% confidence interval and 80% power to the study. Statistical significance was kept at $p<0.05$. Descriptive statistics was performed in terms of mean, standard deviation.

Results

Primary cell culture

After establishing a primary cell culture from biopsy specimens, the cells were characterized for fibroblast cell lineages. The CD90 surface marker was used to verify the cultivated cells' mesenchymal origin. The positivity with CD90 indicated the mesenchymal origin of the fibroblast cell type, as shown in Figure 1.

A MTT assay to check cytotoxicity for arecoline was performed using a 96-well plate seeded with 10,000 (1 x 104) normal human oral fibroblasts, which were then treated with arecoline at various doses of 1, 2, 5, 10, 15, 25, 20, and 25µg/ml. At concentrations of 5–20 µg/ml, arecoline showed a proliferative effect and no cell death. 15 µg/ml concentration showed good results. So, 15 µg/ml concentration of arecoline was used to induce fibrosis in oral human fibroblast. The cells were scanty and started losing their spindle shape, as shown in Figure 2.

On in vitro evaluation of *Baicalin* extract's anti-inflammatory and antifibrotic properties following pre-treatment in an arecoline-induced human oral fibroblast cell line model. A MTT assay was done for *Baicalin* to determine its non-toxic concentration. To determine a safe dose of *Baicalin* without causing cell death, cells were treated with concentrations of 1, 2, 5, 10, 15, 20, 25, and 50µg/ml. The values showed the proliferative nature of *Baicalin* at concentrations of 20–25 µg/ml.

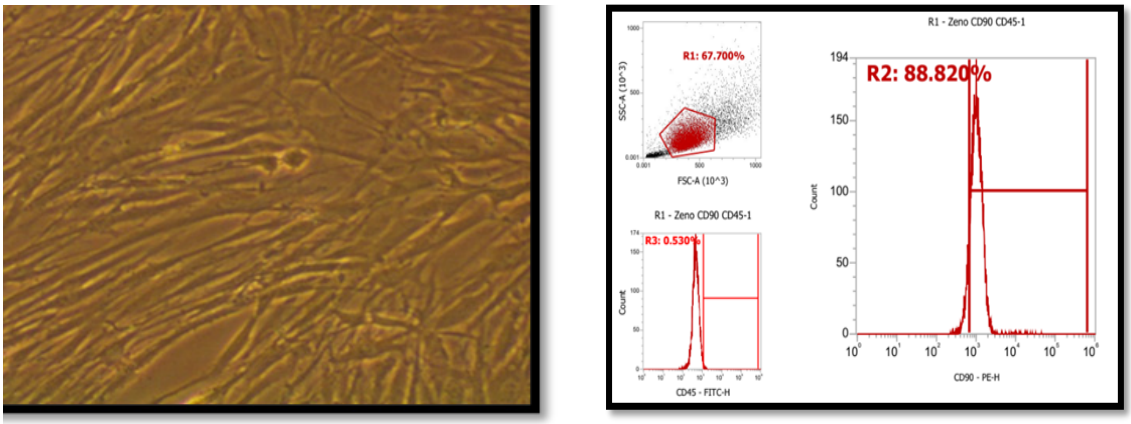

Figure 1. Shows Primary Fibroblast before Treatment with Arecoline and on Right Side it Shows Cells Characterized with CD 90 Fibroblast Surface Marker.

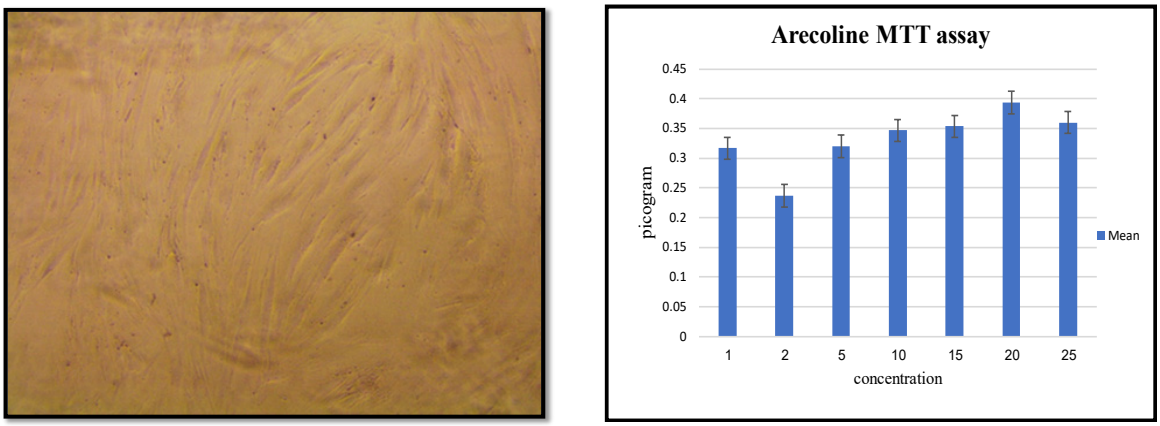

Figure 2. Shows Cells Treated with Arecoline and Right-Side Inhibitory Concentration (IC_{50}) for Arecoline after Determining the Percentage of Viable Cells.

In-vitro evaluation of *Baicalin* extract's anti-inflammatory and antifibrotic properties following pre-treatment in an arecoline-induced human oral fibroblast cell line model.

After successfully establishing primary cell culture and treating it with various concentrations of arecoline, we evaluated the above herbs for their antifibrotic properties.

We evaluated the MTT cytotoxic assay for all herbs

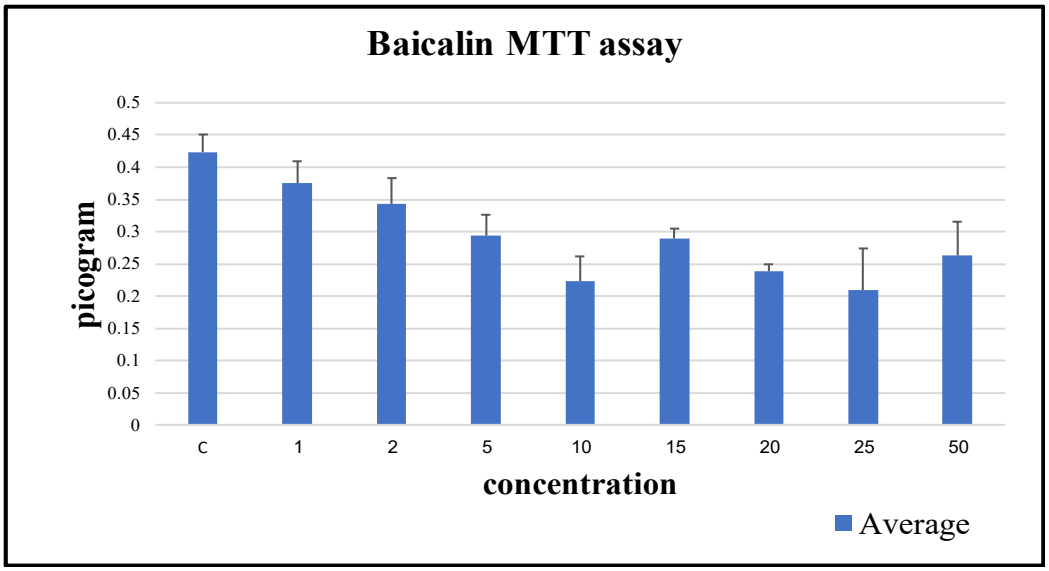

Figure 3. MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) for *Baicalin* to Check Viability of Cells

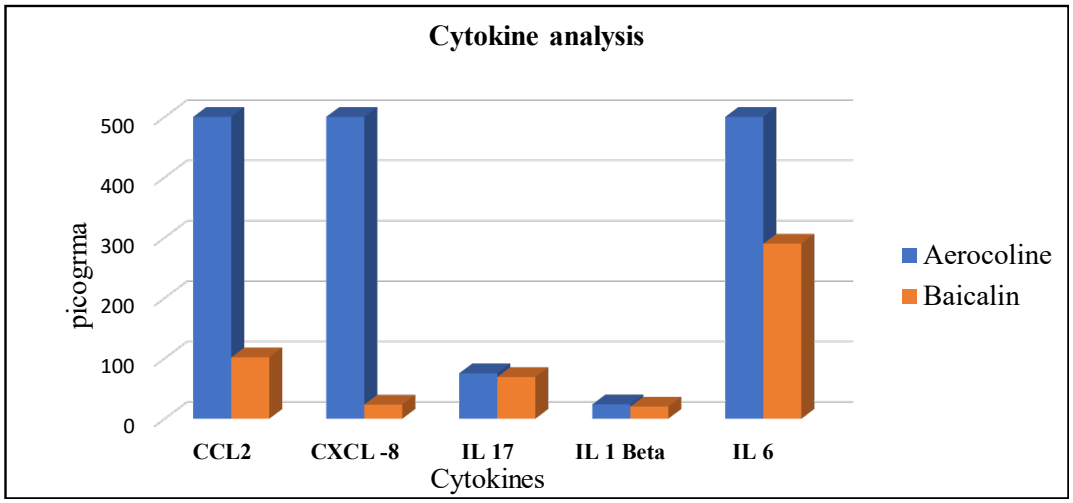

Figure 4. Cytokine Analysis after Treatment with Arecoline and *Baicalin*

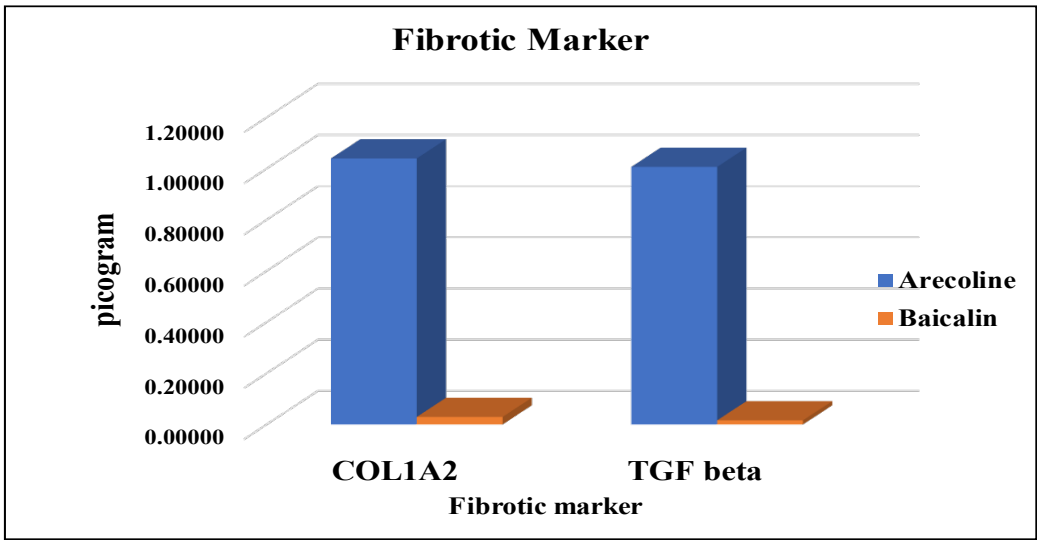

Figure 5. Fibrotic Marker Analysis after Treatment with Arecoline and *Baicalin*

and the expression of molecular markers and fibrotic markers on arecoline-induced primary fibroblast cells. Cells treated with *Baicalin* show no change in morphology at 25µg/ml of *Baicalin* proving its nontoxicity to cells, as shown in Figure 3.

Assessment of inflammatory on pre-treatment with *Baicalin* extract on arecoline induced fibrosis in oral fibroblast. In six-well plates, five lakh (5 X 10^5) cells were planted to show the anti-inflammatory effects of ethanolic extracts. When cultured fibroblasts reached 50 to 60% confluency, they were either left untreated (control) or treated with ethanolic extracts of *Baicalin* at concentrations of 1, 2, 5, 10, 15, 20, 25, and 50 µg/ml. Following a 48-hour incubation period, fibrosis was produced in the cells using 25 µg/mL of arecoline. An overall substantial downregulation of inflammatory markers and an overexpression of anti-inflammatory markers were analysed. We studied CCL2, CXCL-8, IL17, IL-beta, and IL-6, as all these markers have shown upregulation in OSMF (Figure 4).

Arecoline at a concentration of 25µg/ml significantly upregulated the expression of CCL2, CXCL-8, IL17, IL1 beta, and IL6. When treated with a nontoxic dose of *Baicalin*, all the above inflammatory cytokines were downregulated, which was statistically significant (p value <0.05). [13] stated that all TGF –β1 controls the breakdown of collagen by activating the genes for tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAI). The most significant component of the ECM is collagen. Fibroblasts, endothelial cells (EC), and certain epithelial cells can all release collagen. TGF-β1strongly promotes the synthesis and deposit of ECM [14]. COL1A1, COL1A2, collagenase-1, TGF-β1, LOX, and *CST3* genotypes were associated with the greatest risk of OSMF in the high-exposure group, according to [10] and previous research. In both low- and high-exposure groups, there was a clear correlation between the genotype distribution of the TGF-β1 and *CST3* genes and the risk of OSMF [10]. So, we examined TGF-β and COL1A2 genes through RTPCR and studied if *Baicalin* could have an inhibitory effect on these genes.

Our study showed similar results where there was significant upregulation of TGF –β1 and COL1A2 by arecoline, explaining its role in the formation of fibrosis

in OSMF.*Baicalin* significantly downregulated regulated TGF –β1 and COL1A2 by 5 folds with a p value of 0.045 (p value < 0.05), proving that these herbs have an anti-fibrotic effect, as shown in Figure 5.

Discussion

Oral submucous fibrosis is typically observed in India or Southeast Asia and is unquestionably linked to betel nut chewing, although it can sporadically occur in people who do not chew betel nuts. Daily chewing causes changes in frictional tissue, chronic inflammation, and the production of fibrogenic cytokines, including transforming growth factor. Recent research has linked areca nut usage to the development of oral submucous fibrosis in a dose-dependent manner. Oral submucous fibrosis patients will have dysplastic alterations in 7% to 26% of cases, and 7% to 13% of these patients will go on to develop squamous cell carcinoma [15]. This condition is marked by trismus, burning sensation, loss of tongue movement, loss of gustatory sensibility, and blanching and rigidity of the oral mucosa. The Indian community accounts for the majority of these instances [16].

The biggest drawback of corticosteroids is the fibrosis that is brought on by the needle puncture needed for medication administration and their long-term adverse effect when given systemically. Long-term use of the topical formulation is ineffective. Despite the different OSMF therapy regimens now in use, none of them are 100 percent successful in every instance. Despite being effective in treating OSMF, several pharmaceuticals have been discovered to cause side effects and recurrences [17].

The goal of Ayurveda medicine is to treat the patient as an organic whole, which entails the prudent use of medications, diets, and certain practices [18]. Since ancient times, people have used plant-based medications, and they continue to do so now as a crucial part of the Indian healthcare system. Due to either improved results or more patient compliance, the usage of herbal extracts or commercially accessible herbal extracts has recently increased [19].

In the study by Latief et al. [20], at least fifty medicinal plants and the byproducts of those plants, which included *Baicalin*, showed antifibrotic action in animal models, making them the most likely candidates to provide therapeutic liver protection. However, a lot more effort is still required to investigate molecular pathways and find possible uses for these medications in order to broaden the scope of biomedical research. Almost all of these herbs acted by inhibiting TGF-β1, a crucial factor in liver inflammation. Additionally, it inhibits α-SMA, which stops hepatic stellate cells from activating and lessens hepatic fibrosis [20].

Arecoline, the most prevalent areca alkaloid, has been proposed as a probable carcinogen after being discovered to be cytotoxic and genotoxic in many cell types. They looked at how arecoline affected epithelial and fibroblast cell lines. They discovered that at greater doses of arecoline over 48 hours, a considerable number of cells were detached and spherical in form. The number of rounded cells rose proportionately to the arecoline concentration, indicating that arecoline had a dose-dependent impact on the cells. Furthermore, large amounts of arecoline caused cell death [21]. Similarly, our study showed that fibroblasts became scanty and lost their spindle shape when treated with arecoline, indicating its cytotoxic effect.

Examined the antifibrotic efficacy of flavonoids and nonflavonoids identified in 21 Chinese herbs in vitro in a related but more thorough experiment. Fibrosis was induced in rat kidney fibroblasts using TGF-β1. Five substances were identified in their research as having antifibrotic potential: quercetin, baicalein, *Baicalin*, salvianolic acid B, and emodin. These compounds inhibit collagen 1 and alpha-smooth muscle actin at the protein and mRNA levels, as well as total collagen accumulation as determined by spectrophotometry [22]. As compared to our study, *Baicalin* also showed similar results by downregulating TGF-β and COL1A2 genes, which are responsible for collagen accumulation and fibrosis.

In an *in-vivo* investigation, showed that TNF-α subcutaneous perfusion induces localized proliferation of fibroblasts, epidermal cells, and capillaries with substantial hydroxyproline upregulation. Both IL-6 and IL-8 (stromal activators) have been linked to the development of fibrosis, which results in an increase in the synthesis of type I and III collagens [23].

Xiao-Dong Peng, [24] *Baicalin* appears to have some therapeutic benefits in hepatic fibrosis, most likely through immunoregulation of the imbalance of profibrotic and antifibrotic cytokines. Serum TGF-β1, TNF-α, and IL-6 levels in the model group were considerably higher than in the normal control group ($P < 0.01$). Treatment with 70 mg/kg *Baicalin* significantly suppressed upregulation ($P < 0.01$) [24]. Our study also proved that *Baicalin* plays a role in reducing fibrosis by downregulating cytokines and acting as an antifibrotic agent by reducing expression of TGF-β1 and COL1A2, which was statistically significant.

Cytokines like CCL 2 exhibit chemotactic activity for monocytes and basophils, and studies have shown CCL 2 could be responsible for the pathogenesis of OSMF by recruiting myofibroblast [25]. CXCL 8, also called IL 8, is realized early post injury mainly by macrophages. A study by Yadahalli et al. [26] shows a transient increase in CXCL 8 in OSMF as compared to normal tissue [26]. IL-17, IL-6 and IL-1 beta are potent inflammatory cytokines in OSMF studies by Hague et al. [23] Our study also showed all these cytokines and fibrotic markers were upregulated by arecoline, indicating its role in the pathogenesis of OSMF, and downregulated on treatment with *Baicalin*, explaining its role as an antifibrotic agent.

Cui et al. [13] stated that all TGF –β1 controls the breakdown of collagen by activating the genes for tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAI). Fibroblasts, endothelial cells (EC), and certain epithelial cells can all release collagen. TGF-β1strongly promotes the synthesis and deposit of ECM [24] *COL1A1, COL1A2,* collagenase-1, TGF-β1, LOX, and *CST3* genotypes were associated with the greatest risk of OSMF in the high-exposure group, according to Chiu's study (2002) and previous research. In both low- and high-exposure groups, there was a clear

correlation between the genotype distribution of the TGF-β1 and *CST3* genes and the risk of OSMF [10].

To the best of our knowledge, this is the first study of its type to show that *Baicalin* exhibits an antifibrotic potential in Oral submucous fibrosis. We believe that the ability of *Baicalin* to display effective anti-inflammatory and antifibrotic action might be one of the mechanisms involved in the considerable downregulation of the most important fibrogenic markers, TGFβ1 and COL1A2. Collagen quantification studies and more animal studies needed to be conducted to prove its potential role.

In conclusion, there is no one specific and highly effective treatment for tissue fibrosis. Therefore, it is essential to create an alternative management system. The discovery of ethanolic leaf extracts of *Baicalin* that significantly reduced arecoline-induced inflammatory mediated fibrosis in a human buccal fibroblast cell line model was a novel and important finding in the current study. Ethanolic extracts of *Baicalin* dramatically reduced all inflammatory markers used in this study, downregulating the fibrotic markers TGF-β1 and COL1A2, marking its potential role as an anti-fibrotic and anti-inflammatory agent.

Limitation of the study

Because the fibroblasts were not obtained from a private entity as compared to many other studies, the current study had fewer limitations as primary cell cultures were generated from fibroblasts of oral tissues. The only drawback was that fibrosis was induced on cells by arecoline, which may not resemble the precise fibrosis; thus, cells from OSMF patient's biopsy specimens may have been better possibilities. Second, because the study was done in vitro, confounding variables that may arise in the oral environment could not be evaluated. Notwithstanding its limitations, the current work opens up new paths for investigation and provides a foundation for future research in this field.

Author Contribution Statement

Pallavi Channe (PP) and Mahesh Chavan, conceiving the research idea and writing original draft for thesis work. Ramesh Bhonde (RB) and Supriya Kheur (SP) Head of the Regenerative Medicine Laboratory Department where cell cultural and tests were recruited. Supriya Kheur helped as thesis directors and did conceptualization, conceiving the research idea, supervision and validation. Pallavi Channe (PP) interpreted the data and drafted the manuscript under the guidance of Mahesh Chavan (MC). All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The data and materials that support the findings of this study are available on request.

Scientific approval

The thesis was approved by scientific committee of the institution as a PhD thesis.

Ethics approval

This study was approved by the Institutional Ethical Committee (IEC) in DYPV/252/2019.

Conflict of interest

All the authors declare that no conflict of interest.

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