5.1 Collection, authentication and preparation of crude ethanolic extracts of the three selected plant species.

5.1.1 Collection of the three selected plant species

Aerial parts of *Centella asiatica* L. (*C. asiatica* L.), *Ocimum basilicum* L. (*O. basilicum* L.) and *Origanum vulgare* L. subsp *hirtum* (*O. vulgare* L.) leaves were collected from seasonal herbs, Tuticorin, Tamil Nadu. The impurities were removed and the leaves of the plant were included in the study.

Figure 5.1: Leaves of *C. asiatica* L. (a), *O. basilicum* L. (b) and *O. vulgare* L. (c)
5.1.2 Microscopic and anatomical characterization of the three selected plant species

Microscopic characteristics of *C. asiatica* L., *O. basilicum* L. and *O. vulgare* L. subsp *hirtum* leaves were observed and authenticated by Prof Jayaraman, Director of Institute of Herbal Botany, Tambaram, Tamil Nadu. The authentication certificates are enclosed (Annexure).

**Figure 5.2: Microscopic and anatomical features of C asiatica L.** Leaves of *C. asiatica* L. (a), 10X view, TS of lamina (La) and midrib (MR) along with vascular bundle (VB) [Xylem (X) and Phloem (Ph)] (b), 40X view, of lamina & midrib showing subsidiary (SC), epidermal cells (EC) and glandular trichromes (GT) (c), 10X view, TS showing vein islets (VI) and vein terminations (VT) (d)
In Fig 5.2: The leaf has thick and prominent plano-convex midrib and thick lamina. The adaxial (AdS) epidermal layer of the lamina has rectangular thick cells, abaxial epidermis is thin having narrow cylindrical or spindle shaped cells; both epidermal layers are stomatiferous. Vascular system is multistranded; vascular bundles are collateral with xylem (X) elements situated in the inner region and phloem (Ph) in the outer region of the bundles (Fig b & c). Venation is reticulate, vein islets (VI) are wide, vein terminations (VT) are unbranched (simple) long and slender (Fig d). Powder microscopy revealed epidermal cells fairly wide and elongated. Their anticlinal (right angle) walls are wavy. The stomata are diffuse in distribution; diacytic type. Each stoma has two subsidiary cells at right angles to the guard cells.
Figure 5.3: Microscopic and anatomical features of *O basilicum* L. Leaves of *O basilicum* L. (a), 10X view, TS of lamina(La) and midrib(MR) along with vascular bundle (VB) [Xylem (X) and Phloem (Ph)] (b), 40X view, TS of lamina & midrib showing subsidiary (SC), epidermal cells (EC) and glandular trichomes (GT) (c), 10X view, TS showing vein islets (VI) and vein terminations (VT) (d).
In Fig 5.3: The leaf consists of adaxial (AdS) concavity and abaxial prominent midrib (bowl shaped) with the lamina directed towards upper side. The epidermal layer is thin with small, thick walled cell having eclinate outer walls. The ground tissue is parenchymatous. The lamina is dorsi-ventral; the adaxial epidermis is slightly thick with elliptical and cylindrical cells, abaxial epidermis includes thin and squarish small cells with well distributed stomata (diacytic type) with two subsidiary cells at right angles to the guard cells. Peltate types of glandular epidermal trichomes frequently seen (Fig b & c). Venetian pattern (paradermal section) is sparsely reticulate, vein-islets are wide and polygonal, Vein-terminations wavy, long and slender; xylem and phloem elements also seen (Fig d). Epidermal trichomes (Glandular and non-glandular trichomes) were seen under powder microscopy.
Figure 5.4: Microscopic and anatomical features of *O. vulgare* L. subsp *hirtum*. Leaves of *O. vulgare* L. (a), 10X view, TS of lamina(La) and midrib (MR) along with vascular bundle (VB) [Xylem (X) and Phloem (Ph)] (b), 40X view, TS of lamina & midrib showing epidermal cells (EC) and glandular trichomes (GT) (c), 10X view, TS showing vein islets (VI) and vein terminations (VT) (d)
In Fig 5.4: The leaf consists of fairly prominent midrib (planoconvex) with flat adaxial (AdS) side and semicircular abaxial part. The epidermal layer of the midrib is thick with squarish, highly thick walled cells. The lamina is flat on the adaxial side and undulates on the abaxial side; the adaxial epidermis and abaxial epidermis (stomatiferous) have thick, rectangular cells with thick cuticle. Peltate types of glandular trichomes are frequently seen (Fig b & c). On observing the paradermal sections of the lamina; adaxial epidermis is apostomatic and abaxial epidermal layer is densely stomatiferous. Venetian pattern (paradermal section) is sparsely reticulate, vein-islets are wide and polygonal, Vein-terminations wavy, long and slender (Fig d). Powder microscopy evaluation of fragments of leaf demonstrated epidermal glands (peltate type), leaf margins demonstrated non-glandular trichromes (unicellular or multicellular), and epidermal peelings demonstrated densely distributed stomata with two subsidiary cells.
5.1.3 Preparation of crude ethanolic leaf extracts

Shade dried and powdered leaves of *C asiatica* L., *O basilicum* L. and *O vulgare* L. were macerated in 100% ethanol. The pooled extracts were collected and concentrated for routine use in further experiments.

**Figure 5.5:** Crude ethanolic leaf extract of *C asiatica* L. (LECA), Leaves of *C asiatica* L. (a), shade dried leaves of *C asiatica* L. (b), final crude ethanolic leaf extract of *C asiatica* L. (c)

**Figure 5.6:** Crude ethanolic leaf extract of *O basilicum* L. (LEOB), Leaves of *O basilicum* L. (d), shade dried leaves of *O basilicum* L. (e), final crude ethanolic leaf extract of *O basilicum* L. (f)

**Figure 5.7:** Crude ethanolic leaf extract of *O vulgare* L. (LEOV), Leaves of *O vulgare* L. (g), shade dried leaves of *O vulgare* L. (h), final crude ethanolic leaf extract of *O vulgare* L. (i)
5.1.4 Assessment of physical characteristics

*C asiatica* L., *O basilicum* L. and *O vulgare* L. were assessed for their various physical parameters from time of purchase of the plant material to the final extract preparation (Table 5.1).

<table>
<thead>
<tr>
<th>PHYSICAL PROPERTIES</th>
<th><em>C asiatica</em> L.</th>
<th><em>O vulgare</em> L.</th>
<th><em>O basilicum</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of plant material on purchase</td>
<td>3 kg</td>
<td>4 kg</td>
<td>6 kg</td>
</tr>
<tr>
<td>Weight of plant material on removal of impurities</td>
<td>2.5 kg</td>
<td>3.5 kg</td>
<td>4.5 kg</td>
</tr>
<tr>
<td>Weight of dried plant material</td>
<td>140 g</td>
<td>180 g</td>
<td>216 g</td>
</tr>
<tr>
<td>Weight of powdered plant material</td>
<td>140 g</td>
<td>160 g</td>
<td>200 g</td>
</tr>
<tr>
<td>Volume of pooled extracts</td>
<td>1.5 L</td>
<td>1.0 L</td>
<td>2.2 L</td>
</tr>
<tr>
<td>Final yield of extracts</td>
<td>115 g</td>
<td>73 g</td>
<td>102 g</td>
</tr>
<tr>
<td>Colour of plant material on purchase</td>
<td>Bright green</td>
<td>Bright green</td>
<td>Bright green</td>
</tr>
<tr>
<td>Colour of plant material on drying</td>
<td>Brown green</td>
<td>Brown green</td>
<td>Brown green</td>
</tr>
<tr>
<td>Colour of pooled extract</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Dark green</td>
</tr>
<tr>
<td>Colour of final extract</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Dark green</td>
</tr>
<tr>
<td>Taste of extract</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter - mint</td>
</tr>
<tr>
<td>Smell of extract</td>
<td>Aromatic</td>
<td>Aromatic</td>
<td>Aromatic</td>
</tr>
</tbody>
</table>
Table 5.1: Depicts the physical properties of *C. asiatica* L., *O. basilicum* L. and *O. vulgare* L. subsp *hirtum* during different phases of extraction.

*C. asiatica* L. weighed 3 kg on purchase and yielded a final extract of 115 g. The final extract was dark green in colour, bitter in taste and had an aromatic smell.

*O. basilicum* L. weighed 6 kg on purchase and yielded a final extract of 102 g. The final extract was dark green in colour, bitter mint in taste and had an aromatic smell.

*O. vulgare* L. subsp *hirtum* weighed 4 kg on purchase and yielded a final extract of 73 g. The final extract was dark green in colour, bitter in taste and had an aromatic smell.
5.2 In-vitro biochemical analysis and assessment of antioxidant potential of the three selected plant species

The extent of oxidative damage caused by reactive oxygen species (ROS) can be exacerbated by a decreased efficiency of antioxidant defense mechanism of the body. Literature has reported downregulation of betacarotene levels in patients with progressive stages of OSMF (Aggarwal et al., 2011; Riana et al., 2005) and increase in inducible nitric oxide synthase (iNOS) in the early stages of OSMF (Rajendran and Varkey, 2007 & 2011). Hence anti-oxidant activity for ethanolic leaf extracts of Casiatica L., O basilicum L. and O vulgare L. was determined.

5.2.1 In-vitro preliminary biochemical analysis of the prepared crude ethanolic leaf extracts of Casiatica L., O basilicum L. and O vulgare L.

The antioxidant activity exhibited by medicinal plants has been attributed to the presence of active secondary metabolites (flavanoids, polyphenols and terpenoids), these biologically active compounds combat oxidative stress by quenching reactive oxygen species. Preliminary biochemical analysis was performed to indicate the presence of phytochemical constituents in ethanolic leaf extracts of Casiatica L., O basilicum L. and O vulgare L.
Table 5.2: *In-vitro* biochemical analysis of crude ethanolic leaf extracts of *C. asiatica* L. (LECA), *O. vulgare* L. subsp. *hirtum* (LEOV) and *O. basilicum* L. (LEOB)

<table>
<thead>
<tr>
<th>Phytochemical constituents</th>
<th>Colour Indicating the presence of active constituents</th>
<th><em>C. asiatica</em> L.</th>
<th><em>O. vulgare</em> L.</th>
<th><em>O. basilicum</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triterpenoids</td>
<td>Pink</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroidal terpenoids</td>
<td>Brown ring</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Dark yellow</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Green</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Dark green</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Blue, Green, Red</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>Bluish green</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>White precipitate</td>
<td>-</td>
<td>Mild +</td>
<td>Mild +</td>
</tr>
</tbody>
</table>
In-vitro Biochemical analysis & Antioxidant assays  Chapter 5.2

Table 5.2 depicts the phytochemical constituents in crude ethanolic leaf extracts of *C asiatica* L. (LECA), *O vulgare* L. subsp *hirtum* (LEOV) and *O basilicum* L. (LEOB).

Ethanolic LECA showed positivity for steroidal terpenoids, flavonoids, steroids, glycosides, carbohydrates and polyphenols. Our results were concurrent with studies done by Brinkhaus *et al.* in 2000 who demonstrated the presence of large amounts of flavanoids and triterpenoids, particularly madecassic acid, asiatic acid and asiaticoside. Similar observation was made by Hashim in 2011 who stated that the leaves of *C asiatica* L. comprises of large pools of biologically active constituents like triterpenes e.g asiaticoside, asiatic acid; and a high total phenolic content which is contributed to the presence of flavonoids. The amount of constituents present varies depending upon the location and diverse environmental conditions.

Ethanolic LEOV showed positivity for triterpenoids, flavonoids, glycosides, carbohydrates, polyphenols and a mild positivity for tannins. Our results were concurrent with studies done by Ryszard *et al.* in 2009 who observed positivity of phenolic constituents like thymol, carvacrol, benzyl alcohol and eugenol in the prepared extracts of *O vulgare* L. subsp *hirtum*. Bendini *et al.* in 2002, reported the presence of phenolic compounds and flavonoids namely luteolin, herbacetin and quercetin in the ethanolic extracts of *O vulgare* L.

Ethanolic LEOB showed positivity for triterpenoids, flavonoids, glycosides, carbohydrates, polyphenols and a mild positivity for tannins. Our results were concurrent with studies done by Kaurinovic *et al.* in 2011, who observed the presence of polyphenols and flavanoids in ethanolic extracts of *O basilicum* L; he also commented on the type of solvent used for extraction which could affect the
antioxidant, anti-inflammatory and antimicrobial activities of the herb. Similar results were reported by Hussain et al. in 2008, who demonstrated the presence of essential volatile oils (terpenes) like linalool in the plant extract.

5.2.2 In-vitro evaluation of the antioxidant activity of the prepared crude ethanolic leaf extracts using five assay systems

Oxidative stress plays an important role in oral tissue fibrosis; arecoline promotes formation of reactive oxygen species leading to imbalance in cytokine microenvironment. On evaluation, very low levels of antioxidant vitamins have been observed in OSMF individuals.

10 mg of the extract was dissolved in 1 mL of dimethyl sulphoxide and 9 mL of water and shaken well. Serial dilutions of the extracts (1000 µg, 800 µg, 400 µg, 200 µg, 100 µg, 50 µg, 10 µg) were prepared and tested for their antioxidant property.

Antioxidant activity of the ethanolic LECA, LEOV and LEOB were assessed by determining:

a) Total Antioxidant Activity
b) Reducing Power
c) Nitric oxide scavenging activity assay
d) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay
e) Superoxide radical scavenging activity assay.
Figure 5.8: Depicting the total antioxidant activity of standard (Quercetin) and crude ethanolic leaf extracts of *C. asiatica* L. (LECA), *O. vulgare* L. subsp *hirtum* (LEOV) and *O. basilicum* L. (LEOB)

Values are mean ± Standard Deviation of triplicates. All the extracts exhibited significant antioxidant activity in a dose dependent manner with p < 0.01**

In Fig 5.8: The standard, *C. asiatica* L., *O. vulgare* L. subsp *hirtum* and *O. basilicum* L. ethanolic leaf extracts showed potent antioxidant activity. The standard, ethanolic LEOV and LEOB showed a dose dependent response with maximum activity at a concentration of 1000 µg/mL and minimum activity at 10 µg/mL, whereas ethanolic LECA showed increase in activity from 1000 µg/mL and reached its maximum at 200 µg/mL followed by a steady decrease with minimum activity at 10 µg/mL. With regard to the total antioxidant assay (Fig 5.8), ethanolic LEOV exhibited the highest antioxidant activity at a concentration of 1000 µg/mL.
Figure 5.9: Depicting 2, 2-Diphenyl-1-picrylhydrazyl antioxidant assay of standard (ascorbic acid) and crude ethanolic leaf extracts of *C asiatica* L. (LECA), *O vulgare* L. subsp *hirtum* (LEOV) and *O basilicum* L. (LEOB)

Values are mean ± Standard Deviation of triplicates. All the extracts exhibited significant antioxidant activity in a dose dependent manner with p < 0.01**

**In Fig 5.9:** The standard, *C asiatica* L., *O vulgare* L. subsp *hirtum* and *O basilicum* L., ethanolic leaf extracts exhibited a potent antioxidant activity in a dose dependent response with maximum activity at a concentration of 1000 µg/mL and minimum activity at 10 µg/mL. With regard to the DPPH assay (Fig 5.9), ethanolic LEOB exhibited the highest antioxidant activity at a concentration of 1000 µg/mL.
Figure 5.10: Depicting the Nitric oxide radical scavenging activity of standard (ascorbic acid) and crude ethanolic leaf extracts of *C asiatica* L. (LECA), *O vulgare* L. subsp *hirtum* (LEOV) and *O basilicum* L. (LEOB)

Values are mean ± Standard Deviation of triplicates. All the extracts exhibited significant antioxidant activity in a dose dependent manner with $p < 0.01^{**}$

In Fig 5.10: The standard, *C asiatica* L., *O vulgare* L. subsp *hirtum* and *O basilicum* L., ethanolic leaf extracts exhibited a potent antioxidant activity in a dose dependent response with maximum activity at a concentration of 1000 µg/mL and minimum activity at 10 µg/mL. With regard to the nitric oxide radical scavenging assay (Fig 5.10), ethanolic LECA exhibited highest radical scavenging activity at a concentration of 1000 µg/mL.
Figure 5.11: Depicting the Reducing power of standard (Quercetin) and crude ethanolic leaf extracts of *C asiat*eca* L. (LECA), *O vulg*are* L. subsp hirt*um (LEOV) and *O basil*icum* L. (LEOB)

![Reducing Power Assay](image)

Values are mean ± Standard Deviation of triplicates. All the extracts exhibited significant antioxidant activity in a dose dependent manner with $p < 0.01^{**}$

In Fig 5.11: The standard, *C asiat*eca* L., *O vulg*are* L. subsp hirt*um and *O basil*icum* L., ethanolic leaf extracts exhibited a potent antioxidant activity in a dose dependent response with maximum activity at a concentration of 1000 $\mu$g/mL and minimum activity at 10 $\mu$g/mL. With regard to the reducing power estimation method (Fig 5.11) ethanolic LEOV exhibited the highest antioxidant activity at a concentration of 1000 $\mu$g/mL.
Figure 5.12: Depicting the Superoxide radical scavenging activity of standard (ascorbic acid) and crude ethanolic leaf extracts of *C asiatica* L. (LECA), *O vulgare* L. subsp *hirtum* (LEOV) and *O basilicum* L. (LEOB).

Values are mean ± Standard Deviation of triplicates. All the extracts exhibited significant antioxidant activity in a dose dependent manner with \( p < 0.01 \) **

**In Fig 5.12:** The standard, *C asiatica* L., *O vulgare* L. subsp *hirtum* and *O basilicum* L., ethanolic leaf extracts exhibited a potent antioxidant activity in a dose dependent response with maximum activity at a concentration of 1000 µg/mL and minimum activity at 10 µg/mL. With regard to the superoxide radical scavenging activity (Fig 5.12), ethanolic LECA exhibited highest radical scavenging activity with a concentration of 1000 µg/mL.
Table 5.3: Antioxidant assays of ethanolic leaf extracts compared at 1000 µg/mL of extracts with 1000 µg/mL of standards (as % of control)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>TYPE OF ASSAY</th>
<th>Total antioxidant % of Quercetin activity (OD 695 nm)</th>
<th>Reducing power % of Quercetin activity (OD 700 nm)</th>
<th>DPPH % Ascorbic activity</th>
<th>Nitric oxide radical scavenging assay % Ascorbic activity</th>
<th>Superoxide radical scavenging % Ascorbic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>C. asiatica</em> L.</td>
<td></td>
<td>0.08</td>
<td>0.07</td>
<td>0.85</td>
<td>0.86</td>
<td>1.9</td>
</tr>
<tr>
<td>2. <em>O. basilicum</em> L.</td>
<td></td>
<td>0.39</td>
<td>0.07</td>
<td>1</td>
<td>0.83</td>
<td>1.07</td>
</tr>
<tr>
<td>3. <em>O. vulgare</em> L.</td>
<td></td>
<td>0.5</td>
<td>0.15</td>
<td>0.86</td>
<td>0.85</td>
<td>1.71</td>
</tr>
</tbody>
</table>

*In-vitro* human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis
5.3 Quantification of secondary metabolites in the crude ethanolic leaf extracts of *C asiatica* L., *O basilicum* L. and *O vulgare* L.

Leaf extracts of *C asiatica* L. (LECA), *O basilicum* L. (LEOB) and *O vulgare* L. subsp *hirtum* (LEOV) were reported to have significant antioxidant potential (Adtani *et al*., 2014; Cervato *et al*., 2000; Chauhan *et al*., 2010; Gulcin *et al*., 2007; Huda-Faujan *et al*., 2007; Kacaniova *et al*., 2012; Khanum *et al*., 2011; Obayed *et al*., 2009; Shafique *et al*., 2011; Syed *et al*., 2009). Asiatic acid, linalool and thymol, active secondary metabolites in these plant species, were reported to have antioxidant, anti-inflammatory and antifibrotic activities in *in-vitro* and *in-vivo* models (Abdullah *et al*., 2009; Balasubramaniam and Anuradha, 2011; Dong *et al*., 2004; Hashim, 2011; Huang *et al*., 2011; Liang *et al*., 2014; Okoye *et al*., 2014; Ryszard *et al*., 2009).

Table 5.3 shows that the ethanolic leaf extract of *O vulgare* L. had a good total antioxidant (0.5%) and reducing power activity (0.15%). 2,2, DPPH activity of ethanolic extract of *O basilicum* L. was nearly as effective as standard ascorbic acid. Among the three extracts tested for nitric oxide scavenging and superoxide radical scavenging activity, the ethanolic leaf extract of *C asiatica* L. was the best (with 0.86% and 1.9% of ascorbic acid equivalents, respectively).

In conclusion all three extracts showed desirable antioxidant properties in the biochemical assays used. Hence we attempted to quantify the functional secondary metabolites in the ethanolic extracts of these species using HPTLC and GC/MS. Standards were procured from Sigma Chemical Co (USA) for quantification. The standardized ethanolic extracts in terms of a known reference compound could then be used in the cell line based assays.
5.3.1 Quantification using HPTLC for asiatic acid and thymol in the ethanolic leaf extracts of *C asiatica* L. and *O vulgare* L. (Xie et al., 2014)

5.3.1.1 *C asiatica* L.: The leaves of *C asiatica* L. were shade dried and a stock solution of the ethanolic LECA (50 mg/mL) was prepared in ethanol. Variable concentrations of standard asiatic acid (2, 4, 6, 8 and 10 µl) from a stock of 1 mg/mL, and ethanolic LECA (2, 4, 6 µl) from a stock of 50 mg/mL were spotted on HPTLC plates. The plates were developed using solvent system that comprised of (toluene: ethyl acetate: formic acid: methanol) in the ratio of (7:3:0.5:0.2) and scanned at 203 nm. The results of the densitogram of ethanolic LECA and asiatic acid are shown in Fig 5.13.

Figure 5.13: Shows the densitogram of the crude ethanolic leaf extract of *C asiatica* L. and the standard asiatic acid (Rf 0.24) (a); the absorbance of known amounts (b); linearity of the spotted amounts (c) and the peak area of interest (d).
The UV spectrograph in (Fig 5.13 a), clearly shows that the ethanolic LECA contained about 7 bands with the predominance of asiatic acid at $R_f$ 0.24. The linear regression analysis data for the calibration plots demonstrated linearity with $r = 0.99209$, with respect to peak area, in the concentration range (2, 4, 6, 8 and 10 µl) per spot. The calibration curve plotted against concentration was seen to be linear ($Y= -215.436 + 962.274 * X$; correlation coefficient ($r$) = 0.99209; $sdv = 8.00\%$) (Fig 5.13 c). Based on the chromatogram peak area for the standard and sample (Fig 5.13 b & d) it was estimated that 0.8 g of asiatic acid was present in 100 g of ethanolic LECA. Ethanolic LECA was further evaluated for its anti-inflammatory and antifibrotic potential in cell line based model using stock concentration of 5 mg/mL (ethanolic LECA) with calculated equivalence of 4.8 mg/mL of asiatic acid.

5.3.1.2 *O vulgare* L. **subsp hirtum**: The leaves of *O vulgare* L. were shade dried and a stock solution of ethanolic LEOV (50 mg/mL) and thymol (1 mg/mL) was prepared in ethanol.
Figure 5.14: Shows the densitogram of the crude ethanolic leaf extract of *O. vulgare* L. and standard thymol (R_f 0.75) (a); the absorbance of known amounts (b); linearity of the spotted amounts (c) and the peak area of interest (d)
HPTLC validation for thymol in the crude ethanolic LEOV was carried out (Xie et al., 2014). The results presented in Fig 5.14 showed the \( R_f \) of 0.75 for the molecule eluted with toluene: ethyl acetate (9:2). Samples had mixtures including thymol at \( R_f \) 0.75 well separated. The dilutions (2, 4, 6 µl) of the standard compound showing defined peak areas (Fig 5.14 b) and its linearity (Fig 5.14 c) were used to compute the relative concentrations of the active compound in the extracts (Fig 5.14 d).

The linear regression analysis data for the calibration plots demonstrated linearity with \( r = 0.98571 \), with respect to peak area, in the concentration range (2, 4, 6, 8 and 10 µl) per spot. The calibration curve plotted against concentration was seen to be linear (\( Y = 5337.696 + 1604.018 \times X \); correlation coefficient (\( r \)) = 0.98571; sdv = 6.69%) (Fig 5.14 c). Based on the chromatogram peak area for the standard and sample (Fig 5.14 b & d) it was estimated that 1.2 g of thymol was present in 100 g of ethanolic LEOV. Ethanolic LEOV was further evaluated for its anti-inflammatory and antifibrotic potential in cell line based model using stock concentration of 5 mg/mL (ethanolic LEOV) with calculated equivalence of thymol at 1.5 mg/mL.

5.3.2 Quantification using GC/MS for linalool in the ethanolic leaf extracts of *O basilicum* L.

5.3.2.1 *O basilicum* L.: The leaves of *O basilicum* L. were shade dried. GC/MS was performed for quantifying linalool in ethanolic LEOB as it is a volatile compound with a lower molecular weight.
Figure 5.15: GC/MS of crude ethanolic leaf extract of *O basilicum* L. (LEOB) and standard linalool in which the compound with Retention time 6.95 was identified
The results of the densitogram of ethanolic LEOB and linalool are shown in Fig 5.15. On evaluating the standard linalool as a reference, a retention time of 6.95 was observed (Fig a). On analyzing ethanolic LEOB for the presence of linalool, a similar peak with a retention time of 6.95 was seen confirming the presence of linalool (Fig b). On calculation, 0.2 g of linalool was present in 200 g of ethanolic LEOB. Ethanolic LEOB was further evaluated for its anti-inflammatory and antifibrotic potential in cell line based model using stock concentration of 5 mg/mL (ethanolic LEOB) with calculated equivalence of linalool weighing 1.5 mg/mL.

5.3.3 Conclusion

In the present experimental study, functionally active secondary metabolites were quantified in the ethanolic leaf extracts of three selected plant species (*C asiatica* L., *O basilicum* L. and *O vulgare* L. subsp *hirtum*) using HPTLC and GC/MS. On HPTLC analysis; 0.8 g of asiatic acid was present in 100 g of ethanolic LECA and 1.2 g of thymol was present in 100 g of ethanolic LEOV with a Rf of 0.24 and 0.75 respectively. GC/MS analysis quantified 0.2 g of linalool in 200 g of ethanolic LEOB with a retention time of 6.95. The presence of the above active metabolites in the ethanolic leaf extract of plant species could help us in testing their role in inhibiting inflammation and fibrosis.
5.4. Characterizing fibroblast cell type established from Human buccal cells

Processed fibroblast cell lines [gingival, dermal, uterine, bladder, foreskin and 3T3 (Swiss albino mouse embryo)] (ATCC cell lines) are available, but they do not classify as suitable for studying the mechanisms involved in OSMF. The initial stages of OSMF, are the fibrotic bands at the retromolar region that progress to the buccal mucosa gradually involving the soft palate, uvula, faucial pillars and sometimes the pharynx (Chole et al., 2012; Shafer et al., 2009). Induction of fibrosis in buccal cells during arecanut chewing and arecoline association in this process (association 17.6%) was clearly demonstrated in Taiwanese population (Yang et al., 2001). Arecoline was reported to induce TGFβ/Smad signalling pathway in buccal fibroblast cell lines leading to increased collagen deposition mimicking early stages of OSMF (Khan, 2011). Therefore, studies on cell line based model of human buccal cells would be closer to the in-vivo response of arecoline induced fibrosis and in identifying specific points during the early stages of fibrosis development. Hence, we attempted to isolate and seed primary cell cultures from human buccal cells. Primary fibroblast cultures were assessed for morphology using microscopy (shape & population), for chromosomal abnormalities by karyotyping and determining the cell type and origin using immunocytochemistry to demonstrate the in-vitro fibroblast differentiation.

5.4.1 Standardizing protocol for obtaining primary cultures from buccal samples

Media to multiply and expand the population of fibroblast were optimized. A total of six samples were processed (vide material & methods 4.5.3). Viable cells obtained from three of the samples were contaminated and showed poor fibroblast morphology, while three samples were established successfully. Of the three samples, yield of viable cells ranged from $10^5$ to $10^6$/mL requiring seeding with adjusted appropriate volumes of harvested cells at the rate of $10^3$/mL. The morphology of established cultures is shown in Fig 5.16 (a-c).
Figure 5.16: Establishment and morphology of primary cell cultures of human oral buccal fibroblasts showing seeded cells (a) 3 days post inoculation (b) and confluency on the 9th day (c)
The tissue was obtained from histologically normal surgical third molar areas of subjects after obtaining informed consent. The results presented in Fig 5.16 showed five hundred to thousand (500 - 1000) number of cells in DMEM. Round, live cells typical of mesenchymal origin were seen in (Fig 5.16 a). Microscopy of the cultures on incubation for 72 hours showed attached monolayer of spindle shaped fibroblasts in FBS (Fig 5.16 b). Fibroblasts showed complete (80%) confluency on day 9 (Fig 5.16 c). Such differentiated fibroblasts were used for karyotyping analysis and subculturing cells on coverslips for immunocytochemistry.

5.4.2 Genetic characteristics of established fibroblasts

Karyotyping was performed (vide material & methods 4.5.4) to rule out any chromosomal abnormalities in the established primary fibroblast cells. G-banding of chromosomes from all 3 samples of cultured buccal fibroblast cells showed normal chromosomal morphologies in the fibroblasts and no chromosomal abnormalities (Fig 5.17).

**Figure 5.17: Karyogram from cultured human buccal fibroblasts using Giemsa staining**
Developing Human buccal fibroblast cell line model  

Chapter 5.4

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

Figure 5.18: Immunofluorescence staining of cultured human buccal cells leading to the formation of homogenous fibroblast cells. Vimentin (b & c) staining indicated mesenchymal origin, while s100a4 (e & f) detected the intracellular filaments in fibroblasts. Surface protien receptor (g) TGFβR1, indicated the fibrotic nature of the differentiated cells, while phalloidin (h) was used to show the presence of actin filaments in such cells. DAPI staining of nucleated cells confirmed the viability of the growing cells (a & d)
5.4.3 Immunocytochemistry of fibroblast cells established using fibroblast specific markers

Characterization was performed to ensure and confirm the cell type and origin. To confirm the mesenchymal origin of the cultured cells, vimentin staining was performed (Goodpaster et al., 2008). Filament associated calcium binding protein is characteristic of fibroblastic phenotype (Nishitani et al., 2005) and can be visualized using s100a4. Transforming growth factor beta receptor (TGFβR1), a potent fibrotic mediator (Komuta et al., 2010) indicates its presence on the cell surface of differentiated cells. Phalloidin is a dye that binds to the actin filaments present in contractile cells like fibroblasts (Verderame et al., 1980).

At the end of the 9th day, trypsinized cultures from culture flasks were seeded onto small rounded glass cover slips placed in culture plates containing growth media (DMEM, 5% FBS and PSA). On observing attachment and 40 - 50% confluency (5th day), the cells were fixed in methanol (-20°C) and prepared for staining with the respective antibodies and dyes.

In Fig 5.18: Immunofluorescent analysis of cultured cells showed normal fibroblast growth in cultures. Microscopy of the cultures showed specific fibroblast staining with vimentin (b & c), and s100a4 (e & f). Microscopy of cultures showed positive cytoplasmic staining for phalloidin within the fibroblast cells and TGFβR1 on the fibroblast cell membrane (g-i). The nucleus of fibroblast cells stained positive with DAPI (a & d). Thus using these immunomarkers we established normal fibroblast primary cultures in-vitro. The positivity with vimentin indicated the mesenchymal origin of the fibroblast cell type, the positivity with s100a4 indicated the contractile property of the fibroblasts as s100a4 binds to the actin filaments present in the cell type. Such cultures of the primary fibroblasts were then used for studies on induction of fibrosis with arecoline (pre-stage leading to OSMF) and screening for antifibrotic agents.
5.5 Human buccal fibroblast (HBF) cell line model induced with arecoline

The human buccal fibroblast cell cultures established in the previous chapter could now serve as the model for studying inflammation mediated inducible fibrosis. Upregulation of proinflammatory cytokine expression (IL-1β, IL-6 and TNFα) was reported in OSMF tissues using immunohistochemistry (Haque et al., 1998 and 2000). Gingival fibroblasts showed an enhanced expression of fibrotic markers, TGFβ1, COL1A2 and COL3A1 (Khan, 2011; Khan et al., 2012) when induced with arecanut constituents using microarray assays. We therefore, studied the proinflammatory cytokines (IL-1β, IL-6 and TNFα), anti-inflammatory cytokine (IL-10) and fibrotic markers (TGFβ1, COL1A2 and COL3A1) in an arecoline inducible fibrosis model.

Characterized and established HBF’s (Fig 5.17 & 5.18) were used to develop an experimental in-vitro model for OSMF, using arecoline for inducing fibrosis. Progression of fibrosis in the cultured cells treated with arecoline was optimized by:

a. Determining the cytotoxic (IC50) values for arecoline

b. Expression of molecular markers of inflammation (IL-1β, IL-6, TNFα & IL-10) and fibrosis (TGFβ1, COL1A2 and COL3A1) using RT-PCR

c. Changes in morphology of induced fibrotic cells
5.5.1 MTT cytotoxicity assay

To determine the IC\textsubscript{50} concentration of arecoline: Cell viability and proliferation was monitored by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay at 590 nm. Ten thousand (1 X 10\textsuperscript{4}) normal HBF’s were seeded per well in a 96 well plate and incubated with various concentrations of arecoline (0, 5, 10, 15, 25, 50, 100 and 200 µg/mL) (Shieh et al., 2004) for 48 hours. The proportions of viable cells were determined and the inhibitory concentration (IC\textsubscript{50}) for arecoline is shown in Figure 5.19.

Figure 5.19: Cytotoxicity of arecoline to human buccal fibroblast cells using MTT assay

![Figure 5.19: Cytotoxicity of arecoline to human buccal fibroblast cells using MTT assay](image)

Arecoline at 200 µg/mL was toxic to the cells and killed 70% of the fibroblasts at the end of 48 hours. At 50 µg/mL or 25 µg/mL (p < 0.05*) percentage of cell death was 50% and 46% respectively. Hence, IC\textsubscript{50} for arecoline on HBF’s was determined to be 25 µg/mL. This concentration was used for further experiments.
5.5.2 *In-vitro* semiquantitative assessment of inflammatory (IL-1β, IL-6, TNFα and IL-10) markers post arecoline treatment using reverse transcriptase polymerase chain reaction.

To demonstrate the inflammation induced by arecoline on HBF’s, five lakh cells (5 X 10⁵) were seeded in 6 well plates. Upon attaining 50 - 60% confluency HBF’s were untreated (control, 0 µg/mL) or treated with 5, 10, 15, 25 & 50 µg/mL of arecoline and incubated for 48 hours. At the end of 48 hours HBF’s were evaluated for the expression levels of inflammatory markers using two step semiquantitative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene for expression. An overall significant upregulation of inflammatory markers and downregulation of anti-inflammatory marker was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemeneyi) test was used to analyze statistically significant and effective concentrations of arecoline.
In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

**Figure 5.20:** Increase in expression of proinflammatory and decrease in anti-inflammatory markers in fibroblasts treated with varying concentrations of arecoline

![Gene expression analysis](image)

**Figure 5.21:** Increase in expression of proinflammatory and decrease in anti-inflammatory markers in fibroblasts treated with varying concentrations of arecoline

![Graphical representation](image)
Data presented in Fig 5.20 & 5.21, demonstrated that HBFs treated with arecoline (0 - 50 µg/mL) for 48 hours showed uniform expression of actin (494 bp). Anti-inflammatory cytokine IL-10 showed reduction of expression with increasing concentrations of arecoline treatment. IL-10 activity decreased 2 - 3.8 folds at 25 µg/mL and 50 µg/mL thereby inducing inflammation 48 hours post arecoline treatment. On the contrary, proinflammatory markers IL-1β, IL-6 and TNFα showed significantly increased expression. IL-1β increased 50 folds; IL-6 activity increased 9 - 10 folds and TNFα by 7 folds at 25 µg/mL and 50 µg/mL respectively on arecoline treatment. IL-1β expression as seen in Fig 5.20 was upregulated by 25 folds even with 5 µg/mL post arecoline treatment. While for IL-6, 7.4 fold increase was seen with 15 µg/mL treatment. TNFα was induced markedly by 3 fold with 10 µg/mL of arecoline.

Treatment with arecoline for 48 hours remarkably elevated the mRNA expression levels of proinflammatory cytokines IL-1β, IL-6, TNFα and substantially downregulated the level of anti-inflammatory cytokine IL-10 in a concentration dependent manner; thereby contributing to inflammation. Significant upregulation was observed at 25 µg/mL & 50 µg/mL of arecoline. Hence screening compounds for anti-inflammatory activity could be carried out using arecoline at concentration of 25 µg/mL in cultured HBF’s.

An overall significant upregulation of inflammatory markers (IL-1β, IL-6 (p = 0.005, < 0.01**) & TNFα (p = 0.006, < 0.01**) and downregulation of anti-inflammatory marker, IL-10 (p = 0.006, < 0.01**) on treatment with different concentrations of arecoline for 48 hours was observed. Maximum upregulation was observed at statistically significant concentration of 25 µg/mL for IL-1β (p = 0.007,
< 0.01**) and 50 µg/mL for IL-6 (p = 0.007, < 0.01**) and TNFα (p = 0.01, < 0.05*). Maximum downregulation was observed at significant concentration of 50 µg/mL for IL-10 (p = 0.007, < 0.01**).

5.5.3 *In-vitro* semiquantitative assessment of fibrotic (TGFβ1, COL1A2 and COL3A1) markers post arecoline treatment using reverse transcriptase polymerase chain reaction.

To demonstrate the fibrosis induced by arecoline on HBF’s, five lakh cells (5 X 10^5) were seeded in 6 well plates. Upon attaining 50 - 60% confluency HBF’s were untreated (control 0 µg/mL) or treated with (5, 10, 15, 25 & 50 µg/mL) of arecoline and incubated for 48 hours. At the end of 48 hours HBF’s were evaluated for the expression levels of fibrotic markers using two step semiquantative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene. An overall significant upregulation of fibrotic markers was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemenyi) test was used to analyze statistically significant and effective concentrations of arecoline.
Figure 5.22: Increase in expression of fibrotic markers in fibroblasts treated with varying concentrations of arecoline

Figure 5.23: Increase in expression of fibrotic markers in fibroblasts treated with varying concentrations of arecoline
Data presented in Fig 5.22 & 5.23, demonstrated that HBF’s incubated with arecoline at increasing concentrations (0 - 50 µg/mL) for 48 hours were normal showing uniform expression of actin (630 bp) levels. Fibrogenic mediators TGFβ1, COL1A2 and COL3A1 showed upregulation of expression with increasing arecoline treatment. TGFβ1 activity increased by 3 to 5 folds, COL1A2 by 8 to 10 folds and COL3A1 by 20 folds at 25 µg/mL and 50 µg/mL respectively post arecoline treatment. COL1A2 and TGFβ1 expression as seen in Fig 5.22 was upregulated even with 5 µg/mL of arecoline, while COL3A1 was induced markedly with 10 µg/mL of arecoline. Arecoline treated HBF’s produce reactive oxygen species (ROS). ROS activate the inflammatory cascade by upregulating proinflammatory cytokines, IL-1, IL-6, IL-8 and TNFα. These cytokines further stimulate fibroblast proliferation causing increased collagen production. ROS simultaneously activate NF-κB, JNK, p38 MAPK and phosphorylates SMAD2, leading to upregulation of growth factors PDGF, FGF, TGFβ and CTGF. TGFβ is a potent stimulator and main trigger for increased collagen production (Ekanayaka and Tilakaratne, 2013).

Apart from the above mechanisms arecoline influences deposition of ECM by attenuating collagen degradation proteins (MMP’s) and upregulating inhibitors of collagen breakdown (TIMPs). This imbalance in equilibrium produced by arecoline sequentially leads to increased collagen production (COL1A2 and COL3A1), a hallmark of the disease (Ekanayaka and Tilakaratne, 2013).

Treatment with arecoline at increasing concentrations 0 – 50 µg/mL for 48 hours, remarkably elevated the mRNA expression levels of fibrogenic mediator TGFβ1 and ECM elements COL1A2 and COL3A1 in a concentration dependent manner; thereby contributing to inflammation triggered TGFβ fibrogenesis. An
overall significance of upregulated fibrotic markers (p = 0.005, < 0.01**) on
treatment with arecoline was observed. All fibrotic markers; TGFβ1, COL1A2 and
COL3A1 were statistically significantly upregulated at 50 µg/mL of arecoline (p=
0.0076, < 0.01**). Significant upregulation was observed at 25 µg/mL & 50 µg/mL
by arecoline. Thus a concentration of 25 µg/mL was chosen for further experiments to
induce fibrosis in cell lines (48 hours).

5.5.4 Morphological assessment of HBFs and qualitative assessment of collagen
deposition on induction with arecoline using Masson trichrome staining

Masson trichrome is a connective tissue stain, used to differentiate between
collagen and smooth muscle in tumors and the increase in collagen deposition in
fibrotic conditions. It helps to qualitatively assess the effects of antifibrotic agents on
extracellular matrix deposition (Xu et al., 2013).

Ten thousand (1 X 10⁴) cells were seeded onto round glass cover slips in 12
well plates containing growth media. On attaining 40% - 50% confluency, cells
treated with 25 µg/mL of arecoline and incubated for 48 hours were fixed with 3.7%
formaldehyde. Staining was carried out stepwise according to the protocol
standardized by Lillehei Heart Institute, University of Minnesota, USA (vide material
& methods 4.5.6). The fibrotic effects of arecoline were documented under a bright
field microscope.
Figure 5.24: Qualitative assessment of collagen deposition and fibroblast morphology in cultured cells treated with 25 µg/mL of arecoline and stained with Masson trichrome stain. Normal spindle shaped cells are seen in monolayer (a) while distorted cells were seen with arecoline treatment (b)

In Fig 5.24: The fibrotic effects of arecoline on HBF’s were significantly more with 25 µg/mL treatment. Masson trichrome staining revealed fibroblasts with spindle morphology and scanty quantity of ECM (faint collagen staining) to be present in the untreated (control) group (a). In contrast, HBFs treated with arecoline appeared distorted, rounded and stained, strongly positive for collagen (b) demonstrating excessive deposition of the ECM.
5.5.5 Summary

(1) Arecoline was used to develop an in-vitro cell line model for inducing fibrosis in cultured buccal cells. On MTT cytotoxicity analysis (48 hours), non-toxic concentrations of arecoline were determined.

(2) Arecoline at 25 µg/mL induced distorted and rounded fibroblast morphology and led to excessive collagen deposition in the ECM (strong positive collagen staining) (Fig 5.24).

(3) Such treated cells also expressed inflammatory genes (IL-1β, IL-6, and TNFα) increasingly while downregulating anti-inflammatory cytokine, IL-10.

(4) Collagen deposition increased during the induction of fibrosis as fibrotic marker genes (TGFβ1, COL1A2 and COL3A1) were upregulated (p = 0.005, < 0.01**).

(5) Thus, arecoline effectively, in a dose dependent manner, upregulated the mRNA expression levels of both inflammatory (IL-1β, IL-6, TNFα) and fibrotic (TGFβ1, COL1A2 and COL3A1) markers concomitantly downregulating the anti-inflammatory marker (IL-10). Such a regulated expression of intracellular molecular proteins is reflective of the known pathways of inflammatory mediated TGFβ fibrogenesis.

(6) The present experiments helped us establish, a working in-vitro OSMF model and to determine the end points for inflammation and fibrosis post arecoline treatment. Under these experimental culture conditions the effects of plant extracts and pure compounds were evaluated.
5.6. In-vitro assessment of anti-inflammatory and antifibrotic potential of ethanolic leaf extract of C asiatica L. and asiatic acid on pretreatment in an arecoline induced human buccal fibroblast (HBF) cell line model.

On successfully establishing an experimental in-vitro model for OSMF, by inducing inflammation and fibrosis with arecoline, ethanolic leaf extract of C asiatica L. (LECA) and the pure compound asiatic acid were tested for their anti-inflammatory and antifibrotic potential. As shown in literature, leaf extract of C asiatica L., was known to impart anti-inflammatory activity in in-vivo models at 100 mg/kg b.w. of mice by inhibiting IL-6 and TNFα (Roy et al., 2013) and antifibrotic activity by downregulating TGFβ1, COL1 and COL3 (Widegrow, 2011).

Its pentacyclic triterpenoid asiatic acid was reported as an antifibrotic agent as it reduces collagen synthesis at various concentrations of 5.5 - 2000 µM in HSC-T6 cell line model (Dong et al., 2004). It is also known to target and inhibit proinflammatory cytokines (IL-1β, IL-6 and TNFα) in in-vitro cancer cell lines (Yadav et al., 2010). Since fibrosis results from continuous inflammatory insult to buccal cells, the ability of ethanolic LECA and asiatic acid to reduce inflammation and/or fibrosis was evaluated. The activities of ethanolic LECA and asiatic acid were characterized by:

a. Determining the cytotoxic (IC50) values for ethanolic LECA and asiatic acid.

b. Expression of molecular markers of inflammation (IL-1β, IL-6, TNFα & IL-10) and fibrosis (TGFβ1, COL1A2 & COL3A1) on pretreatment with ethanolic LECA and asiatic acid in arecoline induced buccal fibroblast cell line model.

c. Changes in morphology of induced fibrotic cells on pretreatment with asiatic acid.
5.6.1 MTT cytotoxicity assay

Determination of inhibitory concentration (IC\textsubscript{50}) for ethanolic LECA and asiatic acid

Cell viability and proliferation was monitored by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay at 590 nm. Ten thousand (1 X 10\textsuperscript{4}) normal HBF’s were seeded per well in 96 well plate and incubated with various concentrations of ethanolic LECA (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) and asiatic acid (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µM) (Dinesh \textit{et al.}, 2014) for 48 hours. The proportions of viable cells were determined and the inhibitory concentration (IC\textsubscript{50}) for ethanolic LECA and asiatic acid is shown in Figure 5.25 (a & b).

\textit{Figure 5.25: Determining cytotoxic concentration (IC\textsubscript{50}) of crude ethanolic leaf extract of \textit{C asiatica} L. and asiatic acid 48 hours post treatment}
Ethanolic LECA and asiatic acid induced cytotoxicity to HBFs at a concentration above 125 µg/mL and 125 µM respectively (p < 0.05*) during 48 hours incubation (Fig 5.25 a & b). At concentrations of 250, 500 & 1000 µg/mL of ethanolic LECA, fibroblast cell proliferation was inhibited and cell death upto 56.9%, 72.8% and 65.1% was observed. Whereas at 250, 500 & 1000 µM of asiatic acid, fibroblast cell proliferation was inhibited and cell death upto 45%, 43% and 42% was seen.

Thus, for testing the anti-inflammatory and antifibrotic activity of ethanolic LECA and asiatic acid in arecoline induced OSMF cell line model, concentrations of 7.8 - 125 µg/mL and 7.8 - 125 µM were used routinely.

5.6.2 Evaluation of molecular markers of inflammation and fibrosis using RT-PCR on pretreatment with ethanolic leaf extract of C asiatica L. (LECA) and asiatic acid

HBFs cell line model pretreated for 48 hours with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LECA and asiatic acid (7.8 - 125 µM) were induced and incubated for 48 hours with arecoline (25 µg/mL) for fibrosis. The ability of ethanolic LECA and asiatic acid to protect cells from inflammation and fibrosis was assessed using RT-PCR.

5.6.2.1 In-vitro semiquantitative assessment of inflammatory markers on pretreatment with ethanolic leaf extract of C asiatica L. (LECA) and asiatic acid in arecoline induced fibrosis in HBF

To demonstrate the anti-inflammatory activity of ethanolic LECA and asiatic acid five lakh cells (5 X 10⁵) were seeded in 6 well plates. Upon attaining 50 - 60% confluence HBF’s were untreated (control; 0 µg/mL) or treated with ethanolic LECA.
(7.8, 15.6, 31.25, 62.5, 125 µg/mL) or asiatic acid (7.8, 15.6, 31.25, 62.5, 125 µM).

Cells then were incubated for 48 hours and fibrosis was induced with 25 µg/mL of arecoline. Indomethacin, a known anti-inflammatory drug; was used as a positive control. At the end of 48 hours HBF’s were evaluated for the expression levels of inflammatory markers using two step semiquantative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene.

An overall significant downregulation of inflammatory markers and upregulation of anti-inflammatory marker was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemeneyi) test was used to analyze statistically significant and effective concentrations of ethanolic LECA and asiatic acid.
Figure 5.26: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with ethanolic leaf extract of *C. asiatica* L. in arecoline induced cell line model

![Figure 5.26](image)

Figure 5.27: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with ethanolic leaf extract of *C. asiatica* L. in arecoline induced cell line model

![Figure 5.27](image)
5.6.2.1.1 Activity of ethanolic leaf extract of *C asiatica* L.

Data presented in Fig 5.26 & 5.27, demonstrated that HBFs pretreated with ethanolic LECA at increasing concentrations (7.8 - 125 µg/mL) for 48 hours were normal showing uniform expression of β actin (494 bp). Arecoline (25 µg/mL) downregulated the anti-inflammatory marker (IL-10) by 3.8 folds when compared to untreated group (Fig 5.20). On treatment with 31.25 µg/mL of ethanolic LECA, almost 2 fold increase in IL-10 expression was observed, in such arecoline treated cells. While with 62.5 µg/mL and 125 µg/mL the increase observed was 3 folds and 4 folds respectively. Indomethacin (standard drug) at 50 µM showed anti-inflammatory effect by upregulating IL-10 expression upto 11.3 folds.

On the contrary, arecoline (25 µg/mL) upregulated proinflammatory markers (IL-1β, IL-6 and TNFα) when compared to baseline (untreated) (Fig 5.20). On treatment with 31.25 µg/mL of ethanolic LECA, almost 2 fold decrease in IL-1β expression was observed, whereas on treatment with 62.5 µg/mL and 125 µg/mL the decrease in expression observed was 3 fold and 6 fold respectively. Indomethacin (50 µM) showed anti-inflammatory effect by decreasing IL-1β levels by 13 folds.

IL-6, on treatment with 15.6 µg/mL of ethanolic LECA, almost 1.7 fold decrease in expression was observed, whereas with 62.5 µg/mL and 125 µg/mL the decrease in expression observed was 24 fold and 61 fold respectively. Indomethacin (50 µM) showed anti-inflammatory effect by decreasing IL-6 levels by 94 folds.

TNFα, on treatment with 31.25 µg/mL of ethanolic LECA, 2.5 fold decrease in expression was observed. On treatment with 62.5 µg/mL and 125 µg/mL the
decrease in expression observed was 5 fold and 7 fold respectively. Indomethacin (50 µM) exhibited anti-inflammatory effect by decreasing TNFα levels upto 13 folds.

Inflammatory markers (IL-1β, IL-6 and TNFα) were upregulated in arecoline treated HBF’s (Fig 5.20) while indomethacin suppressed arecoline induced overexpression of IL-1β by 13 fold, IL-6 by 94 fold and TNFα by 13 fold.

In comparison to the above results the effective concentrations of ethanolic LECA were 62.5 µg/mL and 125 µg/mL, which downregulated IL-10 levels upto 3 & 4 fold, upregulated IL-1β by 3 & 6 fold, IL-6 by 24 & 61 fold and TNFα by 5 and 7 folds.

An overall significant downregulation of inflammatory markers (IL-1β, IL-6 & TNFα) and upregulation of anti-inflammatory marker (IL-10) on treatment with different concentrations of ethanolic LECA was observed with (p = 0.003, < 0.01**), on comparison with arecoline induced group. Ethanolic LECA significantly downregulated IL-1β (p = 0.048, < 0.05*), IL-16 (0.027, < 0.05*) & TNFα (p = 0.007, <0.01**) and upregulated IL-10 (p = 0.007, < 0.01**) at a significant concentration of 125 µg/mL. The standard drug, indomethacin at 50 µM also exhibited a significant downregulation of proinflammatory markers IL-1β (p = 0.007, < 0.01**), IL-6 (p = 0.014, < 0.05*), TNFα (p = 0.007, < 0.01**) and significant upregulation of anti-inflammatory cytokine IL-10 (p = 0.048, < 0.05*).
Figure 5.28: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with asiatic acid in arecoline induced cell line model.

![Graph showing expression levels of inflammatory markers with asiatic acid treatment](image)

Figure 5.29: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with asiatic acid in arecoline induced cell line model.

![Graph showing relative gene expression](image)
5.6.2.1.2 Activity of asiatic acid

Arecoline (25 µg/mL) induced inflammation by downregulating IL-10 by 3.8 fold on comparison with control (Fig 5.20). In fig 5.28 & 5.29, on treatment with 31.25 µM of asiatic acid, 1.7 fold increase in IL-10 level was observed, whereas at higher concentrations of 62.5 and 125 µM almost a 2 fold increase was observed. Indomethacin (50 µM) significantly upregulated IL-10 level by 3.7 fold.

On the contrary, treatment with arecoline (25 µg/mL) upregulated proinflammatory cytokines, IL-1β (50 fold), IL-6 (9 fold) and TNFα (7 fold) (Fig 5.20). On treatment with a minimum concentration of 7.8 µM of asiatic acid, an efficient downregulation by 2 folds in IL-1β level was observed. Asiatic acid exhibited anti-inflammatory effect at 15.6 and 31.25 µM by decreasing IL-1β levels by 3 folds, 4 fold at 62.5 µM and by 22 folds at 125 µM. Indomethacin (50 µM) demonstrated significant anti-inflammatory activity by reducing IL-1β level by 39 folds.

Asiatic acid (7.8 - 31.25 µM) was found to reduce IL-6 by 2 folds. At higher concentrations of 62.5 and 125 µM, asiatic acid decreased IL-6 levels by 3 and 4 folds respectively. Indomethacin (50 µM) demonstrated an effective reduction by 9 folds.

On treatment with 7.8 µM of asiatic acid, almost 3 fold decrease was observed in TNFα level. A significant reduction of 22 fold was seen at 31.25 µM, 33 fold at 62.5 µM and 43 folds at 125 µM. Indomethacin (50 µM) downregulated the inflammatory response by reducing TNFα level by 55 folds. Thus, asiatic acid exhibited significant anti-inflammatory activity at minimum concentration of 31.25 µM for all markers evaluated.
An overall significant downregulation of inflammatory markers (IL-1β, IL-6 & TNFα) and upregulation of anti-inflammatory marker (IL-10) on treatment with different concentrations of asiatic acid was observed with (p = 0.003, < 0.01**), on comparison with arecoline induced group. Asiatic acid at 125 µM significantly downregulated IL-1β, IL-6 and TNFα (p = 0.048, < 0.05*). The standard drug, indomethacin at 50 µM, exhibited a significant downregulation of proinflammatory markers IL-1β, IL-6 & TNFα (p = 0.007, < 0.01**).

5.6.2.2 In vitro semiquantitative assessment of fibrotic markers on pretreatment with ethanolic leaf extract of C asiatica L. (LECA) and asiatic acid on arecoline induced fibrosis in HBF.

Five lakh cells (5 X 10⁵) were seeded in 6 well plates and upon attaining 50 - 60% confluency HBF’s were either untreated (control; 0 µg/mL) or treated with (7.8, 15.6, 31.25, 62.5, 125 µg/mL) of ethanolic LECA or (7.8, 15.6, 31.25, 62.5, 125 µM) of asiatic acid and were incubated for 48 hours. HBF’s were then induced with fibrosis on treatment with 25 µg/mL of arecoline for 48 hours. At the end of incubation HBF’s were evaluated for the expression levels of fibrotic markers using two step semiquantitative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene. An overall significant downregulation of fibrotic markers was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemenyi) test was used to analyze statistically significant concentration of ethanolic LECA and asiatic acid that downregulated the fibrotic markers effectively.
Figure 5.30: Fold decrease in fibrotic marker expression in arecoline induced HBFs cell line model pretreated with ethanolic leaf extract of *C. asiatica* L.

![Figure 5.30](image1)

Figure 5.31: Fold decrease in fibrotic marker expression in arecoline induced HBFs cell line model pretreated with ethanolic leaf extract of *C. asiatica* L.

![Figure 5.31](image2)
5.6.2.2.1 Activity of ethanolic leaf extract of *C asiatica* L. (LECA) on fibrosis

On observation from figure 5.30 & 5.31 (baseline control vs. induced control), arecoline (25 µg/mL) induced fibrosis in HBF’s by upregulating TGFβ1 expression 24 folds, COL1A2 by 66 folds and COL3A1 by 84 folds when compared with baseline (untreated). Data shown in Fig 5.30 & 5.31 indicated 1.7 folds reduction in TGFβ1 levels on treatment with 7.8 µg/mL of ethanolic LECA. Maximum reduction was observed with 31.25 µg/ml, 62.5 µg/mL and 125 µg/mL (5, 14 and 109 folds respectively) when compared with induced control.

Downregulation of COL1A2 by almost 1.5 fold was observed at 15.6 µg/mL. Effective downregulation by 2 fold and 4 fold was observed at 62.5 µg/mL and 125 µg/mL when compared to the induced control. COL3A1 expression was downregulated by almost 2 folds at 7.8 µg/mL, 15.6 µg/mL and 31.25 µg/mL. Effective reduction by 4 fold and 198 fold was observed at higher concentrations of 62.5 µg/mL and 125 µg/mL of extracts.

At 125 µg/mL of extracts arecoline induced cells showed near baseline levels of expression in TGFβ1, COL1A2 and COL3A1. An overall significant downregulation of fibrotic markers (TGFβ1, COL1A2 and COL3A1) on treatment with different concentrations of ethanolic LECA was observed with (p = 0.003, < 0.01**), on comparison with arecoline induced group. TGFβ1, COL3A1 & COL1A2 significantly downregulated at 125 µg/mL (p = 0.007, < 0.01** and p = 0.048, < 0.05*) respectively.
Figure 5.32: Fold decrease in fibrotic marker expression in arecoline induced HBFs cell line model pretreated with asiatic acid

Figure 5.33: Fold decrease in fibrotic marker expression in arecoline induced HBFs cell line model pretreated with asiatic acid
5.6.2.2.2 Activity of asiatic acid

Arecoline (25 µg/mL) induced fibrosis in HBF’s was demonstrated as it upregulated TGFβ1 expression by 149 fold, COL1A2 by 100 folds and COL3A1 by 118 folds when compared with baseline (untreated) (Fig 5.32 & 5.33).

On treatment with asiatic acid, effective reduction in levels of TGFβ1 by almost 40 folds was observed at 7.8 µM. By 7 fold at 15.6 µM and by 6 fold at 31.25 µM. At higher concentrations of 62.5 µM and 125 µM a minimum reduction of 3 fold was observed. COL1A2 levels efficiently reduced by 13 to 14 folds at 7.8, 15.6 and 31.25 µM of asiatic acid. 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. A minimal reduction of 1 fold was observed at higher concentrations of 62.5 µM and 125 µM. A significant observation from this data was in effectiveness of asiatic acid to reduce the expression of fibrotic markers to a minimum of 1 fold beyond 31.25 µM.

Overall, reduction in levels of TGFβ1 (40 fold at 7.8 µM, 7 fold at 15.6 µM and 6 fold at 31.25 µM), COL1A2 (13 fold at 7.8 µM and 14 fold at 15.6 µM and 31.25 µM), COL3A1 (12-14 fold at 15.6 & 31.25 µM) was observed. An overall significant downregulation of fibrotic markers on treatment with different concentrations of asiatic acid was observed (p = 0.003, < 0.01**), on comparison with arecoline induced group. Asiatic acid statistically significantly downregulated TGFβ1 at 7.8 µM, COL1A2 and COL3A1 at 31.25 µM (p = 0.048, < 0.05*).
5.6.3 Morphological assessment of human buccal fibroblasts (HBFs) and qualitative assessment of collagen, post arecoline treatment and on pretreatment with asiatic acid.

Masson trichrome staining was performed to observe fibroblast morphology and qualitatively assess collagen deposition in the ECM post arecoline treatment and compare it with asiatic acid pretreated cells. From the data shown in Fig 5.32, effective concentration of asiatic acid that downregulated the expression of fibrotic markers was 7.8 and 15.6 µM. Hence, to assess morphological restoration of arecoline induced fibrosis, HBF’s were pretreated with 15.6 µM asiatic acid for 48 hours.

Ten thousand (1 X 10⁴) cells were seeded onto round glass cover slips in 12 well plates containing growth media. On attaining 40% - 50% confluency, cells pretreated with 15.6 µM of asiatic acid for 48 hours were further treated with 25 µg/ml of arecoline and incubated for 48 hours. Such treated cells were then fixed with 3.7% formaldehyde. Staining was carried out stepwise according to the protocol standardized by Lillehei Heart Institute, University of Minnesota, USA (vide material & methods 4.5.6). The fibrotic effects of arecoline were documented under a bright field microscope.
Figure 5.34: Qualitative assessment of collagen deposition and fibroblast morphology using Masson trichrome stain in cultured cells treated with 25 µg/mL of arecoline and on pretreatment with 15.6 µM of asiatic acid. Normal spindle shaped cells were seen in a monolayer (a) while distorted cells were seen with arecoline treatment (b). Cells retained their spindled morphology on asiatic acid treatment (c).

In Fig 5.34: Fibroblasts exhibited spindle morphology and small quantities of ECM in the untreated (control) group (a), by contrast it was observed that HBFs treated with arecoline 25 µg/mL appeared distorted, rounded and stained strongly for collagen (b) demonstrating excessive deposition of collagen. In the asiatic acid pretreated group (c), fibroblasts retained their spindle morphology, showed very low collagen staining, and decreased ECM deposition. The group treated with asiatic acid appeared similar to the control group.
5.7 *In-vitro* assessment of anti-inflammatory and antifibrotic potential of ethanolic leaf extract of *O basilicum* L. (LEOB) and linalool on pretreatment in an arecoline induced human buccal fibroblast cell line model

On successfully establishing an experimental *in-vitro* model for OSMF, arecoline induced inflammation and fibrosis. Ethanolic leaf extract of *O basilicum* L. (LEOB) and its major active compound linalool were tested for their anti-inflammatory and antifibrotic potential. Reported in the literature, leaf extracts 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 treatment for 24 hours (Benedec *et al.*, 2007). Ethanolic leaf extract of *O basilicum* L. (200 mg/kg b.w.) was reported as an anti-fibrotic agent on being tested in rats with carbon tetrachloride induced liver fibrosis (Yacout *et al.*, 2011). Its sesquiterpenoid linalool was reported as an antifibrotic and anti-inflammatory agent in reducing diabetes-induced nephropathic changes in the kidney of male wistar rats. Linalool after 72 hours treatment reduced the levels of TNFα, IL-6, TGFβ1 and NF-κB (Balasubramaniam and Anuradha, 2011).

Since fibrosis results from continuous inflammatory insult to buccal cells, the ability of ethanolic leaf extract of *O basilicum* L. (LEOB) and linalool to reduce inflammation and/or fibrosis was evaluated. The activities of ethanolic LEOB and linalool were characterized by:

a. Determining the cytotoxic (IC₅₀) values for ethanolic LEOB and linalool.

b. Expression of molecular markers of inflammation (IL-1β, IL-6, TNFα & IL-10) and fibrosis (TGFβ1, COL1A2 and COL3A1) on pretreatment with ethanolic LEOB and linalool.

c. Monitoring changes in morphology of induced fibrotic cells on pretreatment with linalool.
5.7.1 MTT cytotoxicity assay for ethanolic leaf extract of *O basilicum* L. (LEOB) & linalool

**Determination of inhibitory concentration (IC\textsubscript{50}) for crude ethanolic extracts**

The alleviation of arecoline induced inflammation and fibrosis induction was assayed using ethanolic LEOB and linalool. To determine the concentrations required for the *in-vitro* activity of ethanolic LEOB and linalool, cytotoxicity assay was performed.

Cell viability and proliferation was monitored by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay at 590 nm. Ten thousand (1 X 10\textsuperscript{4}) normal HBF's were seeded per well in 96 well plate and incubated with various concentration of ethanolic LEOB (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) and linalool (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µM) (Dinesh *et al.*, 2014) for 48 hours. The proportions of viable cells were determined. The inhibitory concentration (IC\textsubscript{50}) for ethanolic LEOB and linalool is shown in Figure 5.35.
Figure 5.35: Determining cytotoxic concentration (IC$_{50}$) of crude ethanolic leaf extract of *O. basilicum* L. and linalool

48 hours post treatment

Ethanolic LEOB and linalool induced cytotoxicity to HBFs at a concentration above 125 µg/mL and 125 µM respectively (p < 0.05*) during 48 hours incubation (Fig 5.35 a & b). At concentrations of 250, 500 & 1000 µg/mL of ethanolic LEOB and 250, 500 & 1000 µM of linalool, fibroblast cell proliferation was inhibited upto 54.3%, 56.5%, 60.15% for ethanolic LEOB and 54.4%, 55.21% and 58.8% for linalool respectively. Thus, for testing the anti-inflammatory and antifibrotic activity of ethanolic LEOB and linalool in arecoline induced OSMF cell line model, concentrations of (7.8 - 125 µg/mL and 7.8 - 125 µM) were used routinely.
5.7.2 Evaluation of molecular markers of inflammation and fibrosis using RT-PCR on pretreatment with ethanolic leaf extract of *O basilicum* L. (LEOB) and linalool

HBFs cell line model pretreated with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOB and (7.8 - 125 µM) of linalool were induced with arecoline for fibrosis. The ability of ethanolic LEOB and linalool to protect cells from inflammation and fibrosis was assessed using RT-PCR.

5.7.2.1 *In vitro* semiquantitative assessment of inflammatory markers on pretreatment with ethanolic leaf extract of *O basilicum* L. (LEOB) and linalool on arecoline induced fibrosis in HBF

To demonstrate the anti-inflammatory activity of ethanolic LEOB on pretreatment, five lakh cells (5 X 10^5) were seeded in 6 well plates. Upon attaining 50 - 60% confluency, HBF’s were untreated (control; 0 µg/mL) or treated with (7.8, 15.6, 31.25, 62.5, 125 µg/mL) of ethanolic LEOB and (7.8, 15.6, 31.25, 62.5, 125 µM) of linalool and incubated for 48 hours. Post incubation HBF’s were treated with arecoline (25 µg/mL) and further incubated for 48 hours following which HBF’s were evaluated for the expression levels of inflammatory markers using two step semiquantitative RT-PCR. Indomethacin was used as a positive control. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene.

An overall significant downregulation of inflammatory markers and upregulation of anti-inflammatory marker was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemeneyi) test was used to analyze statistically significant and effective concentrations of ethanolic LEOB and linalool.
Figure 5.36: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with ethanolic leaf extract of *O basilicum* L. in an arecoline induced cell line model

![Figure 5.36](image1.png)

Figure 5.37: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with ethanolic leaf extract of *O basilicum* L. in an arecoline induced cell line model

![Figure 5.37](image2.png)
5.7.2.1.1 Effect of ethanolic leaf extract of \textit{O basilicum} L. (LEOB)

Data presented in Fig 5.36 & 5.37, demonstrated that HBFs pretreated with ethanolic LEOB at increasing concentrations (7.8 - 125 µg/mL) for 48 hours were normal showing uniform expression of β actin (494 bp). Arecoline (25 µg/mL) induced inflammation by downregulating the anti-inflammatory cytokine (IL-10) by 3.8 folds when compared to baseline (untreated) (Fig 5.20).

On treatment with 31.25 µg/mL of ethanolic LEOB almost 0.2 fold increase in expression of IL-10 was observed. Whereas 0.7 fold increase was observed at 125 µg/mL. A similar anti-inflammatory activity (0.7 fold increase) was observed on treatment with standard drug (indomethacin, 50 µM)

On the contrary, arecoline (25 µg/mL) upregulated proinflammatory markers (IL-1β, IL-6 and TNFα) when compared to baseline control (0 µg/mL) (Fig 5.20). On treatment with 31.25 µg/mL of ethanolic LEOB, almost 7 fold decrease in expression of IL-1β was observed, whereas at higher concentrations of 62.5 and 125 µg/mL the decrease in expression observed was 8 fold and 9 fold respectively. On comparison with indomethacin (50 µM) anti-inflammatory effect was observed by downregulating IL-1β upto 7 folds, similar to that exhibited by 31.25 µg/mL of ethanolic LEOB.

Ethanolic LEOB at 31.25 µg/mL, downregulated IL-6 expression by almost 800 fold, exhibiting maximum anti-inflammatory activity at 62.5 and 125 µg/mL (1140 and 1200 fold) decrease. Indomethacin (50 µM), was observed to decrease IL-6 level by 1200 fold.

On treatment with 15.6 and 31.25 µg/mL of ethanolic LEOB, almost 2 fold decrease in TNFα expression was observed. At higher concentration of 62.5 and 125
µg/mL, 16 fold and 12 fold decrease in expression respectively was observed. Indomethacin (50 µM), showed anti-inflammatory effect decreased TNFα expression by 13 folds.

Overall, arecoline induced inflammation in established HBF cell line by upregulating proinflammatory cytokines (IL-1β, IL-6 and TNFα) and downregulating anti-inflammatory cytokine (IL-10) in a dose dependent manner (Fig 5.20). Indomethacin (50 µM) suppressed arecoline induced overexpression by down regulating IL-1β activity by 7 fold, IL-6 by 1200 fold and TNFα by 13 fold, concomitantly upregulating IL-10 levels 0.7 fold. In comparison to the above results the effective concentration of ethanolic LEOB that exhibited effective anti-inflammatory activity ranged from 31.25 µg/mL to 125 µg/mL.

An overall significant downregulation of inflammatory markers; IL-1β (p = 0.004, <0.01**), IL-6 (p = 0.006, < 0.01**) & TNFα (p = 0.003, < 0.01**) and upregulation of anti-inflammatory marker, IL-10 (p = 0.004, < 0.01**) on treatment with different concentrations of ethanolic LEOB was observed on comparison with arecoline induced group. Ethanolic LEOB significantly downregulated IL-1β at 62.5 & 125 µg/mL (p = 0.03, < 0.05* & p = 0.008, < 0.01** respectively), IL-6 at 62.5 & 125 µg/mL (p = 0.03 & p = 0.02, < 0.05* respectively) & TNFα at 62.5 µg/mL (p = 0.007, <0.01**) and upregulated IL-10 at 125 µg/mL (p= 0.048, <0.05*). The standard drug, indomethacin at 50 µM, exhibited a significant downregulation of proinflammatory markers IL-6 (p = 0.01, < 0.05*), TNFα (p = 0.048, < 0.05*) and upregulation of anti-inflammatory cytokine, IL-10 (p = 0.007, < 0.01**).
Figure 5.38: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with linalool in an arecoline induced cell line model.

Figure 5.39: Decrease in expression of proinflammatory and increase in anti-inflammatory markers on pretreatment with linalool in an arecoline induced cell line model.
5.7.2.1.2 Effect of linalool

Arecoline (25 µg/mL) induced inflammation by downregulating the anti-inflammatory cytokine (IL-10) by 3.8 folds when compared to baseline (Fig 5.20). On treatment with 31.25 µM of linalool, 1.8 fold increase in IL-10 level was observed (Fig 5.38 & 5.39). At higher concentrations of 62.5 µM and 125 µM almost 1.9 fold increase was observed. Indomethacin (50 µM) significantly upregulated IL-10 levels by 2 fold.

On the contrary, arecoline (25 µg/mL) upregulated proinflammatory markers (IL-1β, IL-6 and TNFα) by 50 fold, 9 fold and 7 fold respectively (Fig 5.20) thereby inducing inflammation in HBF’s.

On treatment with 7.8 µM of linalool, 1 fold decrease in IL-1β expression was observed. Downregulation by 1 to 1.5 fold was observed at 62.5 µM with maximum downregulation of upto 3.9 fold at 125 µM. On comparison with indomethacin (50 µM), 4.2 fold decrease in IL-1β expression was observed.

One fold decrease of IL-6 expression was observed at 31.25 µM of linalool, with maximum downregulation of upto 1.9 fold and 3.5 fold at 62.5 µM and 125 µM of linalool. On comparison with indomethacin (50 µM), 4.3 fold decrease in IL-6 expression was observed.

TNFα activity decreased by almost 1.2 fold at a minimum concentration of 7.8 µM of linalool, and reduced by 2, 3.2 and 4.3 folds at 31.25 µM, 62.5 µM and 125 µM of linalool respectively. Indomethacin (50 µM) downregulated TNFα expression by 4.9 fold.
Overall, arecoline induced inflammation in established HBF cell line by upregulating proinflammatory cytokines (IL-1β, IL-6 and TNFα) and downregulating anti-inflammatory cytokine (IL-10) in a dose dependent manner (Fig 5.20). Indomethacin (50 µM) suppressed arecoline induced overexpression by downregulating IL-1β activity by 4.2 fold, IL-6 by 4.3 fold and TNFα by 4.9 fold, concomitantly upregulating IL-10 levels by 2 fold. In comparison to the above results the effective concentration of linalool that exhibit effective anti-inflammatory activity ranged from 31.25 µM to 125 µM for TNFα and 62.5 µM - 125 µM for IL-1β, IL-6 and IL-10.

An overall significant downregulation of inflammatory markers (IL-1β, IL-6 & TNFα) and upregulation of anti-inflammatory marker (IL-10) on treatment with different concentrations of linalool was observed with (p = 0.003, < 0.01**), on comparison with arecoline induced group. Linalool at 125 µM significantly downregulated IL-1β, IL-6 (p = 0.048, < 0.05*) and TNFα (p = 0.03, < 0.05*) and upregulated IL-10 (p = 0.01, < 0.05*). The standard drug, indomethacin at 50 µM, exhibited a significant downregulation of proinflammatory markers IL-1β, IL-6 (p = 0.007, < 0.01**), TNFα (p = 0.008, < 0.01**) and upregulated IL-10 (p = 0.007, < 0.01**).

5.7.2.2 In-vitro semiquantitative assessment of fibrotic markers on pretreatment with ethanolic leaf extract of O basilicum L. (LEOB) and linalool on arecoline induced fibrosis in HBF.

To demonstrate the antifibrotic activity of ethanolic LEOB and linalool on pretreatment, five lakh cells (5 X 10⁵) were seeded in 6 well plates. Upon attaining 50 - 60% confluency HBF’s were pretreated with (7.8, 15.6, 31.25, 62.5, 125 µg/mL) of
ethanolic LEOB and (7.8, 15.6, 31.25, 62.5, 125 µM) of linalool and incubated for 48 hours following which HBF’s were arecoline induced (25 µg/mL) or untreated, baseline control (0 µg/mL) for 48 hours. At the end of 48 hours HBF’s were evaluated for the expression levels of fibrotic markers using two step semiquantative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene. An overall significant downregulation of fibrotic markers was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer test was used to analyze statistically significant concentration of ethanolic LEOB and linalool that downregulated the fibrotic markers effectively.
Figure 5.40: Decrease in expression of fibrotic markers on pretreatment with ethanolic leaf extract of *O basilicum* L. in arecoline induced cell line model

Figure 5.41: Fold decrease in fibrotic marker expression in arecoline induced HBFs pretreated with ethanolic leaf extract of *O basilicum* L.
5.7.2.2.1 Effect of ethanolic leaf extract of *O basilicum* L. (LEOB)

On observation arecoline (25 µg/mL) induced fibrosis by upregulating TGFβ1 expression by 103 fold, COL1A2 by 200 fold and COL3A1 by 104 fold when compared to baseline control (0 µg/mL; Fig 5.40 & 5.41). In Fig 5.40 & 5.41, on treatment with ethanolic LEOB, reduction in TGF β1 level by 1.8 fold was observed at a minimum concentration of 7.8 µg/mL. With increasing concentrations, TGF β1 levels reduced further by 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, with maximum antifibrotic activity at 62.5 µg/mL and 125 µg/mL when compared to induced control.

COL1A2 level decreased by almost 2 fold on treatment with 7.8 µg/mL of ethanolic LEOB, further reduction by 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 was observed. Effective downregulation was noted at 62.5 µg/mL and 125 µg/mL when compared to induced control. Reduction in COL3A1 level by almost 2 fold was observed at 7.8 µg/mL and 15.6 µg/mL of ethanolic LEOB, whereas at higher concentration of 62.5 µg/mL and 125 µg/mL further reduction by 4 fold and 6 fold was observed.

Overall, maximum reduction in expression levels of TGFβ1 (18 and 52 fold), COL1A2 (9 and 14 fold) and COL3A1 (4 and 6 fold) was observed at concentrations of 62.5 µg/mL and 125 µg/mL of ethanolic LEOB. An overall significant downregulation of fibrotic markers (TGFβ1, COL1A2 and COL3A1) on treatment with different concentrations of ethanolic LEOB was observed with (p = 0.003, < 0.01**), on comparison with arecoline induced group. TGFβ1, COL1A2 &COL3A1 statistically significantly downregulated at 125 µg/mL (p = 0.048, < 0.05*).
Figure 5.42: Decrease in expression of fibrotic markers on pretreatment with linalool in arecoline induced cell line model

Figure 5.43: Fold decrease in fibrotic marker expression in arecoline induced HBFs pretreated with linalool
5.7.2.2.2 Effect of linalool on arecoline induced HBFs

On observation arecoline (25 µg/mL) induced fibrosis by upregulating TGFβ1 expression by 37 fold, COL1A2 by 55 fold and COL3A1 by 52 fold when compared to baseline control (Fig 5.42 & 5.43).

On treatment with linalool, effective reduction in TGF β1 level by almost 12 fold was observed at lower concentrations of 7.8 µM and 15.6 µM and by 11 fold at 31.25 µM (Fig 5.42 & 5.43). At higher concentration of 62.5 µM and 125 µM, 7 fold and 5 fold decrease was observed when compared to induced control.

COL1A2 levels decreased by 2 fold at concentrations of 7.8 - 31.25 µM of linalool. At higher concentrations of 62.5 µM and 125 µM, linalool exhibited minimum antifibrotic activity decreasing COL1A2 levels by 1 fold only.

A significant reduction was observed in COL3A1 levels by 17 fold at 7.8 µM, 13 fold at 15.6 µM and 15 fold at 31.25 µM. A one fold reduction was observed at the higher concentrations of 62.5 µM and 125 µM tested.

Overall, reduction in expression levels of TGFβ1 (12 fold at 7.8 µM), COL1A2 (2 fold at 7.8 µM) and COL3A1 (17 fold at 7.8 µM) was observed at the lower concentrations predominantly. Increasing concentrations upto 125 µM showed lesser fold downregulation of fibrotic markers. An overall significant downregulation of fibrotic markers on treatment with different concentrations of linalool was observed (p = 0.003, < 0.01**), on comparison with arecoline induced group. Linalool statistically significantly downregulated TGFβ1 at 15.6 µM, COL1A2 and COL3A1 at 7.8 µM (p = 0.048, < 0.05*).
5.7.3 Morphological assessment of human buccal fibroblasts (HBFs) and qualitative assessment of collagen, post arecoline treatment and on pretreatment with linalool.

Masson trichrome staining was performed to observe fibroblast morphology and qualitatively assess collagen deposition in the ECM, post arecoline treatment and compare it with linalool pretreated cells. From the data shown in Fig 5.42, effective concentration of linalool that downregulated the expression of fibrotic markers was 7.8 and 15.6 µM. Hence, to assess morphological restoration of arecoline induced fibrosis, HBF’s were pretreated with 15.6 µM linalool for 48 hours.

Ten thousand ($1 \times 10^4$) cells were seeded onto round glass cover slips in 12 well plates containing growth media. On attaining 40% - 50% confluency, cells pretreated with 15.6 µM of linalool for 48 hours were further treated with 25 µg/mL of arecoline to induce fibrosis and incubated for 48 hours. Such treated cells were then fixed with 3.7% formaldehyde. Staining was carried out stepwise according to the protocol standardized by Lillehei Heart Institute, University of Minnesota, USA (vide material & methods 4.5.6). The fibrotic effects of arecoline were documented under a bright field microscope.
Figure 5.44: Qualitative assessment of collagen deposition and fibroblast morphology using Masson trichrome stain in cultured cells treated with 25 µg/mL of arecoline and on pretreatment with 15.6 µM of linalool. Normal spindle shaped cells are seen in a monolayer (a) while distorted cells were seen with arecoline treatment (b). Cells retained their spindled morphology on linalool treatment (c).

In Fig 5.44: Fibroblasts exhibited spindle morphology and a small quantity of ECM was present in the untreated (control) group (a). In contrast it was observed that HBFs treated with 25 µg/mL of arecoline appeared distorted, rounded and stained strongly positive for collagen (b) demonstrating excessive deposition. In the linalool (c), treated group, fibroblasts retained their spindle morphology, showed very low collagen staining, decreasing ECM deposition. The group treated with linalool appeared similar to the control group.
5.8. *In-vitro* assessment of anti-inflammatory and antifibrotic potential of ethanolic leaf extract of *O vulgare* L. subsp hirtum (LEOV) and thymol on pretreatment in an arecoline induced human buccal fibroblast (HBF) cell line model

On successfully establishing an experimental *in-vitro* model for OSMF, by inducing inflammation and fibrosis using arecoline, ethanolic leaf extract of *O vulgare* L. (LEOV) and its major active compound thymol were tested for their anti-inflammatory and antifibrotic potential. As reported in literature, leaf extract of *O vulgare* L., was known to impart anti-inflammatory activity in *in-vitro* lipopolysaccharide induced macrophage model. At 0.5 mg/mL, *O vulgare* L. downregulated IL-6 and TNFα secretion by 25% and concomitantly upregulated IL-10 levels (Mueller *et al*., 2010).

Thymol (monoterpene) at 100 mg/kg body weight reduced carrageenan induced paw edema at 2, 3 and 4 hours, by inhibiting 35.3% of edema as compared to 47.1% of edema reduction with dexamethasone, thereby exhibiting an anti-inflammatory response (Riella *et al*., 2012). Thymol prevented glomerulosclerosis at 40 mg/kg body weight in high fat diet induced diabetic nephropathy in C57BL/6 mice, inhibiting the activation of TGFβ1 (Saravanant and Leelvinothan, 2016). Since fibrosis results from continuous inflammatory insult to buccal cells, the ability of ethanolic LEOV and thymol to reduce inflammation and/or fibrosis was evaluated. The activities of ethanolic LEOV and thymol were characterized by:

a. Determining the cytotoxic (IC₅₀) values for ethanolic LEOV and thymol.

b. Expression of molecular markers of inflammation (IL-1β, IL-6, TNFα & IL-10) and fibrosis (TGFβ1, COL1A2 & COL3A1) on treatment with ethanolic LEOV and thymol in arecoline induced buccal fibroblast cell line model.

c. Changes in morphology of induced fibrotic cells on treatment with thymol.
5.8.1 MTT cytotoxicity assay

**Determination of inhibitory concentration (IC$_{50}$) for ethanolic LEOV and thymol**

To determine the concentrations required for the *in-vitro* activity of ethanolic LEOV and thymol, cytotoxicity assay was performed. Cell viability and proliferation was monitored by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay at 590 nm. Ten thousand (1 X $10^4$) normal HBF’s were seeded per well in 96 well plate and incubated with various concentrations of ethanolic LEOV (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) and thymol (7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µM) (Dinesh et al., 2014) for 48 hours. The proportions of viable cells were determined and the inhibitory concentration (IC$_{50}$) for ethanolic LEOV and thymol is shown in Fig 5.45 (a & b).

**Figure 5.45: Determining cytotoxic concentration (IC$_{50}$) of crude ethanolic leaf extract of *O vulgare* L. subsp hirtum (LEOV) and thymol 48 hours post treatment**

![Graphs showing cytotoxic concentration of ethanolic LEOV and thymol](image)

Ethanolic LEOV and thymol induced cytotoxicity to HBFs at a concentration above 125 µg/mL and 125 µM respectively (p < 0.05*) during 48 hours incubation (Fig 5.45 a & b). At concentrations of 250, 500 & 1000 µg/mL of ethanolic LEOV, fibroblast cell proliferation was inhibited and cell death upto 54.5%, 57.2% and 60%
was observed. Whereas at 250, 500 & 1000 µM of thymol, fibroblast cell proliferation was inhibited and cell death up to 51.5%, 52.3% and 54.2% was seen.

Thus, for testing the anti-inflammatory and antifibrotic activity of ethanolic LEOV and thymol in arecoline induced OSMF cell line model, concentrations of 7.8 - 125 µg/mL and 7.8 - 125 µM were used routinely.

5.8.2 Evaluation of molecular markers of inflammation and fibrosis using RT-PCR on pretreatment with ethanolic leaf extract of O vulgare L. subsp hirtum (LEOV) and thymol

HBFs cell line model pretreated with non-toxic concentrations (7.8 - 125 µg/mL) of ethanolic LEOV and (7.8 - 125 µM) of thymol were induced with arecoline for fibrosis. The ability of ethanolic LEOV and thymol to protect cells from inflammation and fibrosis was assessed using RT-PCR.

5.8.2.1 In-vitro semiquantitative assessment of inflammatory markers on pretreatment with ethanolic leaf extract of O vulgare L. subsp hirtum (LEOV) and thymol in arecoline induced fibrosis in HBF

To demonstrate the anti-inflammatory activity of ethanolic LEOV and thymol on pretreatment, five lakh cells (5 X 10⁵) were seeded in 6 well plates. Upon attaining 50 - 60% confluency HBF’s were untreated (control; 0 µg/mL) or treated with (7.8, 15.6, 31.25, 62.5, 125 µg/mL) of ethanolic LEOV and (7.8, 15.6, 31.25, 62.5, 125 µM) of thymol. Cells then were incubated for 48 hours and fibrosis was induced with 25 µg/mL of arecoline.

At the end of 48 hours HBF’s were evaluated for the expression levels of inflammatory markers using two step semiquantitative RT-PCR. Indomethacin, a
known anti-inflammatory drug was used as a positive control. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β-actin as an internal housekeeping gene.

An overall significant downregulation of inflammatory markers and upregulation of anti-inflammatory marker was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer (Nemenyi) test was used to analyze statistically significant and effective concentrations of ethanolic LEOV and thymol.
Figure 5.46: Fold decrease in proinflammatory markers and increase in anti-inflammatory marker expression in arecoline induced HBFs pretreated with ethanolic leaf extract of *O vulgare* L. subsp *hirtum*.

Figure 5.47: Fold decrease in proinflammatory markers and increase in anti-inflammatory marker expression in arecoline induced HBFs pretreated with ethanolic leaf extract of *O vulgare* L. subsp *hirtum*. 
5.8.2.1.1 Effect of ethanolic leaf extract of *O vulgare* L. subsp *hirtum* (LEOV)

Data presented in Fig 5.46 & 5.47, demonstrated that HBFs pretreated with ethanolic LEOV at increasing concentrations (7.8 - 125 µg/mL) for 48 hours were normal showing uniform expression of β actin (494 bp). Arecoline (25 µg/mL) induced inflammation by downregulating anti-inflammatory marker (IL-10) 3.8 folds when compared to baseline (Fig 5.20). On treatment with 31.25 µg/mL of ethanolic LEOV, almost 1 fold increase was observed, whereas 1.2 fold and 1.6 fold increase in IL-10 was observed at higher concentrations of 62.5 µg/mL and 125 µg/mL. On comparison with indomethacin (50 µM), 1.7 fold increase in IL-10 expression was observed. Ethanolic LEOV at 62.5 µg/mL and 125 µg/mL exhibited anti-inflammatory activity similar to indomethacin at 50µM.

On the contrary, arecoline (25 µg/mL) upregulated proinflammatory markers (IL-1β, IL-6 and TNFα) when compared to baseline (Fig 5.20). On treatment with 15.6 µg/mL of ethanolic LEOV, almost 1 fold reduction in IL-1β level was observed, a similar reduction by 1.1 fold and 1.2 fold at 62.5 µg/mL and 125 µg/mL was observed. On comparison, indomethacin (50 µM), 1.5 fold decrease in IL-1β level was observed.

Treatment with lower concentrations of 15.6 µg/mL and 31.25 µg/mL of ethanolic LEOV, downregulated IL-6 levels by 15 fold, whereas at higher concentrations of 62.5 µg/mL and 125 µg/mL, a reduction by almost 20 fold and 17 fold was observed. On comparison with indomethacin (50 µM), 17 fold reduction was observed. Ethanolic LEOV showed maximum activity at 62.5 µg/mL, similar to 50 µM of the standard drug.
On treatment with 15.6 μg/mL of ethanolic LEOV, 1.5 fold decrease was observed in TNFα levels, 1.6 fold reduction was observed at 31.25 and 62.5 μg/mL and 1.7 fold at 125 μg/mL. On comparison to indomethacin (50 μM) an almost 2 fold reduction was observed.

Overall, arecoline induced inflammation in established HBF’s cell line by upregulating proinflammatory cytokines (IL-1β, IL-6 and TNFα) and downregulating anti-inflammatory cytokine (IL-10) in a dose dependent manner (Fig 5.20). Indomethacin (50 μM) suppressed arecoline induced overexpression by downregulating IL-1β activity by 1.5 fold, IL-6 by 17 folds and TNFα by 2 fold, concomitantly upregulating IL-10 levels by 1.7 fold. In comparison to the above results the effective concentration of ethanolic LEOV ranged from 31.25 μg/mL - 125 μg/mL for IL-10, 15.6 μg/mL - 125 μg/mL for IL-6 and 125 μg/mL for IL-1β and TNFα.

An overall significant downregulation of inflammatory markers; IL-1β (p = 0.003, < 0.01**), IL-6 (p = 0.02, < 0.05*) & TNFα (p = 0.003, < 0.01**) and upregulation of anti-inflammatory marker, IL-10 (p = 0.004, < 0.01**) on treatment with different concentrations of ethanolic LEOV was observed on comparison with arecoline induced group. Ethanolic LEOV significantly downregulated IL-1β at 62.5 μg/mL (p = 0.002, < 0.01**) & IL-6 at 62.5 μg/mL (p = 0.03, < 0.05*). The standard drug, indomethacin at 50 μM, exhibited a significant downregulation of proinflammatory markers IL-1β (p = 0.03, < 0.05*), TNFα (p = 0.007, < 0.01**) and upregulation of anti-inflammatory cytokine, IL-10 (p = 0.048, < 0.05*).
Figure 5.48: Fold decrease in proinflammatory markers and increase in anti-inflammatory marker expression in arecoline induced HBFs pretreated with thymol

![Graph showing fold change in proinflammatory and anti-inflammatory markers with thymol treatment](image)

Figure 5.49: Fold decrease in proinflammatory markers and increase in anti-inflammatory marker expression in arecoline induced HBFs pretreated with thymol

![Bar chart showing relative gene expression](image)
5.8.2.1.2 Effect of thymol on inflammation

Arecoline (25 µg/mL) induced inflammation by downregulating anti-inflammatory marker (IL-10) by 3.8 fold when compared to baseline (Fig 5.20). On treatment with 7.8 µM, 15.6 µM and 31.25 µM of thymol almost 3 fold increase was observed in IL-10 levels and 4 fold at 62.5 µM and 125 µM. Indomethacin (standard drug) at 50 µM upregulated IL-10 levels by 7 folds (Fig 5.48 & 5.49)

On the contrary, arecoline (25 µg/mL) upregulated proinflammatory markers (IL-1β, IL-6 and TNFα) by 50 fold, 9 fold and 7 fold respectively (Fig 5.20) thereby inducing inflammation in HBF’s after 48 hours treatment.

In Fig 5.48 & 5.49, on treatment with concentrations of 7.8 µM, 15.6 µM and 31.25 µM of thymol, an almost 1 fold decrease was observed in IL-1β levels. At concentrations of 62.5 µM and 125 µM, an almost 3 fold decrease was observed. In comparison to indomethacin (50 µM), a 3.3 fold decrease was observed.

In relation to IL-6, almost 1 fold decrease was observed at 7.8 µM, 15.6 µM, 31.25 µM and 62.5 µM of thymol, whereas at higher concentrations of 125 µM almost 3 fold reduction was observed. In comparison to indomethacin (50 µM), 4 fold reduction in IL-6 level was observed.

Levels of TNFα decreased by almost 1 fold on treatment with lower concentrations of 7.8 µM, 15.6 µM and 31.25 µM, whereas at higher concentrations of 62.5 µM and 125 µM, almost 3 fold reduction was observed. Indomethacin (50 µM) reduced TNFα by 5 folds.
Overall, arecoline induced inflammation in established HBF’s cell line by upregulating proinflammatory cytokines (IL-1β, IL-6 and TNFα) and downregulating anti-inflammatory cytokine (IL-10) in a dose dependent manner (Fig 5.20). Indomethacin (50 µM) suppressed arecoline induced overexpression by downregulating IL-1β activity by 3.3 fold, IL-6 by 4 folds and TNFα by 5 fold, concomitantly upregulating IL-10 levels by 7 fold. In comparison to the above results the effective concentration of thymol that exhibit effective anti-inflammatory activity ranged from 7.8 to 125 µM for IL-10, 31.25 - 125 µM for TNFα , 62.5 - 125 µM for IL-1β, and 125 µM for IL-6.

An overall significant downregulation of inflammatory markers; IL-1β (p = 0.004, < 0.01**), IL-6 & TNFα (p = 0.003, < 0.01**) and upregulation of anti-inflammatory marker, IL-10 (p = 0.003, < 0.01**) on treatment with different concentrations of thymol was observed on comparison with arecoline induced group. Thymol significantly downregulated IL-1β, IL-6 at 125 µM (p = 0.01, < 0.05*) & TNFα at 62.5 & 125 µM (p = 0.03, < 0.05*). Thymol upregulated IL-10 at statistically significant concentration of 62.5 & 125 µM (p = 0.02, < 0.05*). The standard drug, indomethacin at 50 µM, exhibited a significant downregulation of proinflammatory markers IL-1β (p = 0.01, < 0.05*), IL-6 & TNFα (p = 0.002, < 0.01**) and significant upregulation of anti-inflammatory cytokine, IL-10 (p = 0.002, < 0.01**).
5.8.2.2 *In-vitro* semiquantitative assessment of fibrotic markers on pretreatment with ethanolic leaf extract of *O vulgare* L. subsp hirtum (LEOV) and thymol on arecoline induced fibrosis in HBF

To demonstrate the antifibrotic activity of ethanolic LEOV and thymol on pretreatment, five lakh cells (5 X 10^5) were seeded in 6 well plates. Upon attaining 50 - 60% confluency HBF’s were untreated (control) or treated with (7.8, 15.6, 31.25, 62.5, 125 µg/mL) of ethanolic LEOV and (7.8, 15.6, 31.25, 62.5, 125 µM) of thymol were incubated for 48 hours. At the end of 48 hours HBF’s were evaluated for the expression levels of fibrotic markers using two step semiquantitative RT-PCR. The samples were compared with 1kb DNA ladder on a gel and normalization was performed using β actin as an internal housekeeping gene.

An overall significant downregulation of fibrotic markers was analysed using Kruskal Wallis rank sum test. Post Tukey and Kramer test was used to analyze statistically significant concentration of ethanolic LEOV and thymol that downregulated the fibrotic markers effectively.
Figure 5.50: Fold decrease in fibrotic marker expression in arecoline induced HBF cell line model on pretreatment with ethanolic leaf extract of *O vulgare* L. subsp *hirtum*

![Graph showing fold decrease in fibrotic marker expression](image)

Figure 5.51: Fold decrease in fibrotic marker expression in arecoline induced HBF cell line model on pretreatment with ethanolic leaf extract of *O vulgare* L. subsp *hirtum*

![Bar graph showing relative gene expression](image)
5.8.2.2.1 Effect of ethanolic leaf extract of *O. vulgare* L. subsp *hirtum* (LEOV) on fibrosis

On observation arecoline (25 µg/mL) induced fibrosis in HBF’s by upregulating TGFβ1 expression by 22 fold, COL1A2 by 53 folds and COL3A1 by 47 folds when compared with baseline (untreated) (Fig 5.50 & 5.51).

On treatment with ethanolic LEOV (Fig 5.50 & 5.51), reduction in TGFβ1 levels by almost 1.8 fold at concentration of 7.8 µg/mL was observed. Reduction by 5 fold was observed at 15.6 µg/mL and 31.25 µg/mL, whereas at concentrations of 62.5 µg/mL and 125 µg/mL, 6 and 11 fold reduction in TGF β1 level was observed when compared to induced control. Effective concentrations ranged from 7.8 - 125 µg/mL.

COL1A2 levels decreased by almost 2 folds at 7.8 µg/mL and 15.6 µg/mL, by 3 folds at 31.25 µg/mL of ethanolic LEOV, whereas at higher concentrations, reduction by 4 folds was observed at 62.5 and 125 µg/mL. Reduction in COL3A1 level by almost 2 fold was observed at 7.8 µg/mL, 15.6 µg/mL and 31.25 µg/mL, whereas at higher concentration of 62.5 µg/mL and 125 µg/mL, 3 fold and 4 fold reduction was observed.

Overall, maximum reduction in expression levels of TGFβ1 (6 and 11 fold), COL1A2 (4 fold) and COL3A1 (3 and 4 fold) was observed at higher concentration of 62.5 µg/mL and 125 µg/mL of ethanolic LEOV. An overall significant downregulation of fibrotic markers (TGFβ1, COL1A2 and COL3A1) on treatment with different concentrations of ethanolic LEOV was observed (p = 0.003, < 0.01**), on comparison with arecoline induced group. TGFβ1, COL1A2 & COL3A1 statistically significantly downregulated at 125 µg/mL (p = 0.048, < 0.05*).
Figure 5.52: Fold decrease in fibrotic marker expression in arecoline induced HBF cell line model on pretreatment with thymol

Figure 5.53: Fold decrease in fibrotic marker expression in arecoline induced HBF cell line model on pretreatment with thymol
5.8.2.2.2: Effect of thymol on fibrosis

On observation arecoline (25 µg/mL) induced fibrosis in HBF’s by upregulating TGFβ1 expression by 27 fold, COL1A2 by 52 folds and COL3A1 by 60 folds when compared with baseline (untreated) (Fig 5.52 & 5.53).

On treatment with thymol (Fig 5.52 & 5.53), reduction of 1 fold in TGFβ1 level was observed at 7.8 µM, 62.5 µM and 125 µM. Effective reduction by almost 1.4 fold and 1.5 fold was observed at 15.6 µM and 31.25 µM.

A similar reduction by 1 fold was observed in COL1A2 level at concentrations ranging from 7.8 - 125 µM, with maximum reduction by 1.3 fold at 15.6 µM.

COL3A1 levels downregulated by almost 1.6 fold at lower concentrations of 7.8 and 15.6 µM, 1.5 fold at 31.25 µM and 1.4 fold at 62.5 and 125 µM of thymol.

Overall, maximum reduction in expression levels of TGFβ1 (1.5 fold), COL1A2 (1.3 fold) and COL3A1 (1.6 fold) was observed at concentrations of 7.8, 15.6 and 31.25 µM of thymol. An overall significant downregulation of fibrotic markers on treatment with different concentrations of thymol was observed (p = 0.005, < 0.01**), on comparison with arecoline induced group. Thymol statistically significantly downregulated TGFβ1 at 31.25 µM, COL1A2 at 15.6 and COL3A1 at 7.8 µM (p = 0.048, < 0.05*).
5.8.3 Morphological assessment of human buccal fibroblasts (HBFs) and qualitative assessment of collagen, post arecoline treatment and on pretreatment with thymol

Masson trichrome staining was performed to observe fibroblast morphology and qualitatively assess collagen deposition in the extracellular matrix post arecoline treatment and compare it with thymol pretreated cells. From the data shown in Fig 5.52, effective concentration of thymol that downregulated the expression of fibrotic markers was 7.8 and 15.6 µM. Hence, to assess morphological restoration of arecoline induced fibrosis, HBF’s were pretreated with 15.6 µM thymol for 48 hours.

Ten thousand (1 X 10^4) cells were seeded onto round glass cover slips in 12 well plates containing growth media. On attaining 40% - 50% confluence, cells pretreated with 15.6 µM of thymol for 48 hours were further treated with 25 µg/mL of arecoline and incubated for 48 hours. Such treated cells were then fixed with 3.7% formaldehyde. Staining was carried out stepwise according to the protocol standardized by Lillehei Heart Institute, University of Minnesota, USA (vide material & methods 4.5.6). The fibrotic effects of arecoline were documented under a bright field microscope.
Figure 5.54: Qualitative assessment of collagen deposition and fibroblast morphology using Masson trichrome stain in cultured cells treated with 25 µg/mL of arecoline and on pretreatment with 15.6 µM of thymol. Normal spindle shaped cells are seen in a monolayer (a) while distorted cells were seen with arecoline treatment (b). Cells retained their spindled morphology on thymol treatment (c).

In Fig 5.54: Fibroblasts exhibited spindle morphology and small quantities of ECM was present in the untreated (control) group (a), by contrast it was observed that HBFs treated with 25 µg/mL of arecoline appeared distorted, rounded and stained strongly for collagen (b) demonstrating excessive deposition. In the thymol (c), pretreated group, fibroblasts retained their spindle morphology, showed very low collagen staining, and decreased ECM deposition. The group treated with thymol appeared similar to the control group.
Table 5.4: Anti-inflammatory and antifibrotic activity of ethanolic crude leaf extracts in arecoline induced HBF cell line model

<table>
<thead>
<tr>
<th>GENES</th>
<th>MINIMUM EFFECTIVE CONCENTRATIONS (µg/mL) &amp; FOLD DECREASE</th>
<th>( C_{asiatica} ) L.</th>
<th>( O_{basilicum} ) L.</th>
<th>( O_{vulgare} ) L.</th>
<th>Indo. 50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-inflammatory</td>
<td>Arecoline: ↑ Concentration, ↓ Fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>50 31.25 2 13 31.25 7 7 15.6 1 1.5</td>
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<tr>
<td>IL-6</td>
<td>9 15.6 1.7 94 31.25 800 1200 15.6 31.25 15 17</td>
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<tr>
<td>TNF-α</td>
<td>7 31.25 2.5 13 15.6 2 13 15.6 1 2</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Anti-inflammatory</td>
<td>Arecoline: ↓ Concentration, ↑ Fold</td>
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<td></td>
<td></td>
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<td>3.8 31.25 2 11.3 31.25 0.2 0.7 31.25 1 1.7</td>
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<tr>
<td>Fibrogenic</td>
<td>Arecoline: ↑ Concentration, ↓ Fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL1A2</td>
<td>66 15.6 1.5 200 7.8 2 53 7.8 2</td>
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<tr>
<td>COL3A1</td>
<td>84 7.8 2 104 7.8 2 47 7.8 2</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\( \downarrow \) Fold Decrease, \( \uparrow \) Fold Increase, Arec: Arecoline, Concentration, Indo: Indomethacin

*In-vitro* human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis
Table 5.5: Anti-inflammatory and Antifibrotic activity of pure compounds in arecoline induced HBF cell line model

<table>
<thead>
<tr>
<th>GENES</th>
<th>MINIMUM EFFECTIVE CONCENTRATIONS (µM) &amp; FOLD DECREASE</th>
<th>GENES</th>
<th>MINIMUM EFFECTIVE CONCENTRATIONS (µM) &amp; FOLD DECREASE</th>
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<tr>
<td></td>
<td>Asiatic acid</td>
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<td></td>
<td>Concentration</td>
<td>Fold</td>
<td>Concentration</td>
</tr>
<tr>
<td>Pro inflammatory</td>
<td>Arecoline↑</td>
<td>50</td>
<td>Indo. 50 µM</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>2</td>
<td>39</td>
</tr>
<tr>
<td>IL-6</td>
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<td>2</td>
<td>9</td>
</tr>
<tr>
<td>TNF-α</td>
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<td>3</td>
<td>55</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>Arecoline↓</td>
<td>Concentration</td>
<td>Indo. 50 µM</td>
</tr>
<tr>
<td>IL-10</td>
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<td>1.7</td>
<td>31.25</td>
</tr>
<tr>
<td>Fibrogenic</td>
<td>Arecoline↑</td>
<td>Concentration</td>
<td>Indo. 50 µM</td>
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<tr>
<td>COL3A1</td>
<td>118</td>
<td>15.6</td>
<td>12</td>
</tr>
</tbody>
</table>

↓ Fold Decrease, ↑ Fold Increase, Arec: Arecoline, Concent: Concentration, Indo: Indomethacin

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