6.1 Overview of study

Oral Submucous Fibrosis, a collagen metabolic disorder caused primarily due to arecanut chewing is confined to Southeast Asians and Indians with an overall prevalence rate of about 0.02 - 3.2% (Hazarey et al., 2007). It is well recognized and has been classified as a potentially malignant disorder (PMD) of the oral cavity (Warnakulasuriya et al., 2007), with a malignant transformation rate of 7% - 30% (Arakeri and Brennan, 2013). Over 90% of oral malignancies are known to arise from preexisting potentially malignant lesions and conditions (Sultana et al., 2011).

Arecanut (betel nut) chewing causes injury to the oral mucosa. The constant contact produces friction leading to the seepage of toxic alkaloid constituents like arecoline into the underlying submucosa; which undergoes nitrosation giving rise to lethal metabolites. Due to persistent irritation, chronic inflammation sets in, at the site of injury leading to the release of proinflammatory cytokines like IL-1β, IL-6 and TNFα (stromal activators) and profibrogenic mediators (TGFβ1, PDGF, CTGF and bFGF) characterized by the presence of macrophages and lymphocytes (Rajalalitha and Vali, 2005). TGFβ1 stimulates production of downstream molecules (COL1 and COL3) through the TGFβ/Smad signaling pathway. On constant external stimulation, it loses its autocrine activity and produces an imbalance between collagen production and degradation leading to fibrosis. Yet another predisposing factor is the attempt of the immune system to eliminate the toxic metabolites by phagocytosis leading to the production of reactive oxygen species which gives rise to oxidative stress.

Treating patients with OSMF is a challenge. No effective or satisfactory treatment till date has been developed (Chole et al., 2012; Hazarey et al., 2015; Kerr et al., 2011; Sudarshan et al., 2012). The primary objective is to improve the mouth
opening and provide symptomatic relief. Recent studies have recommended discontinuation of habit, physiotherapy, combination therapies of steroids and hyaluronidase, intralesional injections, placental extracts, collagenase and laser surgeries; but none have been effective.

Tissue fibrosis is a condition that lacks a universal effective therapeutic regimen. Therefore there is a need to develop an alternative management; that can be put into clinical application. Diseases that fail to be treated with allopathic medicines are now successfully being treated with ayurvedic preparations (Chiu et al., 2002). Arecoline induced oral mucosa tissue injury enhances chronic inflammation (increase in proinflammatory cytokines IL-1β, IL-6 and TNFα) and fibrogenic mediators (TGFβ1) (Rajalalitha and Vali, 2005). If these stimulatory molecules were inhibited using plants or its constituents with antioxidant, anti-inflammatory and antifibrotic properties, then the balance between collagen production and degradation could help in not only preventing further progression of fibrosis but also in reducing the potential of malignant transformation. A large reservoir of medicinal plants in India, which have potent antioxidant, anti-inflammatory, antimicrobial and anticancer activities attributed to the presence of active constituents such as flavanoids, polyphenols, alkaloids, triterpenoids and aglycones, can be tested for their ability to reduce tissue injuries caused by arecoline.

With this background our study aimed at evaluating the antioxidant, anti-inflammatory and antifibrotic activity of three selected plant species and their active constituents in an arecoline induced fibrosis model. Crude ethanolic extracts (C asiatica L, O basilicum L. and O vulgare L. subsp hirtum) and pure compounds (asiatic acid, linalool and thymol) respectively were chosen for the present study.
6.2 Model for Oral Submucous fibrosis

A model for OSMF was developed as a primary adherent fibroblast cell culture using buccal tissue samples from healthy volunteers. Confluent fibroblast monolayer’s obtained on 3rd day of seeding (Fig 5.16 c) showed normal karyotype (Fig 5.17). The immunohistological pattern of fibroblast cell differentiation was established using specific markers like vimentin, s100a4, TGFβR1 and phalloidin. The spindle shaped fibroblasts and mesenchymal origin of the cultured cells showed positive vimentin staining (Fig 5.18 b). Our results were concurrent with studies performed by Goodpaster et al. in 2008 who reported maximum (100%) staining intensity with vimentin in dermal and lung fibroblasts when compared to monocyte/macrophage, glioma and osteosarcoma cells explaining its specificity for fibroblasts. Mathew et al. in 2011 reported mitotic (FI, FII and FIII) population of fibroblasts cultured from tissues of patients with normal oral mucosa; he observed that FI population of cells have a spindled morphology which stained positive for vimentin. Similar fibroblast morphology was also observed and reported by Bayreuther et al. in 1988 in skin fibroblasts. Studies done by Waal et al. in 1997 reported that fibroblasts cultured from patients with normal mucosa and no arecanut chewing habit were of the FI (spindle) type when compared to the FIII type (large pleomorphic and epitheloid) in OSMF patients.

Fibroblast specific protein (Fsp1) also called as s100a4 (filament associated calcium binding protein) is associated with cells of mesenchymal origin or of fibroblastic phenotype. Cultured human buccal fibroblasts demonstrated s100a4 staining confirming their fibroblastic phenotype (Fig 5.18 d-f). Nishitani et al. in 2005 also demonstrated Fsp1 expression in fibroblasts accumulating in areas with severe renal interstitial fibrosis.
TGFβ a potent fibrotic mediator and a scar inducing factor is known to be upregulated in many fibrotic conditions, its receptors type I and II are present as cell membrane receptors on cells like fibroblasts. In (Fig 5.18 g-i), accumulation of TGFβR1 present on the cultured fibroblasts was demonstrated. A similar study demonstrated TGFβ1 receptors via *in-situ* hybridization (immunofluorescence staining) of meningial fibroblasts (Komuta et al., 2010). The cultured human fibroblasts showed intracellular cytoplasmic staining with phalloidin - a dye specific for actin filaments (Fig 5.18 g-i). Verderame *et al.* in 1980 also demonstrated thick actin filaments stained with phalloidin, well distributed throughout the cytoplasm in a 3T3 rat fibroblast cell line.

Thus, a primary confluent buccal fibroblast culture, which demonstrated mesenchymal origin and fibroblast cell type properties were obtained and used for testing

a) Induction of inflammation and fibrosis with arecoline.

b) Determining the molecular end points of inflammation and fibrosis.

To evaluate the inflammatory and fibrotic effects of arecoline on normal HBFs, non-toxic concentrations of arecoline were estimated as IC$_{50}$ - 50 µg/mL, by performing MTT colorimetric assay. The results from Fig 5.19, showed that arecoline induced cytotoxicity, cell death and inhibited cell proliferation by 49% at 50 µg/mL, 58.2% at 100 µg/mL and 65.7% at 200 µg/mL in a dose dependent manner, at 48 hours post treatment. Our results were concurrent with studies performed by Chang *et al.* in 2001 who observed 30% cell death at 50 µg/mL of arecoline at 24 hour time point. Similarly, Tsai *et al.* in 2003 and Shieh *et al.* in 2004 also observed that
concentrations above 50 µg/mL of arecoline significantly inhibited the proliferation of fibroblasts thereby inducing cytotoxicity at both 24 and 48 hour time points.

6.3 Responses of cultured HBF cells to arecoline

6.3.1 Arecoline treated cells and expression of proinflammatory and anti-inflammatory markers

On treating the cultured HBFs with non – toxic concentrations (0 - 50 µg/mL) of arecoline remarkably elevated mRNA expressions of proinflammatory cytokines (IL-1β by 50 fold, IL-6 by 9 - 10 fold & TNFα by 7 fold) with downregulated anti-inflammatory marker, IL-10 by 3.8 fold was recorded (Fig 5.20 & 5.21). Increase in IL-1β activity was observed at a minimum concentration of 5 µg/mL of arecoline, IL-6 at 15 µg/mL and TNFα at 10 µg/mL was observed over control. Our results were concurrent with studies performed by Haque et al. in 1998 who predominantly observed, T lymphocytes and antigen presenting cells (high CD4:CD8 ratio and MHC II expressing) in the lesional tissue samples of OSMF when compared to normal buccal mucosa tissue samples. He further confirmed the upregulation in the expression levels of IL-1α, IL-1β, IL-6, TGFβ, PDGF and bFGF with a strong intensity in both the epithelium and the underlying connective tissue, using immunoperoxidase staining technique. They concluded that an increase in proinflammatory cytokines and growth factors play an imperative role in the pathogenesis of OSMF. A similar study performed by Haque et al. in 2000 investigated and reported significant increased levels of IL-1β, IL-6, IL-8 and TNFα in peripheral blood mononuclear cells (PBMC) from OSMF patients when compared with control non - stimulated PBMC. Similar observations were reported by Perera et al. in 2007, who showed higher inflammatory response (predominantly neutrophils
and eosinophils) in mice treated with aqueous arecanut extracts for 300 days and infiltration of plasma cells in the latter phase of treatment providing evidence of a chronic inflammatory process in the buccal tissue.

IL-10 is an anti-inflammatory cytokine with similarities in function to interferon gamma (IFNγ); an antifibrotic agent. Haque et al. in 2000 demonstrated markedly low levels of IFNγ in OSMF patients. Similarly, on induction with arecoline our results showed low levels of IL-10, illustrating the development of an inflammatory and fibrotic environment in the cultured buccal fibroblasts. McCauley et al. 1992 focused on the alterations of immunoregulatory cytokines in patients with keloid formation. He reported markedly depressed production of antifibrotic cytokines like IFNγ, IFNα and TNFβ, with a concomitant rise in IL-6 and IL-1β. Increase in the latter cytokines was known to promote and stimulate stromal cells for collagen synthesis.

Time and again several studies have explained the link between inflammation and fibrosis. Chronic inflammation is the result of a constant and persistent stimuli or injury eventually leading to a fibrogenic environment. In cultured lung fibroblasts, Zhang et al. in 1993 showed upregulated mRNA expressions levels of proinflammatory cytokines, IL-1β and TNFα, type I and III collagens and fibronectin. Similarly, Piguet et al. 1990 illustrated in an in-vivo study, that subcutaneous perfusion of TNFα promotes localized proliferation of fibroblasts, epidermal cells and capillaries with significant upregulation in hydroxyproline. Both IL-6 and IL-8 (stromal activators) have been implicated in the development of fibrosis leading to excessive production of type I and III collagens (Hasegawa et al., 1999; Ziegenhagen et al., 1998).
Arecoline, a known fibrosis inducer, was used in the present study to establish a cultured human cell line *in-vitro* model. This model established the responses of the proinflammatory markers IL-1β, IL-6 and TNFα and IL-10 (antiinflammatory marker) by monitoring the mRNA expression levels. Thus far in literature comparisons for the role of these inflammatory cytokines has been reported while studying normal vs. OSMF samples or blood or in animal models. This is the first study in which an arecoline inducible fibrosis model determining the molecular responses has been proposed.

### 6.3.2 Arecoline treated cells and fibrosis

To validate the above mechanism and substantiate OSMF as a collagen metabolic disorder, we tested the effect of arecoline on fibrotic markers. On incubating HBF’s with the determined non toxic concentrations (0 - 50 µg/mL) of arecoline for 48 hours (Fig 5.22 & 5.23), a significant elevation was observed in mRNA expression levels of TGFβ1 by 3-5 fold, COL1A2 by 8-10 fold and COL3A1 by 20 fold at 25 and 50 µg/mL of arecoline, contributing to inflammation triggered fibrogenesis. The initial upregulation of fibrotic markers by almost 2 fold was observed at a minimum concentration of 5 µg/mL for TGFβ1 and 10 µg/mL for COL1A2 and COL3A1.

Our results were concurrent with studies performed by Khan *et al.* in 2012, who evaluated the combined effects of arecanut and TGFβ on human gingival fibroblasts. He observed an enhanced expression of TGFβ target genes namely αSMA and collagen isoforms (COL1A1, COL1A2 AND COL3A1) along with total collagen protein revealed by direct red dye staining. In a similar microarray analysis study performed in OSMF tissues, an upregulation of the downstream molecules of TGFβ
pathway namely COL1A2, COL3A1, COL4A1, LOX, TIMP1 and TIMP3 was shown. The role of inflammatory response and TGFβ in the pathogenesis of malignant transformation of OSMF was thus proposed by Yanjia et al. in 2008.

Gupta et al. in 2008 & Rajalalitha and Vali in 2005 explained the pathogenesis of OSMF. They explained an uncontrolled increase in proinflammatory cytokines (IL-1β, IL-6 & TNFα) and growth factors such as TGFβ, PDGF and bFGF on continuous stimulation and injury to the oral mucosa by arecanut constituents like arecoline. The fibrogenic growth factors were responsible in stimulating excessive production of collagen (predominantly Type I and III) and its conversion into an insoluble form making it resistant to degradation. The imbalance between collagen production and degradation sets in a fibrotic phase, not only in arecoline induced OSMF but also in various other fibrotic diseases that undergo a similar mechanism for fibrogenesis.

In hepatic fibrosis, injury (alcoholic, fatty, viral and autoimmune origin) to hepatic stellate cells (HSC) serves as a mediator of inflammation further activating the fibrogenic cascade. The activated HSC release profibrogenic mediators IL-6 and TGFβ; IL-6 maintains the continuous activation of TGFβ thereby inducing collagen type I and III transcription (Seki and Schwabe, 2015). In renal fibrosis, a clinical and subclinical insult leads to the development of chronic kidney disease (CKD), depending upon the type of stimuli, the organ undergoes repair and recovery by releasing proinflammatory cytokines and chemotactic factors. Monocytes and macrophages release IL-6, TNFα and TGFβ1 stimulating fibrogenic cells (glomerular mesangial cells, fibroblasts and tubular epithelial cells) leading to excessive production of downstream molecules predominantly collagen type I and III (Liu, 2006). In relation to radiation induced oral mucositis, the initial stages of
inflammation are followed by an epithelial phase, ulcerative/infective phase and lastly a fibrotic phase. During the inflammatory phase, secretion of proinflammatory cytokines (IL-1, TNFα and TGFβ1) is followed by a phase of increased vascular permeability which leads to fibrin deposition and excessive collagen production leading to fibrosis (Cooper et al., 1995).

6.3.3 Morphological evaluation of cultured HBFs treated with arecoline

Masson trichrome staining was performed on HBFs to evaluate the collagen deposition depending upon the staining intensity, and to study the fibroblast morphology post arecoline treatment (Fig 5.24). Masson trichrome staining revealed fibroblasts with spindle morphology and scanty quantity of ECM to be present in the untreated (control) group (a), while HBFs treated with 25 µg/mL of arecoline appeared distorted, rounded and stained strongly positive for collagen (b) demonstrating excessive ECM deposition. Our results were consistent with studies performed by Rooban et al. in 2005 who reported changes in collagen and muscle fibers in OSMF using Masson trichrome staining. Mishra et al. in 2015 observed an haphazard arrangement of type 1 collagen in the tissue sections of stage I OSMF, whereas parallel arrangement in relation to surface epithelium in stage II and III OSMF using Masson trichrome staining. He also reported an increase in the density and thickness of type 1 collagen fibers with disease progression. Perera et al. in 2007 demonstrated amorphous areas, an indication of early hyalinization in subepithelial buccal mucosal tissues of mice treated with aqueous arecanut extracts for 300 - 600 days using Masson trichrome staining.
6.4 Arecoline induced HBF cell line for screening anti-inflammatory and antifibrotic activity of medicinally important plants and their active constituents on pretreatment

On successfully establishing primary human buccal fibroblast cell culture, inducing inflammation and fibrosis with arecoline, an \textit{in-vitro} OSMF cell line model was developed which was used to screen the anti-inflammatory and antifibrotic activity of ethanolic leaf extracts of \textit{C asiatica} L. (LECA), \textit{O basilicum} L. (LEOB), \textit{O vulgare} L. (LEOV) and their respective pure compounds asiatic acid, linalool and thymol.

\textbf{6.4.1 \textit{C asiatica} ethanolic leaf extract and asiatic acid}

The non-toxic concentrations for ethanolic LECA and asiatic acid vary according to the cell type on which they have been tested, hours of incubation and culture media used depending on the nature of experiments (Dinesh \textit{et al}., 2014). The anti-inflammatory and antifibrotic activity of ethanolic crude leaf extract of \textit{C asiatica} L. (LECA) and its pure compound asiatic acid were analyzed on cultured cells after determining the non-toxic concentrations by performing the MTT colorimetric assay (Fig 5.25). Three day old cells were treated with ethanolic LECA from 1000 \(\mu\)g/mL to 7.8 \(\mu\)g/mL. Concentrations of 7.8 \(\mu\)g/mL - 125 \(\mu\)g/mL (\(p < 0.05\)*) showed no loss in cell viability (Fig 5.25 a). Hence, protection of cultured fibroblasts treated with 7.8 \(\mu\)g/mL - 125 \(\mu\)g/mL of ethanolic LECA was studied by the expression of molecular markers induced with arecoline.

Asiatic acid at concentrations tested (1000 \(\mu\)M - 7.8 \(\mu\)M), did not cause loss of cell viability at concentrations of (7.8 - 125 \(\mu\)M) (Fig 5.25 b). Our results were...
consistent with studies done by Dong et al. in 2004 who reported the non toxic concentrations of asiatic acid ranging from 5.5 µM - 2000 µM in HSC-T6 cells.

**6.4.1.1 Molecular marker expression analysis of protected HBF cells induced by arecoline: Anti-inflammatory and antifibrotic activity of ethanolic leaf extract of *C asiatica* L. and asiatic acid**

The anti-inflammatory activity of plant extracts has been attributed to their efficacy of scavenging radical species. With regard to the total antioxidant assay, 2,2-Diphenyl-1-dipicrylhydrazyl assay, nitric oxide radical scavenging activity, reducing power, and superoxide radical scavenging activity ethanolic LECA exhibited potent radical scavenging activity as compared to the standard with \( p < 0.01** \).

With regard to (Fig 5.8, 5.9, 5.10, 5.11 & 5.12) the bar diagrams revealed significant antioxidant activity of *C asiatica* L. via the five model systems when compared to the standard. Ethanolic LECA showed good activity (0.86% and 1.9% respectively) as percentage of ascorbic acid tested via Nitric oxide scavenging assay and superoxide radical scavenging assay (Table 5.3). Our results were in accordance to studies done by Chauhan et al., 2010; Huda-Faujan et al., 2007; Obayed et al., 2009 and Syed et al., 2009.

*C asiatica* L. is known to have 0.1% essential oils and volatile constituents. The antioxidant activity of *C asiatica* L. can be attributed to the presence of active constituents such as terpenes (Madecassic acid, asiatic acid, and three asiaticoside; asiaticoside, asiaticoside A and asiaticoside B) and phenolic constituents predominantly being flavonoids as shown in (Table 5.2). Phenolic compounds have redox properties (oxidation-reduction), which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers that play an important role in the
adsorption or neutralization of free radicals. Our results were in accordance to studies done by Brinkhaus et al., 2000; Jamil et al., 2007; Obayed et al., 2009 and Syed et al., 2009.

All assays displayed a dose dependent activity with maximum activity at 1000 µg/mL and minimum activity at 10 µg/mL, except total antioxidant assay where the maximum activity was exhibited at 200 µg/mL. The variation in antioxidant activity at different concentrations could be attributed to the varying amounts of constituents present at different concentrations. This variation in the phenolic content could be attributed to geographical and seasonal variation. Our results were in support of observations made by Hashim in 2011 and Huda-Faujan et al. in 2007.

Pretreatment with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LECA for 48 hours significantly downregulated the mRNA expression of the elevated inflammatory cytokines in arecoline treated cells. The results shown in Fig 5.26, 5.27 and Table 5.4 indicated almost 2 fold decrease for IL-1β at 31.25 µg/mL. IL-6 levels decreased by almost 1.7 fold at 15.6 µg/mL and by 24 and 61 folds at higher concentrations of 62.5 µg/mL and 125 µg/mL respectively. TNFα activity decreased by 2.5 fold at 31.25 µg/mL, 5 fold at 62.5 µg/mL and 7 fold at 125 µg/mL. A striking upregulation of anti-inflammatory cytokine IL-10 by almost 2 folds at 31.25 µg/mL, 3 fold at 62.5 µg/mL and 4 fold at 125 µg/mL was observed. The standard drug indomethacin (50 µM), decreased the levels of IL-1β & TNFα by 13 fold, IL-6 by 94 folds and increased IL-10 levels by 11.3 folds.

Data presented in Fig 5.30, 5.31 and Table 5.4 showed that, pretreatment with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LECA for 48 hours, significantly downregulated the mRNA expression of the elevated fibrotic markers.
TGFβ1 at 7.8 µg/mL showed reduction of expression by 1.7 folds; with maximum reduction by 5, 14 and 109 folds at 31.25 µg/mL, 62.5 µg/mL & 125 µg/mL respectively. COL1A2 level decreased by almost 1.5 fold at 15.6 µg/mL and by 2 and 4 fold at 62.5 µg/mL and 125 µg/mL. COL3A1 levels reduced by almost 2 fold at 7.8 µg/mL, 15.6 µg/mL and 31.25 µg/mL with a further reduction of 4 fold and 198 fold at 62.5 µg/mL and 125 µg/mL.

Anti-inflammatory activity was observed by Anilkumar in 2010, who reported that water extracts of *C asiatica* L. elicited a dose dependent anti-inflammatory activity at 2 mg/kg b.w. when compared to standard mefenamic acid. He attributed the anti-inflammatory activity to the presence of bioactive terpenoids (asiatic acid and madecassic acid). Roy et al. in 2013 reported *in-vivo* anti-inflammatory activity of ethanolic extract of *C asiatica* L. by downregulating proinflammatory cytokines IL-6 and TNFα at 100 mg/kg b.w. in LPS stimulated inflammation in rats. Widegrow in 2011 recognized that fibrotic diseases occur due to constant competition between proinflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) and profibrotic agents (TGFβ1, PDGF, CTGF and bFGF). To downregulate the events in fibrosis it is important to inhibit the activity of inflammatory cytokines like IL-6 and TNFα which are potent stromal activators. In his review he highlighted the anti-inflammatory potential of *C asiatica* L. by downregulating IL-6.

Taking into account the link between inflammation and fibrosis; in our study, *in-vitro* experimental model results were concurrent with observations made by Widegrow in 2011 who demonstrated a decrease in TGFβ1, COL1A2, COL3A1 and IL-6 levels. His study focused on the Smad pathway of fibrosis, successfully demonstrating an increase in Smad 7 (TGFβ inhibitor), decrease in Smad2, 3 and 4...
complex and inhibition of iNOS production. *C. asiatica* L. was listed as an herb that regulates TGFβ1 which in turn balances collagen type I/III ratio in the ECM of fibrotic diseases. Roy *et al.* in 2013 demonstrated antifibrotic activity in dimethylnitrosamine induced liver fibrosis. Antifibrotic activity of *C. asiatica* L. was explored and reported by Zhang *et al.* in 2013. Treatment with *C. asiatica* L. upregulated the mRNA expression of the antifibrotic cytokine (HGF) at higher concentrations, thereby suppressing the expression of TGFβ1, deactivating the enhanced expression of Smad proteins, blocking the TGFβ/Smad signaling pathway in a dose dependent manner in renal tubular interstitial fibrosis (TIF). *C. asiatica* L. successfully inhibited fibroblast proliferation and collagen. The above discussed literature studies explain the importance of inhibiting inflammatory cytokines to in turn obtain a potent antifibrotic mechanism.

In our study, fresh leaves of *C. asiatica* L. were used for experiments, as maximum active constituents (triterpenoids, polyphenols and flavanoids) are harboured in the leaves of the plant (Hashim, 2011). Anti-inflammatory and antifibrotic activities of *C. asiatica* L. have been attributed to the presence of active terpenoids such as asiatic acid, asiaticoside and madecassic acid (Roy *et al.*, 2013). The data presented in HPTLC (Fig 5.13) showed the presence of asiatic acid in our ethanolic extracts at effective concentrations of 7.8 μg/mL - 125 μg/mL.

With the existing literature we evaluated the anti-inflammatory and antifibrotic activity of a triterpenoid aglycone, asiatic acid on an arecoline induced fibrosis cell line model. Detection of asiatic acid and standardization of active compound in the ethanolic LECA was done by HPTLC. The ethanolic LECA (Fig 5.13 a), contained the predominance of asiatic acid at Rf 0.24. Asiatic acid
calibration curve was observed to be linear (Fig 5.13c) and 0.8 g of asiatic acid was calculated to be present per 100 g of ethanolic LECA (Fig 5.13 b & d). Studies performed by Gupta et al. in 2014 reported 0.4 g of asiatic acid in methanolic extract of *C asiatica* L. from Varanasi with R_f 0.51 using a solvent system comprising of (toluene: ethyl acetate: formic acid) in the ratio of 5:5:1 by HPTLC method. Similarly Joshi et al. in 2012 reported 0.002 g of asiatic acid in methanolic extract of *C asiatica* L. with R_f 0.33 using a solvent system comprising of (toluene: ethyl acetate: chloroform: formic acid) in the ratio (6:6:4:1). The variation in the quantity and R_f value of asiatic acid observed, could be due to the different solvent systems used in various ratios (Roy et al., 2013).

Pretreatment of HBFs with non-toxic concentrations (7.8 - 125 µM) of asiatic acid for 48 hours, significantly downregulated the mRNA expression of the elevated inflammatory cytokines by almost 2 fold for IL-1β at 7.8 µM, 3 folds at 15.6 and 31.25 µM, 4 fold at 62.5 µM and by 22 fold at 125 µM (Fig 5.28, 5.29 & Table 5.5). IL-6 level decreased by almost 2 fold at 7.8 to 31.25 µM. Maximum reduction by 3 fold and 4 fold was observed at 62.5 and 125 µM. TNFα decreased by almost 3 fold at 7.8 µM., with maximum reduction of 22 fold at 31.25 µM, 33 fold at 62.5 µM and 43 fold at 125 µM. A striking upregulation of the downregulated anti-inflammatory cytokine IL-10 by almost 1.7 fold at 31.25 µM. was observed, with maximum upregulation by almost 2 fold at 62.5 µM and 125 µM. The standard drug indomethacin (50 µM), decreased IL-1β by 39 fold, IL-6 by 9 folds, TNFα by 55 fold, increasing IL-10 levels by 3.7 folds. Asiatic acid exhibited anti-inflammatory activity at higher concentrations of 62.5 µM and 125 µM.
With regard to fibrotic markers (Fig 5.32, 5.33 and Table 5.5) TGFβ1 levels declined by almost 40 folds at 7.8 µM, 7 fold at 15.6 µM, 6 fold at 31.25 µM, whereas at higher concentrations of 62.5 and 125 µM TGF β1 levels declined by 3 fold. COL1A2 levels reduced by 13 fold and 14 fold at 7.8 µM, 15.6 µM and 31.25 µM of asiatic acid, whereas at higher concentrations of 62.5 µM and 125 µM a minimal reduction of 1 fold was observed. A significant reduction was observed in COL3A1 levels by 12 fold and 14 fold at 15.6 µM and 31.25 µM, whereas minimal reduction of 1 fold was observed at higher concentrations of 62.5 µM and 125 µM. The expression levels of TGFβ1, COL1A2 and COL3A1 effectively reduced at lower concentrations of 7.8 µM, 15.6 µM and 31.25 µM. This activity of asiatic acid exhibiting antifibrotic activity at lower concentrations when compared to higher concentrations of ethanolic LECA can be attributed to its purity.

In our study, the anti-inflammatory effects of asiatic acid were concurrent with studies reported by Yadav et al. in 2010, who stated that prolonged long standing inflammation is a cause of concern as it leads to PMDs and cancer. He targeted asiatic acid, present in C asiatica L. against inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) and observed an inhibition in the binding of TNFα to its receptor present on the cell surface, thereby downregulating the expression of TNFα in the presence of asiatic acid. A similar study was performed by Huang et al. in 2011 who evaluated asiatic acid in C asiatica L. for its anti-inflammatory effect in a carrageenan induced paw edema model. He observed that asiatic acid at 5-10 mg/kg b.w significantly downregulated the expression levels of NO, IL-1β and TNFα in the serum of rat models. He also reported that an i.p. injection of asiatic acid resulted in diminished neutrophil infiltration into the sites of inflammation similar to indomethacin.
The results of our \textit{in-vitro} antifibrotic activity of asiatic acid were concurrent with studies performed by Tang \textit{et al.} in 2012 who reported inhibition of collagen deposition on treatment with 0, 5, 10, 20 & 30 µM of asiatic acid in TGFβ1 activated HSC cells. Asiatic acid upregulated Smad 7 (inhibition of TGFβ signaling), downregulated Smad 2/3, collagen (type I/III) and inhibited myofibroblast differentiation in a dose dependent manner. In an \textit{in-vivo} rat model, asiatic acid treatment inhibited αSMA and collagen matrix deposition at 0.5, 2 and 10 mg/kg b.w. thereby attenuating CCl\textsubscript{4} induced liver fibrosis. He concluded that asiatic acid can be used as a novel antifibrotic agent inhibiting fibrosis mediated through the TGFβ/Smad signaling pathway. Asiatic acid treatment with 10 - 30 µM downregulated TGFβ1 induced collagen type I formation, upregulated Smad 2/3 antagonist (Smad 7) thereby proving itself as a direct target to TGFβ/Smad signaling pathway mediated by PPAR-γ pathway in keloid fibroblasts activated with TGFβ1 (Bian \textit{et al.}, 2013).

Similar studies were performed by Xu \textit{et al.} in 2013 who investigated the antifibrotic activity of asiatic acid in tubulointerstitial fibrosis in mice with ureteral obstruction. On treating mice with various non toxic concentrations of asiatic acid (1, 4, 16 mg/kg b.w.) for 6 days, he observed that intermediate and high doses of asiatic acid successfully abrogated the increased expression of TGFβ1 and phosphorylated Smad 2/3.

6.4.2 \textit{O basilicum} ethanolic leaf extract and linalool

The non-toxic concentrations for ethanolic LEOB and linalool vary according to the cell type on which they have been tested, hours of incubation and culture media used depending on the nature of experiments (Dinesh \textit{et al.}, 2014). The anti-inflammatory and antifibrotic activity of ethanolic crude leaf extract of \textit{O basilicum} L.
(LEOB) and its pure compound linalool were analyzed after determining the non-toxic concentrations by performing the MTT colorimetric assay (Fig 5.35).

Three day old cells were treated with ethanolic LEOB from 1000 µg/mL to 7.8 µg/mL. Concentrations of 7.8 µg/mL - 125 µg/mL (p < 0.05*) showed no loss in cell viability (Fig 5.35 a). Hence, protection of cultured fibroblasts treated with 7.8 µg/mL - 125 µg/mL of ethanolic LEOB was studied by the expression of molecular markers induced with arecoline. Linalool at concentrations tested (1000 µM - 7.8 µM), did not cause loss of cell viability at concentrations of (7.8 - 125 µM) (Fig 5.35 b).

6.4.2.1 Molecular marker expression analysis of protected HBF cells induced by arecoline: Anti-inflammatory and antifibrotic activity of ethanolic leaf extract of \textit{O basilicum} L. and linalool

The anti-inflammatory activity of plant extracts has been attributed to their efficacy of scavenging radical species. With regard to the total antioxidant assay, 2,2-Diphenyl-1-picrylhydrazyl assay, nitric oxide radical scavenging activity, reducing power, superoxide radical scavenging activity \textit{O basilicum} L. ethanolic leaf extract (LEOB) exhibited potent radical scavenging activity as compared to the standard with p value < 0.01**.

With regard to (Fig 5.8, 5.9, 5.10, 5.11 & 5.12) the bar diagrams revealed potent radical scavenging activity exhibited by ethanolic LEOB. It showed good activity (1%) as percentage of ascorbic acid tested via DPPH radical scavenging assay (Table 5.3). Our results were concurrent with studies performed by Gulcin \textit{et al.}, 2007; Kaurinovic \textit{et al.}, 2011; Shafique \textit{et al.}, 2011 and Tada \textit{et al.}, 1996.
The antioxidant activity of ethanolic LEOB via five model systems can be attributed to the presence of active constituents such as terpenes, polyphenols and flavonoids as shown in (Table 5.2). The major constituents of essential oil in basil are phenylpropanoids and terpenoids such as linalool, eugenol, methyl cinnamate (40%) and methyl chavicol (35%). Thymol, carvacrol and linalool have hydrogen donating ability thereby scavenging reactive oxygen species. The antioxidant activity could also be attributed to the presence of phenolic constituent such as rosmarinic acid which is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid that is believed to be a part of the plant defense system. Flavonoids such as quercetin function as oxygen quenchers. Our results were concurrent with studies done by Abdullah et al., 2009; Lee et al., 2005 and Tada et al., 1996.

Ethanolic LEOB exhibited a dose dependent response with maximum activity at 1000 µg/mL and minimum at 10 µg/mL respectively. The dose dependent response seen by O basilicum L. extracts could be attributed to the increasing amounts of active constituents such as polyphenols and flavonoids with increasing concentration of the extract. With increasing concentration, the hydrogen donating ability of the extract increases. The dose dependent variation could also be attributed to the oxidation of some antioxidant compounds of the extract or due to the low concentration of the extract. Our results were concurrent with observations made by Sarfraz et al., 2011 and Shafique et al., 2011.

Pretreatment with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOB for 48 hours, significantly downregulated the mRNA expression levels of the elevated inflammatory cytokines in arecoline treated cells. The result shown in (Fig 5.36, 5.37 & Table 5.4) indicated almost 7 fold decrease in IL-1β level at 31.25
µg/mL. IL-6 levels reduced by almost 800 fold at 31.25 µg/mL, 1140 fold at 62.5 µg/mL and 1200 fold at 125 µg/mL. TNFα levels decreased by 2 fold at 15.6 µg/mL and 31.25 µg/mL, 16 fold at 62.5 µg/mL and 12 fold at 125 µg/mL. Ethanolic LEOB upregulated the anti-inflammatory cytokine IL-10 by almost 0.2 folds at 31.25 µg/mL and 0.7 fold at 125 µg/mL. The standard drug indomethacin (50 µM), decreased the levels of IL-1β & TNFα by 7 and 13 fold, IL-6 by 1200 folds and increased IL-10 levels by 0.7 folds. The anti-inflammatory activity exhibited by ethanolic LEOB at 125 µg/mL for IL-1β (9 fold), IL-6 (1200 fold) and TNFα (12 fold) was almost similar to that exhibited by indomethacin at 50 µM for IL-1β (10 fold), IL-6 (1200 fold) and TNFα (12 fold). These results strongly suggest that plant extracts contain and exhibit significant anti-inflammatory activity similar to allopathic standard drugs (indomethacin).

Data presented in (Fig 5.40, 5.41 and Table 5.4) showed that pretreatment to HBFs with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOB for 48 hours, significantly downregulated the mRNA expression levels of the elevated fibrotic markers. TGFβ1 at 7.8 µg/mL showed reduction of expression by 1.8 fold, 14 fold at 15.6 µg/mL, 16 fold at 31.25 µg/mL, 18 fold at 62.5 µg/mL and 52 fold at 125 µg/mL. COL1A2 levels decreased by almost 2 fold on treatment with 7.8 µg/mL, 4 fold at 15.6 µg/mL, 5 fold at 31.25 µg/mL, 9 fold at 62.5 µg/mL and 14 fold at 125 µg/mL. COL3A1 level decreased by almost 2 fold at 7.8 µg/mL and 15.6 µg/mL, 4 fold and 6 fold at 62.5 µg/mL and 125 µg/mL.

Ethanolic LEOB exhibited significant anti-inflammatory and antifibrotic activity at higher concentrations of 62.5 µg/mL and 125 µg/mL, although it also showed minimal activity at lower concentrations.
In our study the anti-inflammatory effect of ethanolic LEOB was concurrent with studies performed by Benedec et al. in 2007, who observed that leaf extract of *O basilicum* L. used as tincture (30 mg/100 g b.w) on turpentine induced inflammation in *in-vivo* model (adult male wistar rats), efficiently reduced the total leukocyte count along with reduction in proinflammatory cytokines (IL-1β, IL-6 and TNFα) on 24 hour treatment, the effect observed was stronger when compared to diclofenac sodium. A similar study performed by Selvakkumar et al. in 2007, reported that methanolic extract of *O basilicum* L. at 30 µg/mL downregulated TNFα and IL-1β in LPS induced inflammation in human peripheral blood monocytes. Concurrent results were reported by Meera et al. in 2009, who observed that methanolic extract of *O basilicum* L. at 100 mg/kg b.w. exhibited anti-inflammatory activity in carrageenan induced pedal edema in rats. Phadtare et al. in 2013 reported the anti-inflammatory activity of various species of *O basilicum* L. namely *O basilicum* var. *basilicum*, which at lower doses of 50, 100 and 200 mg/kg body weight decreased carrageenan induced rat paw edema. The anti-inflammatory activity was attributed to the radical scavenging activity of the plant extract consisting of various active constituents namely eugenol, ursolic acid, linalool and oleanolic acid. On similar grounds Mueller et al. in 2010 reported that *O basilicum* L. at 0.2 & 0.5 mg/mL reduced IL-6 and TNFα levels by 25% and upregulated IL-10 levels in LPS stimulated macrophage model.

The antifibrotic effect of ethanolic LEOB observed in our study model was concurrent to studies performed by Dumitriu et al. in 2013, who observed that *O basilicum* L. belonging to the Lamiaceae family efficiently retarded fibroblast turnover by flowcytometry analysis. It also decreased TGFβ secretion and impaired collagen synthesis by upregulating matrix degradation enzymes MMP1 and MMP9;
overall downregulating hydroxyproline content, an indirect measure to quantify collagen in cell culture supernatant. Yacout et al. in 2011 reported that *O. basilicum* L. extract at 200 mg/kg b.w. downregulated CCl₄ induced liver fibrosis in rat model, by increasing liver function enzyme activity and by downregulating the serum marker enzymes such as alkaline phosphatase. It retained tissue architecture and reduced hydroxyproline deposition in hepatocytes thereby preventing hepatic fibrosis. Earlier studies have attributed the anti-inflammatory and antifibrotic activity of *O. basilicum* L. to the presence of essential oils such as eugenol, linalool, thymol, α-caryophylene and the presence of diterpenes and triterpenes (Okoye et al., 2014). In our study, leaves of *O. basilicum* L. were used for extract preparation and experimentation, as the leaves (young & mature) are known to contain the maximum amount of essential oils.

To evaluate the anti-inflammatory and antifibrotic activity of the essential oil linalool; GC/MS was performed to standardize and quantify the amount of linalool in the ethanolic LEOB. The data presented in GC/MS (Fig 5.15 a & b) showed the presence of 0.2% of linalool in 200 g of ethanolic LEOB with a retention time of 6.95. The presence of a small quantity of linalool can be explained because of its volatile nature and low molecular weight, which makes the quantification of the compound arduous. It also largely depends upon the type of solvent, extraction method used and geographical and seasonal variations (Roy et al., 2013). Filho et al. in 2006 reported highest linalool concentration of 69.3% on GC/MS analysis from leaves of *O. basilicum* L. harvested at 16:00 hrs and dried at 40°C. Drying leaves at higher temperatures of 50°C and 60°C decreased linalool content. On the contrary Abduelrahman et al. in 2009 reported low essential oil content (linalool, eugenol, rosmarinic acid) of 0.33% - 0.47% on GC/MS analysis in fresh leaves of *O. basilicum* L. from Sudan. Kasali et al. in 2005 reported 95% of total essential oil obtained by
hydrodistillation of fresh leaves of *O basilicum* L. (Nigeria), 24 compounds were identified by GC/MS analysis of which linalool was 10.8%, methylchavicol (60.3%) and methylcinnamate (6.3%). Earlier studies have reported that essential oils can scavenge free radicals, thereby serving as an effective anti-inflammatory agent. Oxidative burst that occurs in diverse cells (monocytes, macrophages, eosinophils and neutrophils) is a response to inflammation. Reactive oxygen species (O$_2^-$, HO$_2^-$, and ROO$^-$) are produced as a result of phagocytosis and inflammatory processes (NO$, ONOO$) respectively (Miguel, 2010). Thus, linalool can help in combating ROS and inflammatory cytokines, thereby inhibiting the progression of fibrosis.

Pretreatment with non-toxic concentrations (7.8 - 125 µM) of linalool for 48 hours in arecoline treated cells, significantly downregulated the mRNA expression levels of the elevated inflammatory cytokines by almost 1 fold at 7.8 µM, 1.5 fold at 31.25 µM and 62.5 µM and 3.9 fold at 125 µM for IL-1β (Fig 5.38, 5.39 and Table 5.5). IL-6 level decreased by 1 fold at 31.25 µM, 1.9 fold 62.5 µM and 3.5 fold at 125 µM. TNFα decreased by 1.2 fold at 7.8 µM, 2 fold, 3.2 fold and 4.3 fold at 31.25 µM, 62.5 µM and 125 µM of linalool. Linalool treatment concomitantly upregulated the anti-inflammatory cytokine IL-10 by almost 1.8 fold at 31.25 µM and 1.9 fold at 62.5 µM and 125 µM. The standard drug indomethacin (50 µM), downregulated IL-1β & IL-6 by 4 fold, TNFα by 4.9 fold and increased IL-10 by 2 folds. The anti-inflammatory activity exhibited by linalool at 125 µM for IL-1β (3.9 fold), IL-6 (3.5 fold) and TNFα (4.3 fold) was almost similar to that exhibited by indomethacin at 50 µM for IL-1β and IL-6 (4 fold) and TNFα (4.3 fold). Linalool upregulated IL-10 level by 1.9 at 62.5 µM and 125 µM fold same as that of indomethacin at 50 µM. These results strongly suggest that pure compounds contain and exhibit significant anti-
inflammatory activity similar to allopathic standard drugs (indomethacin). Linalool exhibited a significant anti-inflammatory activity at higher concentrations of 62.5 µM and 125 µM, although also showed minimal activity at lower concentration.

On evaluating the antifibrotic activity of linalool, (Fig 5.42, 5.43 and Table 5.5) it was observed that linalool significantly downregulated the mRNA expression levels of COL3A1, COL1A2 and TGFβ1 at a minimum concentration of 7.8 µM. TGFβ1 decreased by almost 12 fold at 7.8 µM and 15.6 µM, 11 fold at 31.25 µM, 7 fold at 62.5 µM and 5 fold at 125 µM. COL1A2 levels also decreased by almost 2 fold at lower concentrations of 7.8 µM, 15.6 µM and 31.25 µM, whereas a decline of only 1 fold was observed at higher concentrations of 62.5 µM and 125 µM. COL3A1 level decreased by 17 fold at 7.8 µM, 13 fold at 15.6 µM and 15 fold at 31.25 µM, whereas a minimal reduction by only 1 fold was observed at higher concentrations of 62.5 µM and 125 µM . A minimal upregulation was observed in the expression levels of COL3A1, COL1A2 and TGFβ1 at higher concentrations of 62.5 µM and 125 µM. Linalool demonstrated antifibrotic activity at lower concentrations of 7.8 µM, 15.6 µM and 31.25 µM. The anti-inflammatory and antifibrotic activity exhibited by linalool at lower concentrations can be attributed to its purity.

The anti-inflammatory and antifibrotic effect of linalool observed in our study model was in concurrence with studies performed by Balasubramaniam and Anuradha in 2011, who reported that linalool treatment for 72 hours in diabetes induced male wistar rats significantly decreased the levels of TNFα, IL-6, TGFβ1 and NF-κB thereby prevented ROS formation and inhibited TGFβ1 mediated production of collagen type I and fibronectin. Okoye et al. in 2014 reported that volatile oils (linalool, eugenol and thymol) at 50 µg/ear demonstrated anti-inflammatory activity.
Discussion

Chapter 6

In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

Inhibited edema) in xylene induced mouse ear edema. He stated that the anti-inflammatory activity of an extract increases with an increase in its volatile oil component. Ku and Lin in 2014 reported that linalool at 500 µM reduced IL-1 & TNFα levels and increased IL-10 level in in-vitro LPS stimulated murine spleenocytes. Miguel in 2010 attributed the anti-inflammatory and antifibrotic activity of linalool not only to its radical scavenging potential (antioxidant activity) but also to its interactions with signaling cascades involving cytokines and regulatory transcription factors.

6.4.3 O vulgare ethanolic leaf extract and thymol

The non-toxic concentrations for ethanolic LEOV and thymol vary according to the cell type on which they have been tested, hours of incubation and culture media used depending on the nature of experiments (Dinesh et al., 2014). The anti-inflammatory and antifibrotic activity of ethanolic crude leaf extract of O vulgare L. (LEOV) and its pure compound, thymol were analyzed after determining the non-toxic concentrations by performing the MTT colorimetric assay (Fig 5.45).

Three day old cells were treated with O vulgare ethanolic leaf extract from 1000 µg/mL to 7.8 µg/mL. Concentrations of 7.8 µg/mL - 125 µg/mL (p < 0.05*) showed no loss in cell viability (Fig 5.45 a). Hence, protection of cultured fibroblasts treated with 7.8 µg/mL - 125 µg/mL of ethanolic LEOV was studied by the expression of molecular markers induced with arecoline.

Thymol at concentrations tested (1000 µM - 7.8 µM), did not cause loss of cell viability at concentrations of (7.8 - 125 µM) (Fig 5.45 b).
6.4.3.1 Molecular marker expression analysis of protected HBF cells induced by arecoline: Anti-inflammatory and antifibrotic activity of ethanolic leaf extract of *O vulgare* L. and thymol

The anti-inflammatory activity of plant extracts has been attributed to their efficacy of scavenging radical species. With regards to the total antioxidant assay, DPPH assay, nitric oxide radical scavenging activity, reducing power, superoxide radical scavenging activity ethanolic leaf extract of *O vulgare* L. (LEOV) exhibited potent radical scavenging activity as compared to the standard with p value < 0.01**.

With regard to (Fig 5.8, 5.9, 5.10, 5.11 & 5.12) the bar diagrams revealed potent radical scavenging activity exhibited by ethanolic LEOV. It showed good activity (0.5% and 0.15%) as percentage of quercetin tested via total antioxidant and reducing power assay (Table 5.3). Our results were concurrent with studies done by Bendini *et al.*, 2002; Cervato *et al.*, 2000; Kacaniova *et al.*, 2012; Khanum *et al.*, 2011; Kulisic *et al.*, 2004 and Meizoso *et al.*, 2009.

The potent antioxidant activity exhibited by ethanolic LEOV can be attributed to the presence of active constituents such as polyphenols and flavonoids as shown in (Table 5.2). Phenolic constituents such as rosmarinic acid, thymol, carvacrol, benzyl alcohol and eugenol in *O vulgare* L. function due to their redox properties which allow them to act as hydrogen donators and singlet oxygen quenchers. Flavonoids such as luteolin, herbacetin and quercetin function as hydrogen donators due to their structure consisting of many hydroxyl groups. Our results were concurrent with studies done by Cervato *et al.*, 2000; Khanum *et al.*, 2011; Kulisic *et al.*, 2004 and Ryszard *et al.*, 2009.
Ethanolic LEOV exhibited a dose dependent relationship with maximum activity at 1000 µg/mL and minimum at 10 µg/mL. The dose dependent response exhibited by ethanolic LEOV can be attributed to the high amount of phenolic constituents present in the extract with increasing concentration. The reductive ability of the extract increases with increased concentration of the extract. Our results were in accordance to the observations made by Gulcin et al., 2011; Kacaniova et al., 2012; Khanum et al., 2011 and Meizoso et al., 2009.

With regard to pretreatment with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOV for 48 hours, significantly downregulated the mRNA expression levels of the elevated inflammatory cytokines in arecoline treated cells. The results shown in (Fig 5.46, 5.47 and Table 5.4) indicated almost 1 fold decrease in IL-1β level at 15.6 µg/mL. IL-6 levels reduced by 15 fold at 15.6 µg/mL and 31.25 µg/mL, 20 fold at 62.5 µg/mL and 17 fold at 125 µg/mL. TNFα decreased by 1.5 fold at 15.6 µg/mL, 1.6 fold at 31.25 µg/mL and 62.5 µg/mL and 1.7 fold at 125 µg/mL. Anti-inflammatory cytokine, IL-10 increased by 1 fold at 31.25 µg/mL, 1.2 and 1.6 folds at 62.5 µg/mL and 125 µg/mL. The standard drug indomethacin (50 µM), decreased the levels of IL-1β by 1.5 fold, IL-6 by 17 fold, TNFα by 2 fold and increased IL-10 levels by 1.7 folds. The anti-inflammatory activity exhibited by ethanolic LEOV at 125 µg/mL by downregulating IL-1β (1.2 fold), IL-6 (17 fold), TNFα (1.7 fold) and upregulating IL-10 (1.6 fold) was almost similar to that exhibited by indomethacin at 50 µM for IL-1β (1.5 fold), IL-6 (17 fold), TNFα (2 fold) and IL-10 (1.7 fold). These results strongly suggest that plant extracts contain and exhibit significant anti-inflammatory activity similar to allopathic standard drugs (indomethacin).
Data presented in (Fig 5.50, 5.51 and Table 5.4), showed that pretreatment with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOV for 48 hours, significantly downregulated the mRNA expression levels of the elevated fibrotic markers. TGFβ1 at 7.8 µg/mL reduced by almost 1.8 fold, 5 fold at 15.6 µg/mL and 31.25 µg/mL, 6 fold and 11 fold at 62.5 µg/mL and 125 µg/mL. COL1A2 levels decreased by almost 2 folds at 7.8 µg/mL and 15.6 µg/mL, by 3 fold at 31.25 µg/mL and by 4 folds at 62.5 and 125 µg/mL. COL3A1 levels reduced by almost 2 fold at 7.8 µg/mL, 15.6 µg/mL and 31.25 µg/mL, 3 fold and 4 fold at 62.5 µg/mL and 125 µg/mL.

Considering the anti-inflammatory activity of ethanolic LEOV our study results were concurrent with experiments performed by Mueller et al. in 2010, who reported that Oregano at a dosage of 0.5 mg/mL decreased the secretion of proinflammatory cytokines IL-6 and TNFα by 25%, simultaneously upregulating the secretion of anti-inflammatory cytokine IL-10; concluding that a diet rich in herbs and spices may contribute in suppressing inflammation. A similar study performed using two fractions (S1 and S2) of O vulgare L. obtained by supercritical fluid extraction on activated THP-1 macrophages (cellular model for atherogenesis) demonstrated a significant reduction in the secretion of proinflammatory cytokines, simultaneously upregulating the anti-inflammatory cytokine IL-10 at a concentration of 30 µg/mL (Ocana et al., 2010). In a comparison established between the anti-inflammatory activities of methanolic extract of O vulgare L. and its most important constituent thymol on activated microglial and mixed glial cells, it was observed that both methanolic extract of O vulgare L. at 2.25 mg/mL and solution of thymol at 0.15 mg/mL exhibited potent anti-inflammatory activity through inhibition of iNOS and TNFα expression. In our study we observed that ethanolic LEOV demonstrated
maximum anti-inflammatory and antifibrotic activity at higher concentrations (62.5 and 125 µg/mL), a recent study by Javadian et al. in 2015 efficiently supports our experimental results. The property of ethanolic extracts exhibiting maximum activity at higher concentration can also be attributed to the high amount of phenolic constituents present in the extract with increasing concentration.

To the best of our knowledge the present study is the first of its kind to demonstrate the antifibrotic activity of ethanolic LEOV on a fibrosis model. We propose that, the potential of ethanolic LEOV to exhibit efficient antioxidant and anti-inflammatory activity could be one of the pathways to demonstrate a significant downregulation of the most crucial fibrogenic marker, (TGFβ1) and its downstream ECM proteins COL3A1 and COL1A2. Studies investigating the antifibrotic activity of different species of Origanum (Origanum syriacum, Origanum elongatum) have been performed on hepatoprotective effects in paracetamol and CCl4 induced liver damage. Origanum species demonstrated therapeutic antifibrotic activity by different mechanisms; such as evaluating levels of glutathione (GSH), glutathione transferase (GST) and superoxide dismutase (Ibrahim et al., 2010). Similarly serum biomarkers (AST, ALT and ALP) have also been evaluated for fibrosis (Douhri et al., 2014), but none have evaluated ethanolic LEOV subsp hirtum for its antifibrotic activity via TGFβ1, COL1A2 AND COL3A1 pathway.

In our study fresh leaves of O vulgare L. were used for experiments, as maximum active constituents (like carvacrol and thymol) are harboured in the leaves of the plants (Hashim, 2011). Earlier studies have attributed the anti-inflammatory and antifibrotic activity of O vulgare L. subsp hirtum to the presence of monoterpenes such as carvacrol and thymol (Bodirlau et al., 2009). To investigate and evaluate the
anti-inflammatory and antifibrotic activity of thymol, detection of thymol and standardization of active compound in the ethanolic LEOV was done by HPTLC.

The data presented in HPTLC (Fig 5.14) showed the presence of thymol in our ethanolic extracts of *O vulgare* L. at effective concentrations. The sample of ethanolic LEOV contained the predominance of thymol at Rf 0.75 (Fig 5.14 a) and 1.2 g of thymol was calculated to be present in 100 g of ethanolic LEOV (Fig 5.14 b & d). The calibration curve plotted against concentration was seen to be linear (Fig 5.14 c). Our results were concurrent with studies performed by Karamanos and Sotiropoulou in 2013, who observed and detected low levels of thymol (0.20% - 1.44%) in Greek oregano (*O vulgare* L. subsp hirtum) cultivated during humid season. The study also highlights, that the variation in the quantities of essentials oils can be attributed to several factors (extraction method, type of solvent, climatic and geographical conditions). Bayramoglu et al. in 2008 reported that the prime aromatic compound of Oregano essential oil was thymol (650-750 mg/mL) quantified by GC/MS analysis. Alma et al. in 2003 reported 2.1% thymol to be present in the essential oil of Syrian Oreganum leaves originating from Turkey. He also observed that Oreganum originating from Israel contained 59.87% of thymol. The variations in the essential oil content were attributed to geographical variations, origin, growing conditions and harvesting methods.

Pretreatment with non - toxic concentrations (7.8 - 125 µM) of thymol for 48 hours, downregulated the mRNA expression levels of the elevated inflammatory cytokines in arecoline treated cells. IL-1β, IL-6 and TNFα levels decreased by almost 1 fold at 7.8 µM, 15.6 µM and 31.25 µM and by 3 fold at 62.5 µM and 125 µM (Fig 5.48, 5.49 and Table 5.5). Thymol treatment concomitantly upregulated the anti-
inflammatory cytokine, IL-10 by almost 3 fold at 7.8 µM, 15.6 µM and 31.25 µM and 4 fold at 62.5 µM and 125 µM. The standard drug indomethacin (50 µM), decreased IL-1β levels by 3.3 fold, IL-6 levels by 4 fold, TNFα by 5 fold and increased IL-10 levels by 7 folds. The above results suggest that indomethacin at 50 µM exhibited better anti-inflammatory activity than thymol, except in relation to IL-1β where thymol downregulated IL-1β by 3 folds at 125 µM and indomethacin downregulated IL-1β by 3.3 fold at 50 µM.

On evaluating the antifibrotic activity of the thymol (Fig 5.52, 5.53 and Table 5.5) it was observed that thymol demonstrated minimal downregulation of the elevated mRNA expression levels of COL3A1, COL1A2 and TGFβ1. TGFβ1 decreased by almost 1 fold at 7.8 µM, 62.5 µM and 125 µM, 1.4 fold at 15.6 µM and 1.5 fold at 31.25 µM. COL1A2 decreased by 1 fold at 7.8 µM, 31.25 µM, 62.5 µM, 125 µM and by 1.3 fold at 15.6 µM. COL3A1 decreased by 1.6 fold at 7.8 µM and 15.6 µM, 1.5 fold at 31.25 µM and 1.4 fold at 62.5 and 125 µM. Thymol exhibited significant antifibrotic activity at lower concentrations of 15.6 µM and 31.25µM. This property of thymol can be attributed to its purity.

Considering the anti-inflammatory activity of thymol, our study results were concurrent with studies performed by Bodirlau et al. in 2009, who reported an inhibition in the production of pro-inflammatory cytokines (IL-1β and IL-6) and prostaglandin on treatment with O vulgare L. He attributed the anti-inflammatory activity to the presence of essential oils; thymol and carvacrol. Similarly, Riella et al. in 2012 reported that thymol at 100 mg/kg b.w. reduced carrageenan induced paw edema & peritonitis by 35.3% and diminished influx of leukocytes. Ershun et al. in
2014 reported that thymol at 4 mg, 8 mg and 16 mg/kg b.w. reduced the number of inflammatory cells and Th2 cytokines in ovalbumin induced mouse asthma.

Liang et al. in 2014 reported thymol as a therapeutic anti-inflammatory agent in LPS stimulated mouse mammary epithelial cells; thymol pretreatment at (10, 20 and 40 µg/mL) for 24 hours, significantly reduced the expression levels of TNFα, IL-6 and IL-1β, it also downregulated the expression levels of iNOS and COX2 in a dose dependent manner. In a combination study performed by Piva et al. in 2015, it was observed that thymol & vanillin (PB) at 5 g/kg for 2 weeks reduced mRNA expression of TNFα, IL-6, IL-1β and TGFβ1, increasing IL-10 in weaned pigs. Yet another study performed by Ku and Lin in 2014, investigated the anti-inflammatory activity of linalool and thymol (monoterpenes) among 27 other terpenoids to inhibit cytokine secretion using murine primary spleenocytes stimulated with an endotoxin LPS. On pretreatment with linalool and thymol for 48 hours an inclination towards Th2 cell type was observed, they significantly increased the expression of IL-10 and decreased the secretion of proinflammatory cytokine IL-1 and TNFα produced from Th1 type cells as a result of LPS stimulation. Thymol and carvacrol at 400 mg/kg b.w. significantly inhibited TNFα secretion in a carrageenan induced pleurisy model, thereby preventing pleural inflammatory exudate formation by 47.3% (Queiroz et al., 2012). A study performed by Javadian et al. in 2015, compared the anti-inflammatory activities of methanolic extract of O vulgare L. and thymol on activated microglial and mixed glial cells. Thymol at 0.15 mg/mL and methanolic extract of O vulgare L. at 2.25 mg/mL respectively effectively inhibited iNOS and TNFα expression.
In our study model, the antifibrotic activity of thymol has been investigated for the first time; no study till date has established a direct link between thymol and fibrotic markers. In a recent study by (Saravanan and Leelevinothan, 2016) reported the inhibition of activation of TGFβ1 in high fat diet induced diabetic nephropathy on thymol treatment. The minimal antifibrotic activity of thymol in our study can be attributed to its ability to scavenge free radicals and to its potent anti-inflammatory effect. Minimal antifibrotic activity of thymol can also be attributed to its healing potential. Wound on the back of rats were dressed with thymol containing collagen based films. Post 7 to 14 days of treatment, prominent wound retraction, improved granulation reaction and better collagen arrangement (Cassia et al., 2013).

6.5 Prevention of fibrosis in cultured cells pretreated with asiatic acid, linalool and thymol.

Data presented in (Figure 5.34, 5.44 & 5.54), showed that fibroblasts exhibited spindle morphology and small quantities of ECM in the untreated (control) group (Fig a), by contrast it was observed that HBFs treated with 25 µg/mL of arecoline appeared distorted, rounded and stained strongly positive for collagen (Fig b) demonstrating excessive deposition of the ECM. In the asiatic acid (Fig 5.34 c), linalool (Fig 5.44 c) and thymol (Fig 5.54 c) treated groups, fibroblasts retained their spindle morphology with a faint positivity for collagen staining, decreasing the ECM deposition. Our study is the first of its kind to evaluate the fibrotic activity post arecoline treatment and antifibrotic activity on pretreatment with minimum concentration (15.6µM) of asiatic acid, linalool and thymol on oral buccal fibroblasts to study the cell morphology and collagen deposition in the ECM qualitatively. Studies performed by Khan et al. in 2012 reported enhanced total collagen protein deposition as an effect of arecanut.
constituents on human gingival fibroblasts via direct red dye staining. A similar study performed by Xu et al. in 2013 reported that kidney sections from mice with tubulointerstitial fibrosis and ureteral obstruction when treated with intermediate doses (1, 4 & 16 mg/kg b.w.) of asiatic acid exerted significant suppressive effects demonstrated by faint blue staining of collagen (Masson trichrome staining) in the ECM, thereby ameliorating fibrosis by preventing fibroblast activation and ECM accumulation mediated by Smad dependent TGFβ pathway.

The above experiments, cumulatively contribute in the understanding of mechanisms of action and therapeutic values of ethanobotanical plants and their respective pure compounds. The present study was valuable in identifying lead compounds and plant species with promising and efficient antioxidant, anti-inflammatory and antifibrotic activity in an oral submucosal fibrosis model. It also helped in establishing a firm association and crosslink between oxidative stress, inflammation and fibrosis.