ORAL SUBMUCOUS FIBROSIS

2.1.1 Introduction

Oral submucous fibrosis (OSMF) has been mentioned in the Indian medical literature, since the time of ‘Sushruta’ who named it as ‘Vidari’. The disease was first reported in 1952 by Schwartz to which he ascribed the descriptive term ‘Atrophica idiopathica (tropica) mucosa oris’ and its precancerous nature was reported by ‘Paymaster’ in 1956. It was in 1953, ‘Joshi’ from Mumbai redesignated the condition as ‘Oral Submucous Fibrosis’. In 1966, Pindborg & Sirsat defined OSMF as ‘an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxtaepithelial inflammatory reaction followed by fibroelastic changes in the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa causing trismus and difficulty in eating’. The WHO definition for an oral precancerous condition was stated as: ‘A generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer (Rajendran, 1994; WHO, 1980). Warnakulasuriya et al. in 2007 termed OSMF as a potentially malignant disorder.

2.1.2 Epidemiology

a. Prevalence: Worldwide estimate in 1996 indicated that 2.5 million people were affected by the disease. In 2002, the statistics from the Indian continent alone was about 5 million people (0.5% of the population of India) (Aziz, 2008)

b. Most prevalent in United Kingdom, South Africa, South East Asian countries.

c. Malignant Transformation Rate: Reported as 7.6 % - 13 % (Sawant et al., 2014).
2.1.3 Etiopathogenesis

The aetiopathogenesis of OSMF was earlier believed to be multifactorial. A number of factors trigger the disease process by causing a juxtaepithelial inflammatory reaction in the oral mucosa. The prime factor recognized responsible for OSMF supported by epidemiological, case control, animal experiments and tissue culture studies is chewing of betel quid (BQ); containing betel leaf, arecanut, tobacco (may or may not be present), slaked lime and other spices. Quid is defined as a substance, or mixture of substances, placed in the mouth, usually containing at least one of the two basic ingredients, tobacco or arecanut, in raw or any manufactured or processed form. In many regions of the world arecanut is an important agricultural product. It is known as supari in Hindi, gua in Bangladesh, puwak in Srilanka, pinang in Malaysia, mak in Thailand (IARC Monographs, 2004).

Table 2.1: Production of arecanut by country since 1961 (in millions of tonnes)

(IARC Monographs, 2004)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>62 995</td>
<td>23 369</td>
<td>25 051</td>
<td>24 120</td>
<td>47 000</td>
</tr>
<tr>
<td>India</td>
<td>120 000</td>
<td>141 000</td>
<td>195 900</td>
<td>238 500</td>
<td>330 000</td>
</tr>
<tr>
<td>Indonesia</td>
<td>13 000</td>
<td>15 000</td>
<td>18 000</td>
<td>22 812</td>
<td>36 200</td>
</tr>
<tr>
<td>Kenya</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Malaysia</td>
<td>6 500</td>
<td>3000</td>
<td>2 500</td>
<td>4000</td>
<td>2500</td>
</tr>
<tr>
<td>Maldives</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>16</td>
<td>37</td>
</tr>
<tr>
<td>Myanmar</td>
<td>8000</td>
<td>19 203</td>
<td>25 807</td>
<td>32 270</td>
<td>51 463</td>
</tr>
<tr>
<td>Taiwan, Chinaa</td>
<td>3718</td>
<td>10 075</td>
<td>24 358</td>
<td>111 090</td>
<td>165 076</td>
</tr>
<tr>
<td>Thailand</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>13 250</td>
<td>20 500</td>
</tr>
<tr>
<td>World</td>
<td>428 428</td>
<td>423 296</td>
<td>583 242</td>
<td>892 316</td>
<td>1 305 732</td>
</tr>
</tbody>
</table>
The fruit is ovoid or oblong with pointed apex, measuring 3-5 cm in length and 2-4 cm in diameter, used fresh or dried by sun drying, basking or roasting. It may also be fermented or boiled and covered with mud for consumption.

**Chemical composition of arecanut:** Arecanut is majorly composed of fats, carbohydrates, proteins, crude fibre, polyphenols (flavonols and tannins), alkaloids and mineral matter. Among the chemical constituents, alkaloids (*arecoline, being the most abundant*), arecaidine, guvacine, isoguvacine and arecolidine are most important biologically (IARC Monographs, 2004).

**Table 2.2:** Ranges in concentration of the chemical constituents of a variety of unprocessed green and ripe areca nuts (IARC Monographs, 2004)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Green (unripe nut)</th>
<th>Ripe nut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content</td>
<td>69.4–74.1</td>
<td>38.9–56.7</td>
</tr>
<tr>
<td>Total polysaccharides</td>
<td>17.3–23.0</td>
<td>17.8–25.7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>6.7–9.4</td>
<td>6.2–7.5</td>
</tr>
<tr>
<td>Fat</td>
<td>8.1–12.0</td>
<td>9.5–15.1</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>8.2–9.8</td>
<td>11.4–15.4</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>17.2–29.8</td>
<td>11.1–17.8</td>
</tr>
<tr>
<td><strong>Arecoline</strong></td>
<td><strong>0.11–0.14</strong></td>
<td><strong>0.12–0.24</strong></td>
</tr>
<tr>
<td>Ash</td>
<td>1.2–2.5</td>
<td>1.1–1.5</td>
</tr>
</tbody>
</table>

**Table 2.3:** Dose-response relationship between frequency of arecanut use and Oral submucous fibrosis (Hazarey et al., 1998; IARC Monograph, 2004)

<table>
<thead>
<tr>
<th>Frequency/day</th>
<th>Cases</th>
<th>Controls</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-users</td>
<td>5</td>
<td>110</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>24</td>
<td>10.1</td>
</tr>
<tr>
<td>2–3</td>
<td>65</td>
<td>42</td>
<td>34.0</td>
</tr>
<tr>
<td>4–5</td>
<td>61</td>
<td>16</td>
<td>83.9</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>58</td>
<td>5</td>
<td>255.2</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>197</td>
<td><em>p</em> for trend &lt; 0.01</td>
</tr>
</tbody>
</table>
Table 2.4: Association between chewing betel quid with and without tobacco and Oral submucous fibrosis (IARC Monograph, 2004)

<table>
<thead>
<tr>
<th>Chewing habit</th>
<th>No. of cases/controls (women and men)</th>
<th>Odds ratio&lt;sup&gt;a&lt;/sup&gt; (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-chewer</td>
<td>9/34 373</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td><strong>Ever chewer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former chewer</td>
<td>161/13 400</td>
<td>44.1 (22.0–88.2)</td>
</tr>
<tr>
<td>Occasional chewer</td>
<td>29/1276</td>
<td>125.2 (56.7–276.3)</td>
</tr>
<tr>
<td>Current chewer</td>
<td>7/2625</td>
<td>12.7 (4.7–34.4)</td>
</tr>
<tr>
<td></td>
<td>125/9499</td>
<td>49.2 (24.3–99.6)</td>
</tr>
<tr>
<td><strong>Frequency of chewing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(times/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–20</td>
<td>114/8991</td>
<td>28.9 (16.5–50.5)</td>
</tr>
<tr>
<td>21–40</td>
<td>30/1443</td>
<td>46.8 (24.3–90.2)</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>8/271</td>
<td>84.3 (32.8–216.8)</td>
</tr>
<tr>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Duration of chewing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–20</td>
<td>88/5971</td>
<td>30.8 (17.6–53.8)</td>
</tr>
<tr>
<td>21–40</td>
<td>54/3470</td>
<td>34.7 (18.6–64.5)</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>9/1217</td>
<td>22.7 (9.0–57.5)</td>
</tr>
<tr>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Arecanut derived N-Nitrosamines

*In-vitro* experiments conclude nitrosation of arecoline during the chewing of betel quid which produces *N*-Nitrosoguvacoline (NGL) and 3-Methylnitrosamino propionaldehyde (MNPN). These compounds have been tested in tumour initiation - promotion experiments and proven to cause lung adenocarcinoma and esophageal papilloma in mice models. The experiments conclude the carcinogenic and toxic potential of arecoline.
Table 2.5: Chemical and physical data of arecanut derived N-Nitrosamines

(IARC Monograph, 2004)

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Chem. Abstr. Name</th>
<th>Molecular formulae and molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methylnitrosamino-propionaldehyde</td>
<td>Propanol, 3-(methyl-nitrosoamino) [MNPA]</td>
<td>C₄H₈N₂O₂, Mol. wt: 116.1</td>
</tr>
<tr>
<td>[85502-23-4]</td>
<td>3-(methylnitrosoamino)-propionaldehyde</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Propanenitrile, 3-(methyl-nitroso-amino) [MNPN]</td>
<td>C₄H₇N₃O, Mol. wt: 113.1</td>
</tr>
<tr>
<td></td>
<td>3-(methylnitrosoamino)-propionitrile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-nitroso-</td>
<td>C₆H₈N₂O₃, Mol. wt: 156.1</td>
</tr>
<tr>
<td></td>
<td>[NGC; nitrosoguvacine]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,2,5,6-tetrahydro-1-nitrosonicotinic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-Nitrosoguvacine</td>
<td>C₇H₁₀N₂O₃, Mol. wt: 170.2</td>
</tr>
<tr>
<td></td>
<td>[55557-01-2]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Pyridinecarboxylic acid, 1,2,5,6-tetra-hydro-1-nitroso-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>methyl ester [NG; NGL; Nitrosoguvacoline]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methyl 1,2,5,6-tetra-hydro-1-nitrosonicotinate</td>
<td></td>
</tr>
</tbody>
</table>
2.1.4 Classification Systems (More et al., 2012)

2.1.4.1 Classification schemes based on clinical features

- Pindborg JJ (1989)
- Lai DR et al. (1995)
- Ranganathan K et al. (2001)
- Rajendran R et al. (2003)

2.1.4.2 Classification based on clinical and functional staging

- Chandaramani B et al. (2012)
- S.M. Haider et al. (2000)

2.1.4.3 Classification based on both clinical features and histopathological features

- Khanna JN and Andrade NN (1995)

2.1.4.4 Classification based on histopathological features

- Pindborg JJ et al. (1966)
- Utsunomiya H et al. (2005)
### Table 2.6: Khanna JN and Andrade NN (1995) (More et al., 2012)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CLINICAL FEATURES</th>
<th>HISTOPATHOLOGY/ MICROSCOPIC APPEARANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Very early cases</td>
<td>Burning sensation, Acute ulcerations and recurrent Stomatitis. Not associated with mouth opening limitation</td>
<td>Fine fibrillar collagen network interspersed with marked edema, blood vessels dilated and congested, Large aggregate of plump, young fibroblasts present with abundant cytoplasm, Inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils. Normal epithelium</td>
</tr>
<tr>
<td>II Early</td>
<td>Buccal mucosa appears mottled and marble like, widespread sheets of fibrosis palpable. Interincisal distance 26-35 mm</td>
<td>Juxtaepithelial hyalinization, collagen present as thickened but separate bundles, blood vessels dilated and congested, young fibroblasts seen in moderate number, inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils and occasional plasma cells, flattening or shortening of epithelial rete pegs evident with varying degree of keratinization.</td>
</tr>
</tbody>
</table>
**Chapter 2.1**

| IV A Advanced | Trismus is severe with interincisal distance of less than 15 mm. The fauces is thickened, shortened and firm to palpation to palpation. Uvula is shrunken and appears as small, fibrous bud. Tongue movements limited. On palpation of lips, circular band felt around entire mouth. | Markedly atrophic with loss of rete pegs. Muscle fibers seen interspersed with thickened and dense collagen fibers. |
| IV B Advanced cases with premalignant and malignant changes | Hyperkeratosis, Leukoplakia, or squamous cell carcinoma can be seen. | Collagen hyalinized as smooth sheet. Extensive fibrosis obliterated the mucosal blood vessels and eliminated the melanocytes. Fibroblasts were markedly absent within the hyalinized zone. Total loss of epithelial rete pegs. Mild to moderate atypia present. Extensive degeneration of muscle fibers evident. |
2.1.6 An overview of proposed mechanisms involved in the pathogenesis of OSMF

2.1.6.1 Localized Trauma:

Sudarshan et al., 2012: Locally arecanut chewing is known to cause trauma and injury to the oral mucosa due to its abrasive nature; placement of betel quid in the buccal vestibule for 15 minutes to 1 hour with a frequency of 5 to 6 times in a day leads to a continuous contact between the quid and the oral mucosa leading to absorption and metabolism of alkaloids in the quid. The aggressiveness is more in individuals with pan masala and gutkha chewing habit due to their micro particulate nature; the probability of particulate adhesion to the traumatized mucosa leads to membrane damage and morphological changes; this continuous local irritation leads to injury related oxidative stress, chronic inflammation and cytokine production. During chronic exposure, these events can lead to preneoplastic lesions in the oral cavity and subsequently to malignancy.

2.1.6.2 Inflammation and its association with Oral submucous fibrosis:

Inflammatory cell activation and recruitment; subsequently follows laying down of extracellular matrix during wound repair; which is a normal phenomenon and a healthy response to tissue damage. These robust and rapid reparative events have been designed so that we can survive in a hostile environment. However we are victims to our evolutionary process, because the end point of repair is abnormal, excessive and poorly organized matrix deposition and fibrosis, which effects normal tissue architecture and ultimately leads to disability of tissues to function.

Recent studies are investigating the molecular mechanisms underlying fibrosis, as there is increasing evidence to suggest that leukocytes can be bad for repair and eventually promote fibrosis. One can understand the role of inflammation during repair not only from studies in skin but also from other organs of the body (Stramer et al., 2007). Within hours of
tissue injury neutrophils are the first cells to be attracted to the wound site, followed by monocytes which mature into macrophages on invading the tissues and finally lymphocytes and mast cells.

Studies have been performed wherein exogenous addition of TGF-β or wild type monocytes drastically increased the levels of fibronectin and collagen expression. This increase in ECM components was attributed to the elevated levels of cytokine (TNFα, IL-1β and IL-6) secretion (Ashcroft et al., 1999). Cytokines regulate two distinct stages of fibrosis (i) fibroblast proliferation and migration (ii) accumulation of collagen matrix. In-vitro both TNFα and IL-1 promote fibroblast proliferation and collagen synthesis (Haque et al., 2000). IL-6 a proinflammatory cytokine has a prominent role in stromal activation; delayed wound healing due to diminished myofibroblast activation was observed in IL-6 null mice models. Myofibroblasts from various tissues produce IL-6, suggesting mesenchymal IL-6 to play a role in facilitating fibrosis, tumoral desmoplasia, epithelial growth and metastasis (Rybinski et al., 2014).

Induction of oral mucosal inflammation by betel quid ingredients is a critical event in the pathogenesis of OSMF (Yadav et al., 2011). Patients suffering from inflammatory or fibrotic conditions such as arthritis, sclerosis and oral submucous fibrosis have benefited with the use IFNγ (antifibrotic agent). Studies have demonstrated inhibition of increased collagen synthesis induced by arecoline on using IFNγ. Intraleisional injections of IFNγ in the oral mucosa have shown an improvement in the mouth opening, decrease in burning sensation and increased suppleness of the buccal mucosa. On histological examination; post treatment with IFNγ showed a decrease in the inflammatory cell infiltrate and an altered cytokine expression (Haque et al., 2001).
Haque et al., 1998: stated that lesional (OSMF) tissue mainly consists of T lymphocytes with high CD4:CD8 ratio and major histocompatibility class II (MHC II) antigen presenting cells. Inflammatory cells produce cytokines and growth factors; thereby promoting fibrosis by inducing fibroblast proliferation, upregulation of collagen synthesis and downregulation of collagen degradation. In the present study immunohistochemical technique was used to demonstrate the expression of inflammatory cytokine and growth factors in OSMF. On evaluation; OSMF tissues stained strongly positive for IL-1α, IL-1β, IL-6, TGF β, PDGF, bFGF, IFN β except IFNγ. The suppression of IFN-γ synthesis, possibly by areca alkaloids, may be fundamental and allow the unchecked production of fibrous tissue, and thus the development of the characteristic clinical and histological features of OSMF.

Haque et al., 2000: reported that continuous local irritation produced by the friction of coarse fibers of areca nut facilitates diffusion of the alkaloids into the sub epithelial connective tissue resulting in juxtaepithelial inflammatory cell infiltration; leading to activation of macrophage and T lymphocytes, thereby increasing the level of cytokines such as IL-6, TNFα, IL-1 and TGFβ; simultaneously reducing the levels of antifibrotic interferon gamma (INF-γ).

Chiu et al., 2001: Tumor necrosis factor-α (TNF-α), situated in the class III region of human leukocyte antigen (HLA), is a mediator with multiple functions, including the regulation of inflammatory reaction and transcriptions of collagen and collagenase. TNF2, the high production allele, was observed to be significantly lower among OSMF group \( n = 166 \) than in areca-chewing controls \( n = 284 \). This finding entails a multifunctional etiological factor of TNFα in OSMF pathogenesis.
**Chiang et al., 2002:** Previous studies have shown that the local and systemic upregulation of inflammatory and fibrogenic cytokines and downregulation of antifibrotic cytokines are central to the pathogenesis of OSMF. The main source of cytokine synthesis is the immunocompetent cells, especially the macrophages and lymphocytes. A study involving immunohistochemical method was used to quantify the T lymphocyte, B lymphocyte and macrophage densities in the epithelium and subepithelial connective tissue of 50 specimens of moderately advanced and advanced OSMF and 10 specimens of normal oral mucosa. On evaluation; results showed a significant increase in the number of T lymphocytes and macrophages and predominance of CD4+ lymphocytes over CD8+ lymphocytes in the subepithelial connective tissue of moderately advanced and advanced cases of OSMF specimens. Thus concluding that cellular immune response may play an important role in the pathogenesis of OSMF.

**Gupta et al., 2008:** reported that patients with oral submucous fibrosis have altered levels of cytokine expression; they demonstrate an increase in transforming growth factor beta (TGF-\(\beta\)), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). On the contrary, they demonstrating decreased levels of IFN\(\gamma\) (antifibrotic agent), thereby promoting collagen synthesis.

The link between inflammation and fibrosis is not straightforward; but it is clear that inflammatory cells do direct fibrogenesis. A thorough understanding of the inflammatory process and the various fibrotic pathways can help establish a relationship. Targeting cytokines like IL-1, IL-6 and TNF\(\alpha\) can be used in practical therapeutic application.
2.1.6.3 Role of Transforming growth factor beta (TGFβ)/Smad signalling and extracellular matrix components in pathogenesis of OSMF.

Fibrosis is defined as overgrowth, hardening, and/or scarring of various tissues and is attributed to excess deposition of extracellular matrix components including collagen. It particularly results from chronic inflammation; most chronic fibrotic disorders have a persistent irritant that mediate the production of proteolytic enzymes, angiogenic factors, growth factors and fibrogenic cytokines which stimulate the deposition of connective tissue elements that progressively remodel and destroy the normal tissue architecture (Wynn, 2008).

Figure 2.1: Persistent stimulus and fibrosis

(ed. Robbins and Cotran, 2014)

The most important fibrogenic mediator and signalling pathway for fibrosis is the TGFβ signalling/serine threonine kinase pathway. The transforming growth factor β (TGFβ) family comprises a large number of structurally associated polypeptide growth factors, each proficient of regulating an interesting array of cellular processes including cell proliferation,
lineage determination, differentiation, motility, adhesion and death. TGFβ and related factors play a prominent role in the homeostasis, development and repair of virtually all tissues in organisms, from fruitfly to human. The TGFβ superfamily of growth factors includes various forms of TGFβ, nodals, activins, Bone morphogenic proteins (BMP), the anti mullerian hormone and several other structurally related factors found in insects, vertebrates and nematodes (Massague, 1998).

TGFβ has three mammalian isoforms (TGFβ1, TGFβ2 & TGFβ3) nearly identical structurally; with a motif consisting of six cysteine residues joined together by three intrachain disulfide bonds that help stabilize β sheet bands. TGFβ is secreted as a latent precursor molecule (LTGFβ) which requires activation for converting into a mature and active form for receptor binding and subsequent activation of signal transduction pathways. LTGFβ is secreted as a large complex which is covalently bound via the latency associated protein (LAP) region to LTGFβ binding protein (LTBP). LAP confers latency, whereas LTBP mediates the binding of TGFβ to the extracellular matrix (ECM) to facilitate proteolytic activation. TGFβ activation is a complex process and requires conformational changes of LTGF-β which is induced either by cleavage of LAP via various proteases such as plamin, plasma transglutaminase, thrombin, or endoglcosylases or via physical interactions of LAP with proteins such as thrombospondin-1 leading to the release of the bioactive, mature TGFβ (Leask and Abraham, 2004).

**Signal transduction machinery**

**TGFβ receptors:** The cellular action of TGFβ superfamily members is initiated on binding to the TGFβ serine/threonine kinase receptors. The TGFβ receptor family comprises of two structurally identical receptors I and II with extracellularly small cysteine rich regions and intracellularly consisting mainly of the kinase domains. Type I receptors have a region rich in
glycine and serine residues (GS domains). Type I and II receptors act sequentially to exert their signal: TGFβ first binds to the TGFβRII (in the cell membrane), thereby recruiting and phosphorylating the GS domains of TGFβRI leading to activation and subsequent intracellular signal transduction. TGFβRIII, a transmembrane proteoglycan also called as Betaglycan does not itself transduce signal, it requires a high affinity binding to TGFβ - TGFβRII (Leask and Abraham, 2004).

**Smad proteins**: After ligand activation, signaling from TGFβRI to the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family. The ligand-specific receptor-activated Smad (R-Smad) are specifically recognized and phosphorylated by the Type I receptors. R-Smads are recruited to the activated TGFβRI by a membrane bound cytoplasmic protein called SARA (Smad anchor for receptor activation). R-Smad include Smad1, Smad5, and Smad8 downstream of the BMP, and Smad2 and Smad3 downstream of TGFβ and activins (Verrecchia and Mauviel et al., 2002).

**Figure 2.2: Detailed structure of Smads**

**Structural domains of an R-Smad**. R-Smad consist of two conserved globular domains known as MH1 (Mad homology 1) and MH2 domains, linked by a linker region. In the basal state, R-Smad remains in an inactive conformation through an auto-inhibitory MH1/MH2 interaction. Phosphorylation of the C-terminal SSXS motif by activated TGFβRI results in R-Smad activation, heteromerization with Smad4, and subsequent translocation into the cell nucleus. The Smad3 domain in MH1 recognizes the DNA sequence CAGAC; the MH2 domain is involved in protein/protein interactions with co-Smad, transcriptional coactivators and corepressors.
R-Smads on being phosphorylated by TGFβR1 form a heteromeric complex with co-
Smad, Smad 4. R-Smad/Smad4 complexes translocate into the nucleus by a mechanism that
involves the cytoplasmic protein importin. This heteromeric complex function as a
transcription factor and binds to the nucleus either directly or in association with other DNA
binding proteins. Maximal affinity of recombinant Smad3 and Smad4 to DNA is observed
with the 5 bp sequence. Smad2, on the other hand, does not bind to the DNA directly, it
requires a nuclear DNA-binding protein of the Fast family to bind to DNA, in association
with Smad4, and activate transcription in response to TGFβ and activins.

**Interaction within the nucleus:** A direct interaction occurs between the Smads and
components of the nuclear pore - in particular, the nucleoporins Nup214 (also known as
CAN) and Nup153. Smad signaling is propagated from receptor to nucleus via the nuclear
pore through a series of competitive protein–protein interactions (Shi and Massague, 2003).

**Nucleocytoplasmic Smad shuffling:** The Smads shuttle both in an uninduced state and
during signaling. In uninduced cells, Smad2 and Smad3 are predominantly cytoplasmic,
whereas Smad4 is distributed throughout the nucleus and cytoplasm. The Smads are not
static, however, but are constantly shuttling between the cytoplasm and the nucleus. Studies
have suggested that Smad nucleocytoplasmic shuttling is independent of transport receptors
and is mediated only through direct interactions with nucleoporins, particularly Nup214 and
Nup153. Smads might be actively imported to and exported from the nucleus by nuclear
transport receptors. Smad4 export requires the nuclear exporter CRM1, which binds to a
nuclear export signal (NES) in the linker region of Smad4. This Smad also contains a basic
bipartite nuclear localization signal (NLS) that binds to importin-a, which in turn interacts
with importin-b to mediate nuclear import (Shi and Massague, 2003).
I-Smads / Inhibitory Smads: A third group of Smad proteins, are the inhibitory Smads (I-Smad), such as Smad6 or Smad7, which prevent R-Smad phosphorylation and subsequent nuclear translocation of R-Smad/Smad4 heterocomplexes. Following target gene transcription, Smad complexes are released from the chromatin and undergo ubiquitination, followed by proteasomal degradation (Massague and Wotton, 2000).

TGFβ/Smad MODULATION OF ECM GENE EXPRESSION

(Verrecchia and Mauviel, 2002)

Recent studies have identified several Smad gene targets like COL1A1, PAI-1, COL1A2, β5 integrin gene, COL3A1, COL5A2, COL6A1, COL6A3, and TIMP-1 using combined cDNA microarray/promoter transactivation approach. A functional interaction between Smad and Sp1 (Aspergillus nuclease) is a critical step for the activation of the above mechanisms. Smad3 and co Smad 4 together with Sp1 and inflammatory cytokine (IL-1), inhibit the transactivation of collagenase (MMP-1), thereby mediating the inhibitory activity of TGFbeta. The inhibition of collagenase prevents collagen degradation and excessive accumulation of the extracellular matrix components.

Smad signaling in wound healing and fibrosis

Smad 3 levels are suppressed during “natural” wound healing, whereas in fibrotic conditions, the presence of a persistent chronic stimulus causes continuous stimulation of TGFβ and activation of the Smad pathway. Patients with systemic sclerotic conditions exhibit abnormal accumulation of various ECM components, predominantly type I and type III collagens and proteoglycans. ECM accumulation is partially also triggered by connective tissue growth factor (CTGF) which is in turn regulated by TGF-β.
Interfering with the fibrotic process

Understanding the mechanisms involved in TGF-β mediated upregulation of ECM gene expression in fibrotic conditions will provide promising and novel approaches to the therapy of these incurable diseases. Sp1 is an important transcription factor, activates all the downstream genes of TGFbeta; therefore altering the function of Sp1 would help us target COL1A2 gene expression and interfere with the pro-fibrotic activity of TGF-β. Agents that could upregulate the expression of Smad 7, inhibit the activation of R-Smads and upregulate MMPs would prove beneficial in targeting the abnormal ECM accumulation.
Selected mechanisms potentially involved in transforming growth factor (TGF)-β1-mediated fibrosis and carcinogenesis. TGFβ is considered to be a “master switch” of the process. The majority of TGFβ is present in the extracellular milieu in a latent form, kept inactive by the latency-associated peptide (LAP), and bound by the latent TGF binding protein (LTBP) (1). Upon release by LTBP, LAP-associated TGFβ is either freed through proteolysis by a variety of enzymes (e.g., plasmin) or is stabilized by membrane-bound integrins (e.g., αvβ6) and directly presented to TGF receptors (2). TGFβ dimers then associate with the type II TGF-β receptor that in turn associates with the type I TGF-β receptor (e.g., ALK5) in a heterodimer (3). The receptor heterodimer becomes activated and initiates a variety of signaling pathways, resulting in both transcriptional and nongenomic signaling. Both Smad-mediated (Smad2 and Smad3) (4) and non-Smad-mediated (5) pathways are involved. Smad-mediated pathways result in activation of TGFβ induced target genes (e.g., α smooth muscle actin, collagen, plasminogen activator inhibitor-1, connective tissue growth factor, and others) as well as inhibition of epithelial genes (e.g., E-cadherin) Non-Smad-mediated pathways are numerous including PI3K/Akt, RhoA, PAR6, and MAPK activation, leading to a host of cellular changes (6). Finally, non-Smad-mediated signaling pathways can interact with Smad-mediated genomic signaling through modulation and activation of transcription factors (e.g., through MAPK) (7).
Studies on TGFβ/Smad pathway, Collagen and OSMF

**Gupta et al., 2008**: explains the pathogenesis of OSMF and the varied interactions between pro-inflammatory cytokines and growth factors (TGFβ, PDGF and bFGF). The continuous stimulation and injury to the oral buccal mucosa by arecoline and arecaidine like constituents in arecanut are the major factors responsible for the induction of the vicious cycle. TGFβ a major profibrogenic mediator stimulates the excessive production of collagens (predominantly type I and type III) and further with the help of crosslinking enzymes such as LOX makes the triple helical protein resistant to degradation. The imbalance between collagen production and degradation sets in a fibrotic phase in the pathogenesis of OSMF.

**Yanjia et al., 2008**: suggested that gene abnormalities in immune response, inflammatory response and EMT induced by TGFβ play an important role in the pathogenesis and malignant transformation of OSMF. The expression profiles of 14,500 genes in human OSMF and normal control were analysed using gene chip array technology; 716 genes were upregulated and 149 genes were downregulated in OSMF tissues. Some of the genes upregulated were PAI-1, MMP3, STAT-1, COL1A2, COL3A1, COL4A1, TNFAIP6, CDC25B, CYP3A5, TGFβ1, COL7A1, LOX, TIMP1 and TIMP3

**Khan, 2011**: To understand the molecular pathogenesis of oral submucous fibrosis (OSMF), gene expression profiling was performed in 10 OSMF tissues against 8 pooled normal tissues using oligonucleotide arrays. Validation employing quantitative real-time PCR and immunohistochemistry confirmed upregulation of transforming growth factor β1 (TGFβ1), TGFBIp, THBS1, SPP1, and TIG1 and downregulation of bone morphogenic protein 7 (BMP7) in OSMF tissues; activation of TGFβ pathway was evident in OSMF as demonstrated by pSMAD2 strong immunoreactivity. These data’s suggest activation of TGF-β signaling and suppression of BMP7 expression in the manifestation of OSMF.
Khan et al., 2012: evaluated the combined effects of arecanut constituents and TGFβ on human gingival fibroblasts using a microarray approach; he observed an enhanced expression of TGFβ target genes namely transglutaminase murine 2 (TGM2), αSMA and collagen isoforms (Col1A1, Col1A2 and Col3A1), concomitantly observed a decrease in TGFβ inhibitor BMP7 (anti-fibrogenic cytokine). He also reported enhanced total collagen protein deposition via direct red dye (Picrosirius) staining.

Collagen production and degradation pathway

Three main events that are modulated by TGFβ which favor collagen production: (Rajalalitha and Vali, 2005; Sudarshan et al., 2012)

Activation of procollagen genes: Collagen being the most abundant protein in the human body plays a critical role as a structural element of the connective tissue. 27 types of collagen have been identified and have been grouped into seven broad categories; major class is the fibrillar collagen, Type I, III and VI form a major part of the connective tissue. Procollagen genes are transcribed and translated to form procollagen monomeric chains (procollagen precursors); these monomers arrange themselves into triple helix aided by disulphide bonds. The procollagen chains are then acted upon by N-C proteinases (PNP and PCP). The newly formed fibrils are then arranged and covalently stabilized into a triple helical structure. TGFβ identifies and targets the genes namely COL1A2, COL3A1, COL6A1, COL6A3 and COL7A1 produced by the fibroblasts.

Elevation of procollagen proteinases levels: Procollagen C proteinases (PCP)/Bone morphogenic protein 1(BMP) and procollagen N proteinases (PNP)

TGFβ treated cells have demonstrated increased levels of PCP and PNP thereby accelerating the formation of fibrils.
2.1.7 Organ Fibrosis

**Introduction:** Multifactorial chronic inflammatory diseases gives rise to progressive and intractable organ fibrosis, in which there is excessive ECM deposition comprising mainly of collagen type 1 and type 3, impairing tissue architecture and function ultimately leading to organ failure. Following tissue injury various organs are affected by organ fibrosis, including the liver, kidney, lungs which is becoming a major cause of death (Kis et al., 2009). Complex biological processes such as macrophage infiltration, secretion of inflammatory mediators and profibrogenic molecules such as TGFβ1 and a shift from Th1 to Th2 phenotype mediate the fibrotic cascade, which ranges from initial epithelial damage to fibroblast induction (Ueha et al., 2012).

**HEPATIC FIBROSIS:**

Hepatic fibrosis is characterized primarily by excessive accumulation of extracellular matrix proteins mainly Type 1 and Type 3 collagen and others such as fibronectin, proteoglycans and laminin in response to liver injury (Stalnikowitz and Weissbrod, 2003). The underlying pathogenic causes for the development of hepatic fibrosis is, first injury to the hepatic stellate cells (alcoholic, fatty, viral and autoimmune origin), second an injury mediated inflammation which further activates fibrogenic signaling cascades leading to fibrogenesis. Injury induced cell death contributes to leukocyte infiltration, release of cytokines (IL-1 and TNFα) and activation of proinflammatory NF-κB signaling pathway which in turn ensure the survival of activated HCS. Chronic inflammatory cells like monocytes and macrophages release inflammatory cytokines such as IL-1β and TNFα that upregulate tissue inhibitor if metalloproteinases (TIMP-1) thereby preventing the extracellular matrix degradation. They also activate HSCs to release the two most important profibrogenic mediators IL-6 and TGFβ. IL-6 maintains the continuous activation of TGFβ whereas, TGF-β induces the transcription of collagen type 1 and type 3 via the Smad
dependent pathways (Seki and Schwabe, 2015). This imbalance between collagen production and degradation is responsible for fibrosis in chronic liver diseases, thus targeting chronic inflammation in context to fibrosis would prove beneficial in preventing the progression of hepatic fibrosis to hepatocellular carcinoma. Studies also outline the role of leptins in the progression of hepatic fibrosis; leptins increase TGFβ secretion from the kupffer cells and sinusoidal endothelial cells thereby enhancing the production of ECM proteins (Honda et al., 2002). In recent few years, oxidative stress (reactive oxygen species) has also been targeted as a plausible etiology for fibrosis (Collel et al., 2001).

RENA L FIBROSIS

Chronic kidney disease (CKD) is a rising epidemic in developing and developed countries. Development of CKD is a result of clinical and subclinical insults including infections, toxins, xenobiotics, immune complexes resulting from autoimmune diseases, mechanical obstruction and genetic disorders. Today the most common cause of CKD however is type 2 diabetes mellitus and ischemic/hypertensive nephropathy (Dufffield, 2014). Depending upon the type of stimuli causing renal injury, the organ prepares itself for repair and recovery by activating resident cells which in turn release proinflammatory cytokines and chemotactic factors. Secretion of chemotactic factors helps monocytes and macrophages to infiltrate bringing in a shift from Th1 to Th2 phenotype further leading to the production of reactive oxygen species thereby creating an environment of oxidative stress, inflammatory cytokines like IL-6 and TNFα which are majorly known for their role for stromal activation, fibrogenic mediators predominantly TGFβ1 and PDGF which stimulate major fibrogenic cells (glomerular mesangial cells, fibroblast and tubular epithelial cells).

The cells with the help of TGFβ1 activate the Smad pathway, leading to the excessive production of downstream proteins like type 1 and type 3 collagen, TIMP1 and PAI. The
parallel action of these proteins promotes ECM deposition and inhibits degradation thereby causing fibrogenesis (Liu, 2006). In the presence of profibrotic mediators the presence of antifibrotic agents like hepatocyte growth factor (HGF) and bone morphogenic proteins (BMP7) play crucial roles. Restoring the balance between pro and antifibrotic signaling could serve as a guiding principle for developing therapeutic strategies.

PULMONARY FIBROSIS

Pulmonary fibrosis is one of the most common causes for the disruption of lung function along with fatal consequences. A number of etiologies such as smoking, occupational exposure (asbestos, metal dusts and silica), viral and bacterial infections, asthma and allergic airway, cystic fibrosis, radiation, chemotherapy have been enlisted as plausible causative factors. Idiopathic pulmonary fibrosis (IPF) is one of the most unique causes of pulmonary fibrosis, the etiology of which is unclear. Studies have stated that the prime factor for fibrosis is a constant stimuli or irritant, a dysregulated healing response evolves into a pathogenic fibrotic response (Wilson and Wynn, 2009). IPF is believed to be preceded by chronic alveolar inflammation following lung injury, on understanding this concept till date the mode of treatment has been anti-inflammatory drugs involving the use of corticosteroids or cytotoxic drugs or immunosuppressive agents (prednisone, cyclophosphamide) but have proven ineffective (Selman et al., 2004). The recent hypothesis for the pathogenesis of IPF focuses on epithelial cells, resident fibroblasts and myofibroblasts. First; differentiation of resident lung fibroblasts directly into myofibroblast under the influence of profibrotic environment. Second; epithelial mesenchymal transition where epithelial cells transform into fibroblasts and further to myofibroblasts. Third; derivation of mesenchymal cells from circulating fibrocytes and other progenitor cells (Scotton, 2007). Apart from the theoretical aspects authors on having performed several experiments have linked both acute (initial) and chronic (late) inflammation and fibrosis. It was observed that the cytokine profile from fluid
or biopsy of Pulmonary fibrosis patients was rich in proinflammatory type 1 cytokines like IL-1β, TNFα, and IL-8. Among the type 1 and type 2 inflammatory cytokines IL-4, IL-13 and TGFβ1 play important roles in exhibiting profibrotic activity through activation, recruitment and proliferation of fibroblasts. TGFβ is one the most studied profibrogenic mediator, association of latency associated polypeptide (LAP) with TGFβ maintains TGFβ in its inactive stage, dissociation of the complex leads to the activation of the Smad/TGFβ signaling pathway (Wilson et al., 2009). The Smad pathway is directly responsible for fibroblast activation, excessive collagen production (type 1 and 3), increase in TIMP1 secretion and decreased MMP2 and MMP9 secretions thereby promoting an imbalance equation between collagen degradation and production. The loss of this equation is responsible for pulmonary fibrosis (Ruiz et al., 2003) Apart from the inflammatory cytokines, an anti-inflammatory cytokine IL-10 derived from T regulatory cells also plays an important role in PF. Polymorphisms in single sequence of IL-10 is observed in patients with PF. Studies have evaluated that there is a stronger inflammatory response in mice deficient with IL-10 leading to excessive subepithelial thickening and extracellular matrix protein component. IL-10 is important as it inhibits the recruitment of inflammatory cells thereby inhibiting fibrogenesis (Garantziotis and Schwartz, 2006).

**RADIATION INDUCED ORAL MUCOSITIS**

Oral mucositis is a tissue reaction to the trauma of radiation and chemotherapy; it is a condition that is best characterized by ulceration, pain and oral erythema. These complications are post several therapeutic procedures involving chemotherapy, radiotherapy or chemoradiotherapy and hemopoietic stem cell transplantation. The pathogenesis of oral mucositis is complex which involves an interaction between cellular, tissue and oral microenvironment. The initial stage/ inflammatory phase is followed by generation of reactive oxygen species, activation of transcription factors like NF-κB, production of
proinflammatory cytokines like IL-1 and TNFα. Increased vascular permeability is observed in irradiated mucocutaneous tissues which further leads to fibrin deposition, excessive collagen production eventuating into fibrosis (Cooper et al., 1995). Presence of oral ulcerations sets in an environment of inflammation, inflammatory cells release several cytokines to promote healing, the constant stimuli (radiation) prevents the healing process, leading to an excessive production of interleukins and mediators such as TGFβ. TGFβ family regulates balance and homeostasis in the human body. TGFβ1 is known to promote fibrogenesis via the Smad dependent pathway, whereas on administration of TGFβ3, a member belonging to the same family reduces the incidence of oral mucositis by negatively regulating epithelial cell proliferation, the effects of which have been examined in – vivo on oral epithelial cells in an hamster model. Studies have shown that topical administration of TGFβ3 prior to radiotherapy or chemotherapy (5-Fluorouracil) significantly reduces the severity of oral mucositis on measuring PCNA and DNA ploidy. These effects are time dependent (Sonis et al., 1997). Ulcerative mucositis also provides a portal for microbial entry (Candidal species, Streptococcus oralis and Streptococcus mitis) leading to local and systemic infections. The pathogenesis of oral mucositis explains the link between initial stages of inflammation followed by epithelial phase, ulcerative/infective phase, healing phase and lastly a fibrotic phase.

2.1.8 Oxidative stress

Oxidative stress plays an important role in tissue fibrosis effecting apoptosis and altering the cytokine microenvironment balance. On evaluation very low levels of antioxidant vitamins have been observed in OSMF individuals; thereby creating oxidative stress that may play a role in progression of OSMF. The extent of oxidative damage caused by ROS can be exacerbated by a decreased efficiency of antioxidant defense mechanism of the body. The adverse effects of ROS are inhibited by cellular antioxidant defense system. The maintenance
of high intracellular antioxidants is considered crucial in providing a reducing environment within the cell further being able to protect the cell against oxidative stress.

**Rajendran and Varkey, 2007**: studied the expression of iNOS immunohistochemically in different grades of epithelial dysplasia in OSMF. Inducible nitric oxide synthase (iNOS) modulates angiogenesis in human models and this information could be extrapolated in elucidating the pathophysiology of OSMF. Significant vasodilation noticed in these cases argues against the concept of ischemic atrophy of the epithelium. Vascularity and iNOS expression helped to explain the vasodilatation noticed (sinusoids) in this disease; NO being a net vasodilator.

**Rajendran et al., 2011**: relates epithelial atrophy in OSMF due to an increase in a signalling molecule; inducible nitric oxide synthase (iNOS). iNOS is proved to have cytotoxic and genotoxic effects, it plays a complex role in cancer, being implicated in tumour progression. He further associated the increase in iNOS with increased angiogenesis in the early stages of OSMF. The study aimed at quantitatively evaluating iNOS expression in OSMF and normal tissue samples. An increase in iNOS expression was observed in OSMF tissues when compared to normal. Upregulation of iNOS leads to an increase in NO expression locally; leads to angiogenesis (increased vascular density, vascular dilatation) and activates wild type p53 gene. Mutations in p53 gene are frequent in oral cancer, thereby supporting the hypothesis that tissue with high level of iNOS could be implicated in neoplastic transformation.

**Riana et al., 2005**: evaluated the clinical profile and serum beta carotene levels in 100 OSMF cases and plotted a relation with different grades of OSMF. Patients considered for the study were predominantly males, 12-78 years age group, burning sensation and inability to open the mouth were the chief complaints. It was observed that with progressive stages of OSMF there

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“In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis”
was a decrease in the serum beta carotene levels (Stage III - 69.9%, Stage II - 76.3%, Stage I - 87.7%).

**Aggarwal et al., 2011:** stated that the extent of oxidative damage caused by ROS can be exacerbated by a decrease in the efficiency of the body's antioxidant defense mechanisms. Beta carotene is an essential precursor of vitamin A or retinol. It is an excellent antioxidant and radical trapping agent, especially for peroxyl and hydroxyl radicals. Aggarwal *et al.* reported that the mean serum beta carotene level was 67 µg/dl, 56.40 µg/dl and 51.33 µg/dl in Groups I (Grade I), Group II (Grade II) and Group III (Grade III), respectively. However, it was 93.02 µg/dl in the control group; the present study demonstrated that beta carotene plays an important role in the pathogenesis of OSMF, and that its level decreases with disease progression.
2.1.8 Overall Pathogenesis

The pathogenesis of OSMF was earlier believed to be multifactorial. It is apparent that fibrosis and hyalinization of subepithelial tissues account for most of the clinical features encountered in this condition. Increased collagen synthesis or reduced collagen degradation as a possible mechanism in the development of the disease (Dyavanagoudar, 2009).

A prominent mediator is transforming growth factor β (TGFβ), in particular seems to be the one that plays a major role in wound repair and fibrosis. It causes the deposition of extracellular matrix (ECM) by increasing the synthesis of matrix proteins like collagen and decreasing its degradation by various stimulating inhibitory mechanisms.
Figure 2.4: The direct influence of areca nut components and molecular events occurring in the disease via the TGFβ pathway

(Rajalalitha and Vali, 2005)
Figure 2.5: Collagen production pathway

(Rajalalitha and Vali, 2005)
**Figure 2.6: Collagen degradation pathway**

(Rajalalitha and Vali, 2005)

- **TGF-β**
  - Activation of TIMP gene
    - Increase of TIMP's
      - Inhibits activated collagenase
        - Decrease in collagenase activity
          - Decrease in collagen degradation
  - Activation of PAI gene
    - Increase PAI
      - Plasminogen
        - Plasmin
          - Procollagenase
            - Collagenase
              - Flavanoids in arecanut

Figure 2.7: Overall effect of activated TGFβ pathway

(Rajlalitha and Vali, 2005)
**Table 2.7: A LIST OF OTHER ETIOLOGICAL FACTORS ASSOCIATED WITH ORAL SUBMUCOUS FIBROSIS: REVIEW**

<table>
<thead>
<tr>
<th>Etiology/ Factor</th>
<th>Literature</th>
<th>Study/Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lysyl oxidase (LOX)</td>
<td>Trivedy <em>et al.</em>, 1999</td>
<td>Increase in LOX expression in early cases of OSMF tissue.</td>
</tr>
<tr>
<td></td>
<td>Sudarshan <em>et al.</em>, 2012</td>
<td>Copper &amp; TGFβ increase production of LOX thereby converting soluble collagen to insoluble making them resistant to degradation.</td>
</tr>
<tr>
<td>2. Plasminogen activating inhibitor (PAI)</td>
<td>Yang <em>et al.</em>, 2007</td>
<td>TGFβ stimulates and increases the production of PAI1, thereby inhibiting MMP activation and collagen degradation.</td>
</tr>
<tr>
<td>3. TIMP’s &amp; MMP’s</td>
<td>Shieh <em>et al.</em>, 2004</td>
<td>TGFβ1 induces TIMP1 production in OSMF tissues thereby inhibiting collagen degradation (inhibiting collagenases).</td>
</tr>
<tr>
<td>4. Cyclooxygenase 2 (COX2)</td>
<td>Tsai <em>et al.</em>, 2003</td>
<td>Treatment with 80 µg/mL of arecoline, upregulates COX2 expression in human buccal fibroblasts</td>
</tr>
<tr>
<td>5. Heat shock protien (HSP)</td>
<td>Yang <em>et al.</em>, 2008</td>
<td>Human buccal fibroblasts treated with 80 µg/ml of arecoline, demonstrate an upregulation in HSP 47 leading to collagen accumulation.</td>
</tr>
<tr>
<td>6. Basic fibroblast growth factor (bFGF)</td>
<td>Bishen <em>et al.</em>, 2008</td>
<td>Increased cellular expression of bFGF was observed in early cases of OSMF, thereby inducing fibroblast activation.</td>
</tr>
<tr>
<td>7. Collagen phagocytic activity</td>
<td>Shieh <em>et al.</em>, 2004</td>
<td>On treatment with arecoline (10-25 µg/mL), safrole (25 µg/mL) and nicotine (50 µg/mL) a significant decrease in fibroblast collagen phagocytic activity was observed in both normal and OSMF tissues.</td>
</tr>
<tr>
<td>8. Saliva</td>
<td>Phatak, 1993</td>
<td>Fibrin production factor in saliva interacts with plasma or fibrinogen in the submucous area of the oral cavity and produces dense fibrosis.</td>
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</tbody>
</table>
In vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

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<tr>
<th>Section</th>
<th>Reference</th>
<th>Description</th>
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<tbody>
<tr>
<td>9. Mast cells</td>
<td>Ankle et al., 2007</td>
<td>Progressive stages of fibrosis, demonstrate increase in mast cell density leading to exponential increase in Microvascular density (MVD).</td>
</tr>
<tr>
<td>10. Hypoxia inducible factor 1α (HIF1α)</td>
<td>Tilakaratne et al., 2006</td>
<td>In advanced stages of OSMF, hypoxia sets in due to constriction of blood vessels, upregulating HIF1α which is correlated to epithelial dysplasia.</td>
</tr>
<tr>
<td>11. Lipids</td>
<td>Sudarshan et al., 2012</td>
<td>Decrease in plasma total cholesterol, HDLC, and triglycerides in OSMF patients.</td>
</tr>
<tr>
<td>12. Micronucleated cells and micronuclei</td>
<td>Holland et al., 2008</td>
<td>High frequency of micronucleated cells and micronuclei in exfoliated cells from buccal mucosa of OSMF patients.</td>
</tr>
<tr>
<td>13. Minerals</td>
<td>Rupak et al., 2012</td>
<td>Low serum iron and hemoglobin levels in patients diagnosed with OSMF.</td>
</tr>
<tr>
<td></td>
<td>Trivedy et al., 2000</td>
<td>Copper content increases in saliva of patients following arecanut chewing for 5-30 minutes.</td>
</tr>
<tr>
<td>14. Genetic &amp; Immunologic factors</td>
<td>Saeed et al., 1997; Caniff et al., 1986.</td>
<td>Raised frequency of HLA-A10, HLA-B7, HLA-DR3, HLA-A24, DRB -11 &amp; DRB3- 0202/3 antigens found in OSMF patients.</td>
</tr>
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<td></td>
<td>Mukherjee et al., 2012</td>
<td>Increased risk of OSMF in men with XRCC3 Thr 241 Met SNPs if exposed to arecanut or smokeless tobacco usage.</td>
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<tr>
<td></td>
<td>Tu et al., 2006</td>
<td>Male arecanut chewers carried 5A allele at -1171 in MMP3 which predisposed them to a threefold risk for OSMF.</td>
</tr>
</tbody>
</table>
15. Connective tissue growth factor (CTGF)  | **Deng et al., 2009**  | Arecoline (0.1 mM) upregulated CTGF expression by 2.5 fold in Human buccal fibroblasts.

16. Keratinocyte growth factor (KGF1)  | **Tsai et al., 2005**  | Arecoline increased KGF1 mRNA expression in a dose dependent manner in OSMF fibroblasts.

17. CCN2  | **Chang et al., 2012**  | Thrombin produced by microtrauma may contribute to the pathogenesis of OSMF by up-regulating CCN2 expression. Inhibited by epigallocatechin-3-gallate.

18. ανβ6  | **Moutasim et al., 2011**  | Over 80% of OSMF-related oral cancers examined had moderate/high ανβ6 expression.

19. Hemeoxygenase -1 (HO-1)  | **Tsai et al., 2009**  | Arecanut contains high iron content and generates ROS during chewing and thereby leads to an upregulation of Hemeoxygenase–1.

20. Microarray analysis  | **Yanjia et al., 2008**  | PAI-1, MMP3, STAT-1, COL1A2, COL3A1, COL4A1, TNFAIP6, CDC25B, CYP3A5, TGFβ1, COL7A1, LOX, TIMP1 and TIMP3 were upregulated in OSMF tissues.

| Li and Jian, 2008  | Individuals with habit of chewing arecanut for more than 4 years, had upregulated genes: Loricrin, Cartilage oligomeric matrix protein (COMP), Cys-X-Cys ligand 9 (CXCL9).

| Chiang et al., 2002  | The presence of serum antigastric parietal cell (GPCA) and antinuclear antigen (ANA) in OSMF patients was associated with daily consumption of arecanut.

| Shin et al., 2004  | Patients with OSMF had a higher frequency of the G allele at position +49 on exon 1 of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4).
<table>
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<tr>
<th>Study (year)</th>
<th>Findings</th>
</tr>
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<tbody>
<tr>
<td>Thangjam <em>et al.</em>, 2009</td>
<td>Upregulation of Transglutaminase-2 (TGM-2) in arecoline induced human gingival fibroblast cells.</td>
</tr>
<tr>
<td>Chung <em>et al.</em>, 2004</td>
<td>The phenotype frequency of allele A6 of major histocompatibility complex (MHC) class I (MICA) subjects with OSMF subjects was significantly higher.</td>
</tr>
<tr>
<td>Sultana <em>et al.</em>, 2011</td>
<td>Few cases of OSMF were found to be positive for p53 overexpression.</td>
</tr>
<tr>
<td>Trivedy <em>et al.</em>, 1998</td>
<td>In OSMF cases, mutations of p53 were majorly observed in exons 6 and 8.</td>
</tr>
<tr>
<td>Rajendran <em>et al.</em>, 1986</td>
<td>Decreased levels of high affinity rosette forming cells (HARFC) and elevated levels of serum IgA, IgD and IgE were found in OSMF.</td>
</tr>
<tr>
<td>Jalouli <em>et al.</em>, 2010</td>
<td>HPV DNA, HSV DNA and EBV DNA were detected from patients with OSMF thereby implying the role of infection in the malignant transformation of OSMF.</td>
</tr>
<tr>
<td>Rajendran <em>et al.</em>, 2009</td>
<td>A positive correlation exists between Rapid urease test (RUT) reactivity (H.Pylori load) and the frequency of mucosal inflammation.</td>
</tr>
<tr>
<td>Pillai <em>et al.</em>, 1990</td>
<td>Interferon treatment recruited active natural killer lymphocytes (AKL) thereby activating their killer potential in response to a stimuli in OSMF.</td>
</tr>
<tr>
<td>Yu <em>et al.</em>, 2013</td>
<td>TGFβ induces s100a4. On treatment of HBFs with arecoline at various concentrations stimulated S100A4 via activation of ERK/NF-κB</td>
</tr>
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</table>
In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

<table>
<thead>
<tr>
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<tr>
<td>Lin <em>et al.</em>, 2005; Tseng <em>et al.</em>, 2007</td>
<td>Arecanut extract activates MAPKs, Akt, &amp; NF-κB in normal oral keratinocytes and downregulates involucrin.</td>
</tr>
<tr>
<td>Sawant <em>et al.</em>, 2014</td>
<td>30% vimentin staining was observed in OSMF tissues.</td>
</tr>
<tr>
<td>Philip <em>et al.</em>, 2014</td>
<td>αSMA stained myofibroblasts expression increased in all grades of OSMF when compared to benign mucosal proliferations.</td>
</tr>
<tr>
<td>Kiran <em>et al.</em>, 2013</td>
<td>Plasma Fibrinogen degradation products (FDP) levels were detected (&gt;200 ng/mL) in all betel nut chewers with OSMF.</td>
</tr>
</tbody>
</table>
2.1.9 Diagnostic Methods for Oral Submucous Fibrosis

2.1.9.1 Clinical Diagnosis: OSMF can predominantly be diagnosed clinically; it has peculiar signs and symptoms. Patients initially report:

1. Burning sensation and vesicle formation.
2. Thick fibrotic bands seen bilaterally on the buccal mucosa, extending till the retromolar area; it may also extend till the faucial pillars in very advanced cases.
3. Blanching along with reduced mouth opening are evident features.
4. Occasionally pigmentation is observed due to the increased tyrosine activity (increased copper levels in arecanut)

2.1.9.2 Routine histopathology: Biopsy specimens can be stained with routine hematoxylin and eosin diagnosis can be carried out.

2.1.9.3 Histological stains: (Mishra et al., 2015)

- Masson’s trichrome stain
- Verhoeff & Von Geison’s stain
- Picrosirius red stain

2.1.10 Treatment Aspects of Oral Submucous Fibrosis

2.1.10.1 Physiotherapy

2.1.10.2 Medical Care

The treatment of patients with oral submucous fibrosis depends on the degree of clinical involvement. If the disease is detected at a very early stage, cessation of the habit is sufficient. Most patients with oral submucous fibrosis present with moderate-to-severe disease. Moderate-to-severe oral submucous fibrosis is irreversible. Medical treatment is symptomatic and predominantly aimed at improving mouth movements.
Treatment strategies include the following:

**Steroids:** In patients with moderate oral submucous fibrosis, weekly submucosal intralesional injections or topical application of steroids may help prevent further damage.

**Placental extracts:** The rationale for using placental extract in patients with oral submucous fibrosis derives from its proposed anti-inflammatory effect hence, preventing or inhibiting mucosal damage. Cessation of areca nut chewing and submucosal administration of aqueous extract of healthy human placental extract (Placentrex) has shown marked improvement of the condition.

**Hyaluronidase:** The use of topical Hyaluronidase has been shown to improve symptoms more quickly than steroids alone. Hyaluronidase can also be added to intralesional steroid preparations. The combination of steroids and topical Hyaluronidase shows better long-term results than either agent used alone.

**IFN-gamma:** This plays a role in the treatment of patients with oral submucous fibrosis because of its immunoregulatory effect. IFN-gamma is a known antifibrotic cytokine. IFN-gamma, through its effect of altering collagen synthesis, appears to be a key factor to the treatment of patients with oral submucous fibrosis, and intralesional injections of the cytokine may have a significant therapeutic effect on oral submucous fibrosis.

**Haque et al., 2001:** investigated the effect of IFN-γ on collagen synthesis by arecoline stimulated OSMF fibroblasts; he observed that arecoline upregulated collagen synthesis in a dose dependent manner upto 50 µg/mL and above 100 µg/mL it was toxic, treatment with IFNγ inhibited the collagen synthesis of stimulated fibroblasts at 10 U/mL concentration. On immunohistochemical analysis; post IFNγ treatment showed a decreased staining for IL-6, IL-1β and PDGF. Clinical trials carried by inducing intralesional injections of IFNγ in OSMF...
patients; considerably improved mouth opening, increased suppleness of the buccal mucosa and reduced burning sensation.

**Lycopene:** Newer studies highlight the benefit of this oral nutritional supplement at a daily dose of 16 mg. Mouth opening in 2 treatment arms (40 patients total) was statistically improved in patients with oral submucous fibrosis. This effect was slightly enhanced with the injection of intralesional betamethasone (two 1-mL ampules of 4 mg each) twice weekly, but the onset of effect was slightly delayed.

**Pentoxyfylline:** It is a tri-substituted methylxanthine; improves microcirculation, decreases platelet aggregation and granulocyte adhesion. It has antithrombin, antiplasmin and fibrinolytic properties; it inhibits activation of T and B cell and promoted natural killer cell activity. Literature studies have reported pentoxyfylline to inhibit burn scar fibroblasts; pentoxyfylline with Vitamin E has shown significant improvement in radiation induced fibrosis. Hence it was used to alleviate the symptoms in OSMF patients.

**Rajendran et al., 2006:** In a pilot study, 14 test subjects with advanced oral submucous fibrosis were given pentoxyfylline at 400 mg 3 times daily who were compared to 15 age and sex matched diseased control subjects. Statistical improvement was noted in all measures of objective (mouth opening, tongue protrusion, and relief from fibrotic bands) and subjective (intolerance to spices, burning sensation of mouth, tinnitus, difficulty in swallowing, and difficulty in speech) symptoms over a 7-month period. Further studies are needed, but this could be used in conjunction with other therapies.

**Mehrotra et al., 2011:** conducted a study on 75 patients diagnosed with OSMF histologically and had habit of chewing arecanut. These patients also complained of difficulty in chewing, repeated ulcers and vesicles in the mouth and burning sensation. Post pentoxyfylline
In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

Chapter 2.1

Oral submucous fibrosis treatment; 25% to 37% improvement was seen in the symptoms; patients were also advised and counseled to eliminate the habit of arecanut chewing.

Aloe vera: It is a mannoprotein; consisting of amino acid called ‘wound healing hormones’.
The polysaccharides present in the gel of the leaves have antioxidant, immunomodulatory and antiinflammatory properties; sterols present inhibit inflammation similar to cortisone without any side effects.

Sudarshan et al., 2012: conducted a study on OSMF patients; he divided them into two groups; group A (receiving aloe vera gel treatment) and group B (receiving antioxidant capsules) for three months. 5 mg of Aloe vera gel was applied bilaterally on the buccal mucosa for three times daily for 3 months. For group B antioxidant capsules were given twice daily for three months. The patients were recalled every 15 days. Different parameters were assessed; in group A, burning sensation reduced to 58% when compared to 36.5% in group B, mouth opening improved by 5.1 mm in group A as compared to 2.5 mm in group B, tongue protrusion improved by 3.1 mm in group A when compared to 1.7 mm in group B. Nausea was the common side effect in patients taking aloe vera treatment; increased appetite was seen in patients consuming antioxidant capsules. Aloe vera treated group had a significant overall response when compared to the group B.

Turmeric: *Curcumin Longa* is a medicinal plant known for its antioxidant, anti-inflammatory and antifibrotic properties; it is broadly used in different systems of medicine which include Ayurveda, Unani and Sidha.

Hastak et al., 1997: In patients with oral submucous fibrosis; an in-vitro study demonstrated a decrease in the number of micronuclei in circulating lymphocytes and exfoliated oral mucosal cells using alcoholic extracts of turmeric, turmeric oil and turmeric oleoresin.
**Hazarey et al., 2015**: demonstrated a statistically significant efficacy of curcumin in OSMF patients. Study groups were broadly divided into curcumin treated and clobetasol propionate treated for three months combined with physiotherapy exercises. The curcumin treated group showed a mouth opening of 5.93 mm when compared to 2.66 mm of the control group. It was concluded that combination treatment modalities (Herbal and physiotherapy) are more efficient when compared to single therapeutic regimen.

**Yadav et al., 2014**: To evaluate the efficacy of curcumin in OSMF patients, an interventional study was performed. Study groups were broadly divided into two. 1st group was administered with two oral curcumin tablets (Turmix 300 mg/day) and 2nd group with intralesional injection of 4 mg of dexamethasone and 1500 IU Hyaluronidase for a period of 3 months. On evaluation it was observed that the burning sensation in both the groups was completely resolved. The interincisal distance improved by 3.13 mm in group 1 and by 1.25 mm in group 2, tongue protrusion improved in group 1 at the end of first month. The study highlighted the importance of herbal medicine in providing symptomatic relief to OSMF patients.

### 2.1.10.3 Surgical Treatment

Surgical treatment is indicated in patients with severe trismus and/or biopsy results revealing dysplastic or neoplastic changes. Surgical modalities that have been used include the following:

- **Simple excision of the fibrous bands**: Excision can result in contracture of the tissue and exacerbation of the condition.

- **Split-thickness skin grafting following bilateral temporalis myotomy or coronoidectomy**: Trismus associated with oral submucous fibrosis may be due to
changes in the temporalis tendon secondary to oral submucous fibrosis; therefore, skin grafts may relieve symptoms.

- Nasolabial flaps and lingual pedicle flaps: surgery to create flaps is performed only in patients with oral submucous fibrosis in whom the tongue is not involved.

- Use of a solid state laser; potassium titanyl phosphate (KTP-532) laser release procedure was found to increase mouth opening range in 9 patients over a 12 month follow-up period in one study.
<table>
<thead>
<tr>
<th>Group</th>
<th>Rationale</th>
<th>Route of administration</th>
<th>Interventions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutrients, micronutrients &amp; antioxidants</strong></td>
<td>Correct deficiency states and promote normal cellular processes present in health that help to protect against adverse events including carcinogenesis</td>
<td>Systemic</td>
<td>Vitamins: A, B, C, D, E, Iodine injections, Minerals Cu, Zn, Mg, Ferrous fumarate, Anti-oxidant: β-carotene, Tea pigment, lycopene, placental extract, Nicotinic acid</td>
</tr>
<tr>
<td><strong>Biogenic stimulation</strong></td>
<td>Homograft stimulates favourable metabolic processes non-fibrotic tissue regeneration</td>
<td>Intralesional injections</td>
<td>Placental extract</td>
</tr>
<tr>
<td><strong>Proteolytic enzymes</strong></td>
<td>Proteolytic enzymes breakdown the inappropriate connective tissue fibrosis</td>
<td>Intralesional injections</td>
<td>Papain, Collagenase, Hyaluronidase, Chymotrypsin</td>
</tr>
</tbody>
</table>
### Oral submucous fibrosis

**Chapter 2.1**

<table>
<thead>
<tr>
<th>Immune modulation</th>
<th>Immune modulation that diminishes pro-fibrotic inflammation and enhances pro-fibrolytic immune mediated pathways</th>
<th>Topical</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intralesional injections</strong></td>
<td></td>
<td>Corticosteroids: Betamethasone, Triamcinolone, Dexamethasone, Methylprednisolone, Betamethasone Hydrocortisone, Interferon Gamma, Levamisole Immune milk from cows immunized with multiple human intestinal bacteria.</td>
</tr>
<tr>
<td><strong>Systemic</strong></td>
<td></td>
<td>Pentoxyfylline Nylidrin hydrochloride Buflomedial hydrochloride Danxuan Koukang</td>
</tr>
<tr>
<td>Promotion of blood flow</td>
<td>Promote blood flow to ischemic tissues via multiple mechanisms including vasodilatation and mild anti-coagulant effects with other biological actions including immunomodulation and anti-oxidant functions</td>
<td>Systemic</td>
</tr>
</tbody>
</table>

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

Oral submucous fibrosis is an irreversible potentially malignant disorder; with a malignant transformation rate of 7-13%. Only if the use of the stimulating factor (Arecanut) is abolished completely can the condition be reversed. OSMF has been seen to be recurrent even after surgical treatment; therefore, formulation of a therapeutic approach is a must. For establishing a proper treatment of OSMF understanding its molecular pathogenesis is important, TGFβ plays an important role in increased deposition and decreased degradation of the extracellular matrix thereby leading to fibrosis. Anti-TGF beta drugs can help in preventing the transformation of this potentially malignant disorder into malignancy.
2.2 Detailed layout of *Centella asiatica* L. and asiatic acid

Traditional medicinal preparations in India date back to about 5000 years and comprise of medicinal plants, minerals and organic matter. These have been derived from rich traditions of ancient civilizations and scientific heritage. Today there are authenticated texts such as Rigveda, Atharvaveda, Charak Samhita and Sushruta Samhita which describe in detail about the importance of selection, preparation and the use of herbs in the right manner (Dubey *et al*., 2004).

2.2.1 *Centella asiatica* Linn

2.2.1.1 Description

*Centella asiatica* L. (*C. asiatica* L.) (Gotu Kola) is an imperative herb in Ayurvedic medicine (Tiwari *et al*., 2011). It is a known medicinal plant that has been employed since prehistoric times, as early as 1200 BC. During the times of ‘Sushruta’ it was referred with a Sanskrit name ‘Manduk – Parni’ as its leaf appears as a standing frog from its backside. *C. asiatica* L. is located at the interface between traditional, modern and scientifically oriented medicine (Brinkhaus *et al*., 2000).

2.2.1.2 Scientific classification

*Centella asiatica* (L.) is a genus of the plant family Apiaceae (Umbelliferae) which contains 20 different species.

<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
<th>NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Eukaryota</td>
</tr>
<tr>
<td>Subkingdom</td>
<td>Embryophyta</td>
</tr>
<tr>
<td>Division</td>
<td>Spermatophyta</td>
</tr>
<tr>
<td>Subdivision</td>
<td>Angiospermae</td>
</tr>
<tr>
<td>Class</td>
<td>Dicotyledoneae</td>
</tr>
<tr>
<td>Subclass</td>
<td>Rosidae</td>
</tr>
</tbody>
</table>
2.2.1.3 Vernacular names

It is known with various other synonyms such as ‘Hydrocotyle asiatica’ L. designation most commonly found, Indian Pennywort in English, Hydrocotyle Asiaticque in French, Idrocotyle in Italian, Brahma – manduki and Brahmi – Buti in Hindi, Tsubo – Kusa in Japanese and Tungchian in Chinese (Brinkhaus et al., 2000).

2.2.1.4 Botanical source and geographical distribution:

*C. asiatica* L. is the most ubiquitous species of Centella. It is most commonly found in Southeast Asia, Sri Lanka, in parts of China, in the western South Sea Islands, Madagascar, South Africa, in the Southeast of the U.S.A, Mexico, Venezuela and Columbia (Jamil et al., 2007).

2.2.1.5 Botanical description

In its macroscopic morphology, *C. asiatica* L. is described as a perennial creeping plant that flowers between August and September; its flowers are light violet in colour. The leaves are cup – shaped, thin and soft, with palmate nerves, measuring about 2 to 5cm in diameter. The leaf margin is crenate or slightly lobed. The petioles are between 5 and 15cm in length, slender and hairless. The brownish green plant has a smell that is similar to tobacco leaves and tastes slightly bitter (Monograph of *Centella asiatica* L. 2007).
In microscopic morphology, the epidermal cells of the upper surface of the leaf are irregular in length with straight or slightly curved walls. The cuticle is thin and densely striated. The stomata are more numerous on the lower surface of the leaf than on the upper surface. The palisade layer of the parenchyma is typically a single row; its cells are wide and short. The petiole contains 7 to 9 vascular bundles, 5 to 7 of which are arranged in a circle in a cross-section. The colourless medullary parenchyma in the middle of the ring of vascular bundles is rich in small supporting cells (Monograph of *Centella asiatica* L. 2007).

2.2.1.6 Parts used: Leaves, root callus and petiole

*Figure 2.8: Illustrates kidney shaped leaves of Centella asiatica L.*

(Eberle, 2014)

2.2.1.7 Chemical composition:

The chemical and active constituents of *C asiatica* L. include 0.1% of essential oils and volatile constituents. It comprises of many other substances which are derived from the metabolism of phenylpropane and acetate, and belong to the flavanoids and terpenes. The most important constituents include triterpenes like madecassic acid, asiatic acid and three asiaticosides; asiaticoside, asiaticoside A and asiaticoside B. *C asiatica* L. comprises of various secondary substances that belong to
groups such as Monoterpenes, Diterpenes, Sesquiterpenes, Alkenes, Alkanes, Phenyl proponoids, Amino acids.

**Table 2.10: Illustrates the secondary metabolites in *Centella asiatica* L.**

<table>
<thead>
<tr>
<th>Secondary substance groups</th>
<th>Secondary substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Monoterpenes</td>
<td>1,8-cineole, limone, borneol, camphene, terpenine-4-ol, borneol acetate, carveol I, carveol II, citronellol acetate, para-cymene, para-cymenol, geraniol acetate, linalool, myrcene, nerol acetate, sabines, terpineolenas.</td>
</tr>
<tr>
<td>B. Diterpenes</td>
<td>Galanal A, galanal B, galanol acetone.</td>
</tr>
<tr>
<td>C. Sisquiterpenes</td>
<td>Bergamotene, bisabolene 2, trans-farnesene, caryophyllenol I, caryophyllene oxide, caryophyllenol II, curcumin (copiaene, trans-farnesene), humulene, santalene</td>
</tr>
<tr>
<td>D. Alkenes</td>
<td>n-heptadecene;</td>
</tr>
<tr>
<td>E. Alkanes</td>
<td>Pentadecane, tridecane</td>
</tr>
<tr>
<td>F. Phenyl – propanoids</td>
<td>Chavicol acetate, 1’-acetoxychavicol acetate, 1’-hydroxychavicol acetate, 4’hydroxy-trans-cinnamaldehyde, 3,4-dimethoxy-trans-cinnamylalcohol, 4-methoxy-trans-cinnamylalcohol, trans-coniferyldiacetate, trans-para – coumaryldiacetate, eugenol acetate, 1’-acetoxyeugenol acetate, eugenol methylether.</td>
</tr>
<tr>
<td>G. Amino acids</td>
<td>Alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, thyrosine.</td>
</tr>
<tr>
<td>H. Terpenoids</td>
<td>Asiaticoside, asiatic acid, madecassic acid, madecassoside</td>
</tr>
</tbody>
</table>
The nutritive analysis of 100 g of leaves of *C. asiatica* L. showed 34 calories, 89.3 g water, 1.6 g protein, 6.9 g carbohydrates, 0.6 g fat, 2 g fibre, 1.6 g ash, 170 mg calcium, 30 mg phosphorous, 3.1 mg iron, 414 mg potassium, 6.58 mg betacarotene, 0.15 mg thiamine, 0.14 mg riboflavin, 1.2 mg niacin, and 4 mg ascorbic acid (Brinkhaus *et al.*, 2000).

### 2.2.1.8 Cultivation

*C. asiatica* L. requires a moist and shady habitat for its growth. It is found most commonly at high altitude ranging between 0 to 2500 meters. Stems and roots are placed in moist sand for its cultivation. The budding plants are transplanted in one to two weeks and harvested 6 months after planting, and at anytime throughout the year (Monograph of *Centella asiatica* L. 2007).

### 2.2.1.9 Medicinal properties and traditional uses

*C. asiatica* L. is used as a nervine tonic, sedative to nerves, carminative, improves appetite, antiseptic, antileprotic. It is used in diseases of skin, nerves and blood. It is also consumed as a tonic for accelerating nervous activity and for improving memory. A special reference has been made in Indian literature to the usefulness of leaves of *C. asiatica* L. as it is most commonly used in the treatment of ulcerations, psoriasis, leprosy, tuberculosis, syphilis, asthma, bronchitis, leucoderma, abdominal disorders, headache, spermatorrhoea and fever. In Chinese medicine Gotu Kula has been used in the treatment of dysentery, urinary calculi, jaundice, vomiting and scabies. In relation to Homeopathic medicine it has been used for skin diseases associated with swelling and itching; it has also been used in inflammation and ulcerations of uterus and eczema (Jamil *et al.*, 2007).
2.2.1.10 Biological and pharmacological studies

On the basis of several pharmacological studies that have been performed in various in-vitro and in vivo test models different biological activities of C. asiatica L. have been observed. The various studies that demonstrated a significant response to the plant were gastric ulcer healing, wound healing; keloid and scar management; oral and topical administration of an alcoholic extract showed to increase cellular proliferation and collagen synthesis at the wound site. Asiaticoside in C. asiatica L. also shows potent antitumor activity; it modulates nitric oxide and tumor necrosis factor α in macrophages thereby promoting apoptosis of cancer cells. Aqueous extract of the herb has been shown to have a significant effect on learning and memory; thereby helping in memory enhancement. C. asiatica L. is also known to have neuroprotective, cardioprotective, hepatoprotective, radioprotective, antidepressant, antipsoriatic, antitubercular, antifilarial, antiviral, antiprotozoal, antispasmodic, anti-inflammatory, antifertility, antioxidant and immunomodulating effects (Jamil et al., 2007).

2.2.1.11 Physicochemical Properties

Dosage

According to the literature the recommended daily dose of C. asiatica L. extracts standardized for asiaticoside, asiatic acid, and madecassic acid is 60 - 120 mg. The recommended daily dosage of crude herb is 0.5 - 6 g (Monograph of Centella asiatica L. 2007).

Side effects and toxicity

According to the established literature alcoholic extracts of C. asiatica L. have shown no side effects at doses of 350 mg/kg i.p to rats. The reported side effects on
consumption of the extract are gastrointestinal upset and nausea. Topical use of the extract has led to rashes (Monograph of *Centella asiatica* L., 2007).

**Contraindication**

*C. asiatica* L. should be avoided during pregnancy as it has an emmenagogue action (Monograph of *Centella asiatica* L., 2007).

### 2.2.2 Asiatic Acid (National Center for Biotechnology Information 2005)

#### 2.2.2.1 Description

A pentacyclic triterpene aglycone

#### 2.2.2.2 Chemical and Physical Properties

- **Chemical Name**: Asiatic acid
- **Molecular formulae**: C_{30}H_{48}O_{5}
- **Molecular weight**: 488.69912 g/mol
- **PubChem CID**: 119034

### 2.2.2.3 2D & 3D STRUCTURE

*Figure 2.9: 2D & 3D structure of asiatic acid*
2.2.2.4 Identification

- IUPAC Name: (1S,2R,4aS,6aR,6aS,6bR,8aR,9R,10R,11R,12aR,14bS)-10,11 dihydroxy-9-(Hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl 2,3,4,5,6,6a,7,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid

2.2.2.5 Experimental properties

- Physical Description: White crystals
- Color: White
- Solubility: In water, 5.98X10⁻² mg/L at 25⁰C
- Vapor pressure: 1.17 X 10⁻¹⁷ mm Hg at 25⁰C
- LogP: log Kow = 5.32

2.2.2.6 Pharmacology and biochemistry

- Pharmacological activities: predominantly used for progressive neurodegenerative disorders, systemic scleroderma, scar and keloid management. It also exhibits anticancer, antimicrobial, antifungal, antioxidant and anti-inflammatory.
- Absorption, Distribution and Excretion: Studies are still ongoing.
- Metabolism/Metabolites: Asiaticoside is converted in vivo to asiatic acid. It is the most active therapeutic ingredient of madecassol.

2.2.2.7 Safety and Hazards

- Inhalation: May be harmful if inhaled. May cause respiratory tract irritation.
- Ingestion: May be harmful if swallowed.
- Skin: May be harmful if absorbed through skin. May cause skin irritation.
- Eyes: May cause eye irritation.
- Flammable

2.2.2.8 Storage conditions

- It should be stored in a dry and well ventilated place.

2.2.2.9 Toxicity: No human clinical trials have been performed

- Asiatic acid was cytotoxic at 100 µM to primary cortical neurons in rats.
2.2.3 Antioxidant activity of *Centella asiatica* L.

**Inamdar et al., 1996:** assessed the presence of varied constituents in *C asiatica* L. The most important biologically active compounds that are responsible for the antioxidant activity are terpenes (Asiatic acid, asiaticoside, madecassoside) and high phenolic content which was contributed by the flavonoids such as quercetin, kaempherol, catechin, rutin, apigenin and naringin. Flavonoids function by quenching free radicals thereby acting as hydrogen donators.

**Brinkhaus et al., 2000:** reported the presence of 0.1% of essential oils and volatile constituents in *C asiatica* L. It comprises of substances which are derived from the metabolism of phenylpropane and acetate. Largely present are flavonoids and terpenes (Madecassic acid, Asiatic acid, and three asiaticoside; asiaticoside, asiaticoside A and asiaticoside B).

**Basile et al., 2005:** commented on the potent antioxidant activity of *C asiatica* L. and attributed it to the presence of polyphenols. Polyphenols have properties of oxidation-reduction which plays an important role in the adsorption or neutralization of free radicals.

**Pittella et al., 2009:** evaluated the antioxidant activity of *C asiatica* L. via the DPPH radical scavenging assay. He observed a potent antioxidant activity exhibited by *C asiatica* L. and attributed this to the presence of phenolic constituents and flavanoids. He stated that flavanoids possess an ideal structure for the scavenging of free radicals, since they present a number of hydroxyls acting as hydrogen-donators which makes them important antioxidant agents.

**Obayed et al., 2009:** evaluated the antioxidant activities of n-hexane, carbon tetrachloride, chloroform and methanol extracts of *C asiatica* L. through the DPPH assay. He observed an increasing antioxidant activity with increasing concentration.
He concluded his study stating that a moderate to potent antioxidant activity was observed in various extracts of *C asiatica* L. at different concentrations.

**Syed et al., 2009:** evaluated and compared the antioxidant activities of essential oil of *C asiatica* L. with Butylated hydroxyanisole in sunflower oil by the DPPH assay. He stated that Butylated hydroxyanisole is a potent carcinogenic agent and therefore there is an increasing interest in the antioxidant activity of natural compounds. He observed that essential oil of *C asiatica* L. showed a potent antioxidant activity as compared to the sunflower oil. He suggested that this potent antioxidant activity may be attributed to the presence of terpenes and phenolics in the essential oil.

**Huda-Faujan et al., 2007:** stated that plants are potential sources of antioxidants, they produce various antioxidative compounds to counteract reactive oxygen species (ROS). He studied the antioxidant activity of methanolic extract of *C asiatica* L. via the reducing power estimation method and compared it with the standard ascorbic acid. He observed that *C asiatica* L. had significant antioxidant activity that could be attributed to the phenolic component, but which could also get altered due to seasonal and geographical variation. Plant phenolics have been found to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition they also have metal chelation potential.

**Chauhan et al., 2010:** stated that plants when subjected to oxidative stress, there is a significant increase in antioxidant enzymes namely catalase and peroxidase. In his study he evaluated the antioxidant activity of ethanolic extract of *C asiatica* L. and observed a significant superoxide free radical scavenging activity when compared to a methanolic extract of *C asiatica* L.
Hashim, 2011: stated that the chemical composition of *C. asiatica* L. plays a very important role in medicinal and nutraceutical applications. *C. asiatica* L. comprises of biologically active components such as triterpenes e.g asiaticoside, asiatic acid, high total phenolic content which is contributed to the presence of flavonoids, rich pools of vitamin C, B1, B2, carotene and Vitamin A. He also suggested that the amount of components can vary depending upon the location and diverse environmental conditions; he stated that the leaves of the plant contain the maximum amount of active constituents. In his study he observed that *C. asiatica* L. leaves reported the highest antioxidant activity by three pathways namely superoxide free radical scavenging activity, inhibition of linoleic acid peroxidation and DPPH radical scavenging activity. Lastly besides the presence of triterpenes, the antioxidant protection effect of *C. asiatica* L. could even be contributed by trace elements such as selenium which stimulate cell rejuvenation.
2.2.4 Anti-inflammatory & antifibrotic activity of *Centella asiatica* L. & Asiatic acid

**Dong et al., 2004**: studied and evaluated the cytotoxic and anti-hepatofibritic activity of various asiatic acid derivatives in rat hepatic stellate cell line (HSC-T6). He explained that fibrosis is characterised by excessive extracellular matrix deposition, predominantly type 1 collagen. The IC<sub>50</sub> values ranged from 5.5 µM to 2000 µM. Exposure of HSC-T6 cells to asiatic acid derivates for 24 hours reduced the collagen synthesis by 14% to 48% measured by hydroxyproline assay. Asiatic acid derivates (Zlx-i-85, 87, 89 & 92) having IC<sub>50</sub> within 25 µM strongly inhibited collagen synthesis. On evaluating TIMP2 and prolyl-4-hydroxylase using RT-PCR, he concluded that the decrease in collagen synthesis can be attributed to the decrease in expression of TIMP2 and prolyl-4-hydroxylase which play an important role in processing collagen at the post-translational level.

**Anilkumar, 2010**: Medicinal plants are a major source of chemical constituents with potential therapeutic effects. He in brief pens down the sequence of events in inflammation (i) Acute transient phase (ii) delayed subacute phase (iii) Chronic proliferative phase. One among the many plants reviewed was *C asiatica* L. which in Ayurveda has been used for many inflammatory conditions. In one of the studies water extract of *C asiatica* L. elicited a dose dependent anti-inflammatory activity at 2 mg/kg b.w concentration when compared to standard mefenamic acid; it showed a further enhanced activity at 10 mg/kg b.w on comparison to standard. The presence of bioactive terpenes (asiatic acid and madecassic acid) in water-methanol extract of *C asiatica* L. contributed to the anti-inflammatory and analgesic activity.

**Yadav et al., 2010**: summarizes the various inflammatory pathways targeted by triterpenoids for the prevention of inflammatory diseases and cancer. Inflammation
plays a dual role; both therapeutic (Acute inflammation) and destructive (Chronic inflammation). Prolonged long standing inflammation is a major cause of concern as it leads to potentially malignant disorders and cancer. There are over 20,000 triterpenoids in nature synthesized by cyclization of squalene. Asiatic acid a potent pentacyclic triterpenoid present in *C asiatica* L. targets tumor necrosis factor (TNF), cytokines (IL-1β, IL-6 and IL-8). IL-1β plays an important role in the inflammatory response against infection by increasing the expression of endothelial adhesion factors, thus allowing infiltration of leukocytes at the site of infection, IL-6 is a proinflammatory cytokine released in response to trauma. IL-8, a member of the CXC chemokine family, functions as a mitogenic, angiogenic, and mutagenic factor promoting cancer progression. Asiatic acid inhibits the NF-κB activation by preventing the binding of TNF to TNF receptor present on the cell surface.

**Widegrow, 2011:** summarizes and explains the cellular/extracellular matrix cross-talk in fibrosis and scar evolution. He targets the Smad signalling pathway, the amalgamation of Smad 2, 3 & 4, TGFβ1 and inflammatory cytokines for the process wound healing, regeneration, repair, fibrotic diseases and scar formation. Fibrotic diseases and scar formation occurs due to a constant competition between proinflammatory (TNFα, IL-6, IL-8 & IL-1β) and profibrotic agents (TGFβ1, CTGF, PDGF & FGF) and their antagonists. His review explains that an ideal antifibrotic agent firstly should upregulate Smad 7 thereby upregulating Smad 3 (antifibrotic growth factor), following decrease in collagen synthesis and inhibition of the Smad pathway. Secondly, it should downregulate TGFβ1; inactivate Smad2/3 causing decreased collagen formation and deposition. One among the many therapeutic agents; *C asiatica* L. was listed as it is known to stimulate TGFβ3, Decrease TGFβ1, decrease matrix deposition including collagen and fibronectin, decrease IL-6,
Smad 7, decrease Smad 2,3 & 4, complex iNOS and COX-2 inhibition. Per se collagen and ECM remodelling, he lists *C asiatica* L. as one the herbs to regulate TGFβ1 to balance collagen type III/I ratios and has an antioxidant activity against pyridinilone linkages.

**Huang et al., 2011**: evaluated asiatic acid, for its antinociioceptive (analgesic) and anti-inflammatory effects. Carrageenan induced paw edema model was used to study the anti-inflammatory effect. Asiatic acid at 5-10 mg/kg b.w, decreased nitric oxide (NO), TNF-α and IL-1β levels in serum at the 5th hour after carrageenan injection. It also decreased carrageenan-induced iNOS, cyclooxygenase (COX-2), and nuclear factor-κB (NF-κB) expressions at the 5th hour in the edema paw model on western blotting analysis. An intraperitoneal (i.p.) injection treatment with asiatic acid resulted in diminished neutrophil infiltration into sites of inflammation as did indomethacin.

**Tang et al., 2012**: studied the activity of asiatic acid in carbon tetrachloride (CCl₄) induced liver fibrosis both *in vitro* and *in vivo*. One of the major causes of liver failure is liver fibrosis; as until today no treatment remains effective. The study involved *in vivo* rat model of liver fibrosis prepared by inducing CCl₄ and *in vitro* by TGFβ1 stimulated rat hepatic stellate cell line (HSC-T6). In the rat model, immunohistochemically asiatic acid inhibited αSMA and collagen matrix expression thereby attenuating CCl₄ induced liver fibrosis. Whereas in the *in vitro* study, asiatic acid upregulated Smad antagonist Smad7, downregulated the expression of Smad2/3, myofibroblast differentiation and collagen matrix expression in a dose dependent manner. Tang *et al.* concluded that asiatic acid can be used as a novel therapeutic agent for liver fibrosis and that the TGFβ/Smad signalling mediated fibrogenesis may be the core mechanism by which asiatic acid inhibits fibrosis.
Roy et al., 2013: summarizes the widespread information on the pharmacological activities of alcoholic and hydroalcoholic extract of *C asiatica* L. With the knowledge of existing literature *C asiatica* L., is known to posses; antimicrobial, anticancer, wound healing, neuroprotective, immunomodulatory, anti-inflammatory, hepatoprotective, insecticidal and antioxidant activity. Major studies are primarily performed using the leaves of the plant and its phytochemical constituents vary in quantity depending upon the type of extraction performed and the geographical conditions. *C.asiatica* L. is rich in amino acids, carbohydrates, phenols (Flavanoids namely Kaempferol, kaempferol-3-o-β-d-glucuronide, castilliferol, quercetin, quercetin-3-o-β-d-glucuronide, castillicetin, apigenin, rutin, luteolin, naringin), terpenoids (asiaticoside, asiatic acid, centelloside, madecassoside, brahmoside, brahminoside, asiaticenolic acid, centelic acid, centoic acid, madecassicacid, terminolic acid and betulic acid), volatile oils and vitamins (Ascorbic acid, nicotinic acid and β-carotene). Among the many the main active constituents are pentacyclic triterpenes (Asiatic acid, madecassic acid, asiaticoside, and madecassoside). Roy et al. reported that ethanolic extract of *C asiatica* L. used at dose of 100 mg/kg b.w in rats demonstrated anti-inflammatory activity when compared to standard ibuprofen. It also demonstrated the same in inflammatory bowel disease in rats. This activity may be attributed to the presence of asiaticoside and asiatic acid; as literature reports that asiaticoside inhibits LPS induced fever and inflammation, including TNFα and IL-6 production. Prolonged inflammation can cause fibrosis of various organs. Due to the above pharmacological actions and presence of active phytoconstituents; studies have demonstrated the anti-fibrotic effect of ethanolic extract of *C asiatica* L. in dimethylnitrosamine induced liver fibrosis. Asiatic acid and madecassic acid have
shown anti-inflammatory effect by the inhibition of enzymes like iNOS, COX-2, IL-6, IL-1β and TNF-α expression through the down-regulation of NF-κB.

**Zhang et al., 2013:** delved the role of herba Centella in relation to the expression of hepatocyte growth factor (HGF); an anti-fibrotic cytokine and monocyte chemotactic protein-1(MCP-1); cytokine that promotes renal tubule interstitial fibrosis (TIF). TIF is the pathological hallmark for end stage renal disease and the result of chronic and persistent renal damage, which further leads to excessive proliferation of the extracellular matrix resulting in fibrosis or sclerosis. Herba Centella has been studied extensively and is known for its large reservoir of pentacyclic terpenoids (asiatic acid, asiaticoside, madecassoside and madecassic acid). It functions by inhibiting ECM proliferation, maintaining the fibre composition by reducing the aminotransferase activity, reducing collagen and acid mucopolysaccharide and lastly by inhibiting the action of TGFβ1. In the present study, Zhang et al. demonstrated the upregulation of HGF and its mRNA expression in rat renal interstitium at a higher concentration in a dose dependent manner, with a simultaneous downregulation of MCP-1 and its mRNA expression using RT-PCR. HGF suppresses the expression of TGFβ1, blocks the TGFβ/Smad signalling pathway, and deactivates the enhanced expression of Smads thereby inhibiting fibroblast proliferation and collagen formation.

**Bian et al., 2013:** studied the antifibrotic activity of asiatic acid on cell proliferation, invasion and collagen synthesis; by inducing normal and keloid fibroblasts with TGFβ1 with or without asiatic acid. Fibrotic and keloid fibroblasts showed elevated levels of TGFβ1, phosphorylated Smad2/3 and a downregulation of Smad 7 (Inhibitory Smad) which antagonises the action of Smad2/3. Asiatic acid demonstrated cell viability at concentrations (10 μM – 30 μM), it downregulated TGFβ1 induced collagen type 1 formation and upregulated Smad2/3 antagonist; Smad
Centella asiatica L. & Asiatic acid

Chapter 2.2

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

7, whereas showed no effect on normal fibroblasts. A direct target of TGFβ/Smad signalling pathway is PAI-1 which is highly expressed in fibrotic and keloid tissue. On treatment with asiatic acid it suppressed PAI-1 upregulation induced by TGFβ1 in keloid fibroblasts. These affects of asiatic acid were mediated by PPAR-γ pathway. 

Xu et al., 2013: performed an in vivo experiment and investigated the antifibrotic activity of asiatic acid in tubulointerstitial fibrosis in mice with ureteral obstruction. Mice were treated with various doses of asiatic acid (1 mg/kg, 4 mg/kg and 16 mg/kg b.w) for six days with oral gavage after the Sham surgery (unilateral ureteral obstruction). On the seventh day they were sacrificed and kidney sections were prepared and stained with Hematoxylin and eosin, periodic acid Schiff’s stain (Analysis of tubular injury) and MTS (evaluating areas of collagen formation). They were also stained with Collagen III and fibronectin antibody. Tubular injury was observed in the renal cortex of the mice administered with the vehicle, while low doses of asiatic acid were observed to exert a significant suppressive effect on tubular injury. Interstitial fibrosis, increased expression of SMA and TGFβ1 and phosphorylation of Smad2/3 were induced by ureteral ligation; however these effects were abrogated by intermediate and high doses of asiatic acid. These results suggested that asiatic acid may ameliorate tubulointerstitial fibrosis by reducing tubular injury, fibroblast activation and ECM accumulation mediated by Smad dependent TGFβ signalling.
2.3 Detailed layout of *Ocimum basilicum* L and linalool

2.3.1 BASIL, *Ocimum basilicum* L., F.Lamiaceae

2.3.1.1 Description

*Ocimum basilicum* L. (*O basilicum* L.), which is also known as French basil or sweet basil, is a member of the Labiatae family (Duke *et al.*, 2006).

2.3.1.2 Varieties of basil

a. *Ocimum basilicum* var. purpurascens is popularly known as Purple Basil

b. *Ocimum basilicum* var. genovese is also called Genovese Basil

c. *Ocimum basilicum* var. Crispum popularly known as Lettuce Leaf Basil

2.3.1.3 Etymology

It is known as the ‘the King of herbs’ where the word ‘King’ is derived from the Greek word ‘basileus’.

2.3.1.4 Taxonomy and scientific classification

Sweet basil is one of the 30 species belonging to the genus Ocimum, it is derived from the Greek word ‘ozo’ which means to smell. In French it is also called as ‘Herbe Royale’.

**Table 2.11: Systematic classification (Taxonomy) of *Ocimum basilicum* L.**

<table>
<thead>
<tr>
<th>SCIENTIFIC CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Phylum</td>
</tr>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Order</td>
</tr>
<tr>
<td>Family</td>
</tr>
<tr>
<td>Species</td>
</tr>
</tbody>
</table>
2.3.1.5 Vernacular names:

In Tamil it is known as ‘tirunittru pachaielai’ and in Hindi it is called as ‘Kali Tulsi’. In English, it is known as Basil, Common Basil or Sweet Basil whereas, in Hindi and Bengali, it is called Babui Tulsi. The plant is known as Badrooj, Hebak or Rihan in Arabic; as Nasabo or Sabje in Gujarati and as Jangli Tulsi in Urdu. Tohrakhurasani and Okimon are the ascribed names of the plant in Persian and Unani. (Bilal et al., 2012).

2.3.1.6 Botanical source and geographic distribution

*Ocimum basilicum* L. is a perennial herb which is a native of and is most commonly distributed to areas of Asia and Africa. Basil was initially brought from India and later grown in Europe in the sixteenth century. It is indigenous to Persia and Sindh and lower hills of Punjab in India. Basil is a rich source of essential oils; it is also believed that if basil is planted around homes and temples, it brings happiness (Parathasarathy et al., 2008)

2.3.1.7 Botanical description

The Genus ‘Ocimum’ is broadly classified into two categories namely ‘Basilicum’ and ‘Sanctum’. Sweet Basil belongs to the basilicum group and grows to a size of 1-2 feet in height. It is an herbaceous branched plant, with stems and twigs that are quadrangular, greenish, purplish or brownish in colour and extipulate leaves that are opposite and decussate measuring around 2 inches in length. The leaves are simple, petiolate, ovate or subovate with serration on entire margin, its flowers are white in colour, conspicuous, small and are arranged as a spicate or recemose inflorescence. These plants are hermaphrodite, seeds are black, ellipsoid and they become mucilaginous when wetted (Parathasarathy et al., 2008)
2.3.1.8 **Parts used:** The whole plant is used as a source of traditional medicine (Bilal *et al.*, 2012).

2.3.1.9 **Active chemical composition**

*Ocimum basilicum* L. revealed the presence of glycoside, gums, mucilage, proteins, amino acids, tannins, phenolic compound, triterpenoids steroids, sterols, saponins, flavones and flavonoids in it. The flavour and smell of basil is largely determined by the varying quantities of major constituents of essential oil found in Sweet Basil. Analyzed essential oils mainly consists oxygenated monoterpenes (60.7-68.9%) followed by sesquiterpenes hydrocarbons (16.0-24.3%) and oxygenated sesquiterpenes (12.0-14.4%).

**Table 2.12: Illustrates the secondary metabolites in *Ocimum basilicum* L.**

<table>
<thead>
<tr>
<th>Secondary metabolite groups</th>
<th>Secondary metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Oxygenated Monoterpenes</td>
<td>linalool, camphor, cis-geraniol and 1,8-cineole.</td>
</tr>
<tr>
<td>b. Sesquiterpene Hydrocarbons</td>
<td>a- bergamotene, b-caryophyllene, germacrene D, c- cadinene and bicyclogermacrene</td>
</tr>
<tr>
<td>c. Oxygenated Sesquiterpenes</td>
<td>epi-a- cadinol and viridiflorol</td>
</tr>
</tbody>
</table>

*In-vitro* human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis
2.3.1.10 Seasonal variation in chemical composition

The chemical composition of basil varies according to the place of cultivation, season of cultivation, season of collection and mode of extraction technique. The maximum amount of oil is found during winter (0.8%) which decreases significantly during summer (0.5%). Samples collected in winter are richer in oxygenated monoterpenes and rich in sesquiterpene hydrocarbons when collected in summer months.

2.3.1.11 Cultivation

Ocimum species sustains its growth in a variety of soils and climatic conditions. It grows well under high rainfall and humid conditions. Ocimum is propagated through seeds, a direct mode of sowing is comparatively better to transplanting. A seeding rate 75 – 250 g/ha should be considered, seeds are mixed with fine sand to ensure even distribution. Sowing is done on a well prepared and levelled land. It is done in long narrow furrows at a spacing of 50 – 60 cm; the seeds are lightly covered by soil and then irrigated. Seeds germinate in two weeks time. No manure is applied for O basilicum L., but application of 20 – 25 kg of N and 10 – 15 kg of P₂O₅ / ha ensures a good vegetative growth, herb and oil yield (Parathasarathy et al., 2008).
2.3.1.12 Harvesting and processing

*O cimum basilicum* L. is raised annually; the first harvest is taken at 90 – 95 days of planting on a bright sunny day. The crop is cut 15 – 20 cm from the ground level. The oil and the yield quality are not diminished upto 6 – 8 hours after harvest (Parathasarathy *et al.*, 2008).

2.3.1.13 Traditional medicinal uses

Traditionally basil was used as a tonic and vermifuge. Basil tea was taken hot for treating flatulence, nausea and dysentery. The oil extract of the plant was used for cold, rhinitis, mental fatigue and spasms. Aqueous extract of the leaves was used in Nigeria for post natal uterine contractions and a cold infusion of the same was used to relieve the severe pain after parturition. It was also given internally for the treatment of cystitis, piles and nephritis.

2.3.1.14 Biological and pharmacological studies

Sweet basil is known for its antioxidant, antimicrobial, antifungal, analgesic, anti-inflammatory, antiulcerogenic, chemomodulatory activity, CNS stimulant activity, CNS activities (sedative, anticonvulsant and anaesthetic), hepatoprotective, hypoglycaemic, hypolipidemic, immunomodulatory, larvicidal and nutritional health benefits. It is also known to have unique health protecting effects due to the presence of flavanoids and volatile oils. Aromatic leaves and flowering parts of *O cimum basilicum* L. are used as a stimulant and tonic agents to treat a large group of ailments such as poor digestion, stomach ache, feverish illnesses, nausea, abdominal cramps, gastroenteritis, migraine, insomnia, depression, gonorrhoea, dysentery, chronic diarrhoea and exhaustion. Topical application of sweet basil is also used in the treatment of acne, loss of smell, insect stings, snake bites and skin infection (Shafique *et al.*, 2011). Lastly *O cimum basilicum* L. is used as a culinary herb in many Italian and Thai cuisines.
2.3.1.15 Physicochemical properties

Dosages

There is insufficient evidence for a recommended dosage for adults and children in the present literature (Ulbricht, 2010).

Side effects

Estragole an important constituent of sweet basil may cause liver damage (Ulbricht, 2010).

Contraindication

Sweet basil is not recommended in pregnant or breastfeeding women (Ulbricht, 2010).
2.3.2 Linalool (National Center for Biotechnology Information 2004)

2.3.2.1 Description

Monoterpene

2.3.2.2 Chemical and Physical Properties

- Chemical Names: Linalool; 3,7-Dimethylocta-1,6-dien-3-ol; 78-70-6; allo-Ocimenol; beta-Linalool
- Molecular formulae: C\textsubscript{10}H\textsubscript{18}O
- Molecular weight: 154.24932 g/mol
- PubChem CID: 6549

2.3.2.3 2D & 3D structure

![Figure 2.12: 2D & 3D structure of linalool](image)

2.3.2.4 Identification

- IUPAC Name: 3,7-dimethylocta-1,6-dien-3-ol

2.3.2.5 Experimental properties

- Physical Description: Liquid; Dry powder, Liquid, Solid
- Color: Colorless liquid
- Odor: Odor similar to that of bergamot oil and French lavender
- Taste: Light, Floral, Spicy, Citrus taste
- Boiling point: 198 °C; 86 °C at 13 mm Hg
• Flash point: 160 °F (71°C)
• Solubility: Soluble in alcohol, ether, fixed oils, propylene glycol; insoluble in glycerin
• Density: 0.858 - 0.868 at 25°C
• Vapor pressure: 0.16 mm Hg at 22 - 25°C
• LogP: log Kow = 2.97
• Stability: Good
• Decomposition: When heated to decomposition emits acrid smoke and irritating fumes.
• Analytical Laboratory Method: GC with flame-ionization detector is used to measure linalool.

2.3.2.6 Pharmacology and biochemistry

• Pharmacological activities: Antihyperalgesic, antinociceptive, antimicrobial, antifungal, antioxidant, anti-inflammatory and acetyl cholinesterase inhibitor.
• Absorption, Distribution and Excretion:
  Permeates human buccal mucosa at a concentration of 46.6% w/W. In rats: linalool is rapidly absorbed from the intestinal tract following oral uptake. Judging from the delay in fecal excretion, intestinal absorption is complete. Subsequent to absorption, linalool is metabolized rapidly, with urinary excretion.
• Metabolism/Metabolites: Linalool is probably converted to geraniol and its metabolites
2.3.2.7 Safety and hazards

- Fire Hazard: Combustible. Gives off irritating or toxic fumes (or gases) in a fire.
- Explosion Hazard: Above 75°C explosive vapour/air mixtures may be formed.
- Skin Hazard: Redness and pain
- Eye Hazard: Redness. Pain

2.3.2.8 Storage conditions

- Should be kept separated from strong oxidants; in a container tightly closed in a dry and well-ventilated place.

2.3.2.9 Toxicity:

- In humans: Linalool more than 45.7% has sedative effects.
  
  Eye irritation is observed at measured vapour concentration of 320 ppm.
  
  Patch test produced allergies in some individuals.

- In animals
  
  i.p injection of linalool at 1200 mg/kg (oily solution) in mouse
  
  Dermal injection of linalool at 610 mg/kg in albino rabbits.
2.3.3 Antioxidant activity of *Ocimum basilicum* L.

**Lee et al., 2005:** assessed the antioxidant activities of phenolic constituents of *Ocimum basilicum* L. (*O basilicum* L.) namely eugenol, thymol, carvacrol and 4-allylphenol with known antioxidant, butylated hydroxytoluene (BHT) and α-tocopherol via hexanal oxidation. He observed that thymol and carvacrol had the maximum antioxidant activity followed by eugenol and 4-allylphenol. He observed that eugenol, thymol and carvacrol inhibited hexanal oxidation.

**Politeo et al., 2007:** evaluated the antioxidant activity of glycosidically bound volatile compounds extracted from basil via GC/MS using the DPPH antioxidant assay. The activity of volatile aglycones and essential oils was compared to the synthetic standard, BHT and eugenol. Essential oils of basil (linalool, eugenol, and carvacrol) demonstrated higher antioxidant activities when compared to volatile aglycones (vanillin, geraniol, lavandulol). Essential oils exhibited activity similar to the standard BHT and eugenol; thereby justifying the potential utilization of herbs and spices for preventing oxidative stress.

**Gulcin et al., 2007:** stated that extraction, characterization and utilization of natural antioxidants may serve to be potent candidates for combating oxidative stress induced diseases such as cancer, immunodeficiency syndrome, heart diseases, stroke, rheumatoid arthritis etc. Exogenous sources such as tobacco smoke and organic solvents gives rise to ROS that includes free radicals such as superoxide anion radicals (O$_2^-$), hydroxyl radicals (OH$^-$); these reactive oxygen species induce oxidative damage to biomolecules such as lipids, proteins, carbohydrates and nucleic acids and cause cellular damage. He suggested that antioxidant compounds that are naturally occurring in plant sources can be identified as free radicals or active oxygen scavengers. Based on this theory he studied the antioxidant activity of water and...
ethanolic extracts of *O basilicum* L. via the reducing power estimation method, DPPH assay and superoxide radical scavenging assay and compared it with the standards. He observed that the ethanolic extracts of *O basilicum* L. demonstrated potent antioxidant activity via all the three mechanisms when compared to the water extract of *O basilicum* L.

**Hussain et al., 2008:** measured the free radical scavenging capacity of the essential oil of *O basilicum* L. via the DPPH radical scavenging assay. He observed a potent antioxidant activity of the essential oil primarily due to the presence of terpenes (Linalool). Terpenes have the ability to donate hydrogen atoms or electrons thereby scavenging the reactive oxygen species.

**Kaurinovic et al., 2011:** evaluated and compared the antioxidant activity of five different extracts (Et₂O, CHCL₃, EtOAc, n-BuOH and H₂O) of *O basilicum* L. and *O vulgare* L. and compared it with the standards via the DPPH assay, nitric oxide assay and superoxide radical scavenging activity. He observed that the ethanolic extract of *O basilicum* L. had a higher antioxidant activity via the DPPH assay and superoxide radical method and that of *O vulgare* L. demonstrated a higher antioxidant activity via the nitric oxide assay. He attributed these variations in the activity due to the varying amounts of phytochemical constituents among the two plants namely polyphenols and flavonoids. Lastly he also commented on the type of solvent used for extraction which could affect the antioxidant activity of herbs.

**Sarfraz et al., 2011:** carried out a study to analyze the antioxidant activity of methanolic extract of Pakistani basil leaves via the DPPH radical scavenging activity. He concluded that the methanolic extracts of Pakistani basil leaves had potent dose dependent inhibitory effect, the inhibition that was attained by the fresh basil leaves sample was 82.46% at a concentration of 200 µg/mL when compared to 61.91% at a
concentration of 50 µg/mL. He stated that this dose dependent variation could be due to oxidation of some antioxidant compounds of the extract or due to the low concentration of the extract.

**Shafique et al., 2011:** conducted a study comparing the antioxidant activity of essential oil of Sweet basil with Butylated hydroxytoluene (BHT) via the DPPH radical scavenging method; he observed that the essential oil of Sweet basil had a higher and potent antioxidant activity at all concentrations when compared to that of BHT. His study depicted that the ability of the extract to scavenge DPPH radical increases significantly with increasing concentration indicating a higher hydrogen donating ability.
2.3.4 Anti-inflammatory and antifibrotic activity of *Ocimum basilicum* L. and Linalool

**Benedec et al., 2007**: investigated the anti-inflammatory effect of *Ocimum basilicum* L. (*O basilicum* L.) in male wistar rats induced with turpentine oil. The experimental animals were divided into three groups, first a positive group with untreated inflammation, second group where the inflammation in animals was treated with *O basilicum* L.; the last group treated with non-steroidal anti-inflammatory drug (NSAID) diclofenac sodium. Animals after treatment were sacrificed and blood was taken from retro-orbital sinus. The following parameters were investigated *in vitro* for: total leukocyte count, differential leukocyte count and phagocytosis expressed as percentage. On comparison to the control group and diclofenac treated group, the *O basilicum* L. treated group significantly decreased the total and differential leukocyte count (polymorphonuclear leukocytes & monocytes). On further analysis *O basilicum* L. extract treated group significantly reduced *in-vitro* phagocytosis in comparison to control group. He explained that acute phase inflammation is marked with increased number of inflammatory cells and inflammatory cytokines (IL-6, IL-1 & TNFα); the *O basilicum* L. extract could significantly decrease the number of total and differential leukocyte count and was stronger on comparison to the diclofenac treated group.

**Selvakkumar et al., 2007**: explained that inflammatory cytokines such as IL-1β and TNFα play very important roles in amplifying the immune response; and inhibition of this mechanism would help as a mode of therapeutic strategy. An experimental study was carried out with human peripheral blood monocytes (PBMCs) where inflammation was induced with lipopolysacchride (LPS) for 12 hours, after incubation the inhibitory effect of methanolic crude extract of *O basilicum* L. on inflammatory...
cytokines was studied using reverse transcriptase polymerase chain reaction. On analysis it was observed that 30 µg/mL of methanolic extract lead to the downregulation of TNFα and IL-1β gene expression. In conclusion anti-inflammatory action was exhibited by crude methanolic extract of *O basilicum* L. by downregulating key proinflammatory cytokines and mediators on LPS induction in PBMCs.

**Meera et al., 2009:** in her review explains that inflammation is a complex biological process. Inflammation plays a dual role; it helps in eliminating infection and promotes wound healing, but at the same time if left unchecked and uncontrolled it leads to inflammatory conditions such as arthritis and fibrosis. She enlists many medicinal plants that have an anti-inflammatory activity like *Cassia fistula* Linn, *Curcuma amada* Roxb. (Zingiberaceae) and *O basilicum* L. (Lamiaceae). A high dosage (500 mg/kg body weight) of methanolic extract of *O basilicum* L. exhibits potent anti-inflammatory activity compared to 300 mg/kg body weight of sodium salicylate in rats as tested by carrageenan-induced pedal edema.

**Phadtare et al., 2013:** reviews that plants of the Lamiaceae family for e.g. *Ocimum sanctum* L. have been used for various ailments such as inflammatory conditions, arthritis, bronchitis, asthma and skin diseases. The aim of the present study was to evaluate the anti-inflammatory potential of *O basilicum* L. species in carrageenan induced paw edema in rats. On experimental analysis it was observed that *O basilicum var. basilicum* decreased carrageenan induced rat paw edema at a dose of 50, 100 and 200 mg/kg b.w thereby possessing potent anti-inflammatory and anti-arthritic effect, whereas *O basilicum var. thyrsiflora* was unable to show activity at lower doses and therefore higher doses may be required. *O basilicum* L. consists of a
pool of oil constituents (Eugenol, ursolic acid and oleanolic acid) which also play an important role in exhibiting anti-inflammatory and anti-arthritic activity.

**Yacout et al., 2011**: studied the anti-fibrotic effect of *O basilicum* L. in CCl₄ induced liver fibrosis and cytotoxicity in rats. Basil is a rich source of polyphenols and flavanoids which have shown to possess antioxidant and anti-inflammatory activity. In the present study both histological and biochemical parameters were studied. CCl₄ induction causes an elevation in the serum marker enzymes ALT, AST and ALP, since liver damage releases these enzymes in the blood circulation. The histological effects in the liver of CCl₄ - treated animals showed many degenerative changes including cytoplasmic vacuolization of the hepatocytes, fatty infiltrations, leucocytic infiltrations, congestion of blood vessels, and fibrosis. The present study demonstrated that basil significantly improved the tissue architecture and increased liver function enzyme activity. When CCl₄ was applied in this experiment to induce liver fibrosis, the level of hydroxyproline in liver significantly increased. Basil and Dimethyl diphenyl bicarboxylate (DDB) were found to be useful for repairing hepatic fibrosis. Improvement of fibrotic changes in the liver and promoting liver regeneration in fibrotic rats was confirmed by the marked reduction of hydroxyproline deposition in hepatocytes. The enlisted activity of *O basilicum* L. may be attributed to its free radical scavenging and antioxidant effect.

**Balasubramaniam and Anuradha, 2011**: studied the effect of Linalool, a plant derived monoterpenic alcohol that rescues the kidney from Diabetes-induced nephropathic changes. Diabetic nephropathy (DN) is caused due to three important mechanisms; firstly, hyperglycemia induced reactive oxygen species (ROS) generated by advanced glycation end product, secondly, proinflammatory cascade activation and lastly, stimulation of mesangial cells to produce extracellular matrix proteins (ECM).
The study explains the association between cytokines and DN. Among the cytokines TNFα promotes ROS, IL-6 mediates glomerular basement membrane thickening, stimulation of TGFβ1 a prosclerotic cytokine causes upregulation of TGFβRII promoting renal deposition of extracellular matrix components like collagen I, IV and fibronectin which further stimulates hypertrophy of mesangial cells inducing renal fibrosis and glomerulonecrosis. The present study analysed the role of linalool as an anti-inflammatory and antifibrotic compound using ELISA, western blotting and reverse transcriptase PCR. On treating the diabetic induced male wistar rats with linalool for 72 hours, it was observed that linalool reduced the levels of TNFα, IL-6, TGFβ1 and NF-κB. These results were further confirmed by histopathological examination, where the major pathologic alteration observed in diabetic kidney is interstitial chronic inflammatory infiltrate; which reduced significantly on treatment with linalool. Therefore; linalool was proposed as a promising anti-inflammatory and antifibrotic adjunctive treatment modality.

Dumitriu et al., 2013: Studied modulation of TGFβ by active compounds from Salvia officinalis (Lamiaceae family). TGFβ is a pleiotropic growth factor synthesised by many cells in an organism. The main function of TGFβ is regulation of tissue homeostasis, by its chemotactic property for fibroblasts, stimulating their proliferation, upregulating ECM proteins synthesis especially collagen. In diseased conditions TGFβ has the ability to impair the balance between activation and inhibition of matrix metalloproteinases (MMP) at the gene level. On performing flowcytometry analysis it was observed that the Lamiaceae plant compound retarded fibroblast turnover, thereby affecting cell division. On performing ELISA on cell culture supernatant for hydroxyproline quantification (An indirect method to quantify total collagen quantity) and measure MMP, it was observed that reduced TGFβ
secretion, impaired collagen synthesis by upregulating MMP1 and MMP9 (gelatinases). The antifibrotic effect was imparted through enzymatic digestion of imperfect fibrillar deposits.

**Okoye et al., 2014:** evaluated the chemical composition and antiinflammatory activity of essential oils from the leaves of *Ocimum basilicum* L. (OB) and *Ocimum gratissimum* L (OG) (Lamiaceae family). These plants are widely distributed aromatic herbs and have been used for ethnomedicinal management of various inflammatory disorders. Two methods were adopted for volatile oil extraction (i) hydrodistillation and (ii) n-hexane extraction technique. It has been previously proved that anti-inflammatory activity increases with increase in the proportion of essential oils like eugenol, linalool, D-fenchone, 1-terpene-4-ol, thymol, alpha-caryophylene and the presence of diterpenes and triterpenes; these oils possess potent antiinflammatory activity. In the present study it was observed that volatile oils extracted using hydrodistillation from OB (OBV) demonstrated better antiinflammatory activity in xylene induced mouse ear edema model when compared to volatile oils extract using n-hexane extract (OBHE). OBV possessed potent antiinflammatory activity as it contained oils like linalool, eucalyptol, germacrene and borneol acetate; which have been previously reported to exhibit antiinflammatory activity. Thus considering the use of Linalool alone or the entire OB plant extract for inflammatory disorders may help curb fibrotic conditions.

**Mueller et al., 2010:** evaluated the antiinflammatory activity of extracts from fruits, herbs and spices. Inflammation plays an important role in various diseases such as rheumatoid arthritis, asthma, atherosclerosis and several other fibrotic conditions. During an inflammatory response, mediators such as proinflammatory cytokines including IL-1, TNFα, IFNγ, IL-6, IL-12, and IL-18 and granulocyte-macrophage
In-vitro human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis

Ocimum basilicum L. & Linalool

Chapter 2.3

In human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis, colony stimulating factor are released; this response is antagonised by anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IFN-α and transforming growth factor β. In the present study LPS-stimulated macrophage was used as a model for testing the various plant extracts for pro or anti-inflammatory activities. The various extracts used were basil, bay leaves, cardamom, cinnamon, ginger, oregano, sage and thyme. On ELISA testing it was observed that basil at a dosage of 0.2 and 0.5 mg/mL reduced the secretion of proinflammatory cytokines IL-6 by 25%, at a dose of 0.2% decreased the secretion of TNFα and increased the secretion of anti-inflammatory cytokine IL-10. Positive control used was cortisol; an anti-inflammatory hormone reduced the secretion of IL-6 and TNFα; and increased the secretion of IL-10 at 50 or 100 nM concentration. These findings further the idea that a diet rich in fruits, vegetables, herbs and spices may contribute to the reduction of inflammation and prove to be preventive against related diseases.
2.4 Detailed layout of *Origanum vulgare* L. subsp *hirtum* and thymol

2.4.1 **ORIGANUM, Origanum vulgare L., F.Lamiaceae**

2.4.1.1 Description

Origanum is an herbaceous plant also known as the ‘Pizza Herb’/ ‘Oregano’ belongs to the Labiatae family.

2.4.1.2 Etymology

In ancient Greece oregano was called as the ‘joy of the mountain’ and was considered as a symbol of joy and happiness.

2.4.1.3 **Subspecies of Origanum** (Chishti et al., 2013)

i. subsp. *vulgare*

ii. subsp. *glandulosum* (Desfontaines) Ietswaart

iii. subsp. *gracile* (Koch) Ietswaart

iv. subsp. *hirtum* (Link) Ietswaart

v. subsp. *viridulum* (Martrin-Donos) Nyman

vi. subsp. *virens* (Hoffmannsegg and Link) Ietswaart

2.4.1.4 **Taxonomy and scientific classification**

Oregano is a perennial herb that belongs to the genus Origanum

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2.4.1.5 Vernacular names

It has various other names such as ‘Mountain mint’, ‘Origani vulgaris herba’, ‘Wild marjoram’ and ‘Zaatar’.

2.4.1.6 Botanical source and geographical distribution

Origanum (Pizza herb) is the herb of spice which is native to Southern Europe. It is known to have a strong aroma that resembles marjoram. Cultivation of Oreganum was started in European countries and was found to grow abundantly in Mexico and is so known as the ‘Mexican sage’. It is known to be the most popular herb in Mediterranean cooking. The herb is traded both as ‘whole’ dried leaves and in ground form. In India, the plant is known to grow in temperate Himalayas from Kashmir to Sikkim at altitudes of 1500 – 3500 m (Parathasarathy et al., 2008)

2.4.1.7 Botanical description

*Origanum vulgare* L. is a branched perennial herb that grows to a height of 0.3 to 1 m tall. Its leaves are broadly ovate, it may be entirely or rarely toothed and it changes to a light green colour when dried. Its flowers are purplish red in colour which occur at the end of stems and branchlets (Parathasarathy et al., 2008)

Figure 2.13: illustrates leaves and flowers of *Origanum vulgare* L. subsp *hirtum*

(Duke, 2015)
2.4.1.8 Active chemical composition

*Origanum vulgare* L. consists of both the volatile and non-volatile phytochemical constituents. The main components of the plant comprise of gamma-terpinene (0.6 - 3.6 %), P-cymene (17.3 - 51.3 %), thymol (0.4 - 42.8%) and carvacrol (1.7 - 69.6%). It was reported that carvacrol was the dominant constituent present in oregano (Parathasarathy *et al.*, 2008).

![Chemical structure for thymol and carvacrol](image)

**Figure 2.14: Chemical structure for thymol and carvacrol**

(Kaurinovic and Popovic, 2012)

2.4.1.9 Cultivation

*Origanum vulgare* L. grows the best in temperate to sub tropical climate. Its growth is propagated by seeds, cuttings, layers or root divisions. *Origanum vulgare* L. seeds have a very small size; therefore the crop is propagated by stem cuttings planted directly in the field. *Origanum vulgare* L. plant if well nourished and looked after multiplies to grand proportions. The plant once grown, its leaves are cut at a blooming stage and dried in shade to be used as a flavouring agent. The dried leaf is approximately 1.5 cm long and light green in color. *Origanum vulgare* L. leaves are either available as crushed or ground form (Parathasarathy *et al.*, 2008).
2.4.1.10 Harvesting and processing: Traditional medicinal uses & pharmacological studies

*Origanum vulgare* L. is known to have various medicinal uses. It has antibacterial, antifungal, antimutagenic, antioxidant, anti-inflammatory, antiparasitic, antithrombin activities. It is widely used in the treatment of acne, asthma, bronchitis; topically used for athlete’s foot, canker sores, gum disease; orally used for dysmenorrheal, headaches, high blood sugar, increased insulin sensitivity, psoriasis, and rheumatoid arthritis (Lee et al., 2005).

2.4.1.11 Physicochemical Properties

**Dosages**

There is insufficient evidence supporting the administration of oregano in children. In adults a recommended dosage of 200 mg of emulsified oil three times daily with meals is prescribed for 6 weeks for infections. In the form of dietary supplement two capsules (unknown dosage) are recommended once or twice daily. A few drops of Oregano oil has been recommended to be added in milk or juice (Ulbricht, 2010).

Oregano oil is also applied topically on the skin, and is also a dominant ingredient in mouthwashes. As an antiseptic 100 g of dried oregano leaf is boiled in 1 L of water for 10 minutes and used for bathing.

**Side effects and warnings** (Ulbricht, 2010)

- Oregano should be avoided in individuals who are known to have allergy or hypersensitivity to oregano. It can lead to swelling of lips and tongue, difficulty in speaking, breathing and swelling of the face.

- Oregano can cause hypoglycaemia.

**Contraindications** (Ulbricht, 2010).

According to historical evidence oregano induces abortions; therefore it should be avoided in pregnancy and during breastfeeding.
2.4.2 **Thymol** (National Center for Biotechnology Information 2005)

2.4.2.1 **Description:**

Monoterpane phenol

2.4.2.2 **Chemical and Physical Properties**

- Chemical Names: THYMOL; 89-83-8; 2-Isopropyl-5-methylphenol; 5-Methyl-2-isopropylphenol; 6-Isopropyl-m-cresol; 3-p-Cymenol
- Molecular formulae: C_{10}H_{14}O
- Molecular weight: 150.21756 g/mol
- PubChem CID: 6989

2.4.2.3 **2D & 3D STRUCTURE**

**Figure 2.15: 2D & 3D structure of thymol**

2.4.2.4 **Identification**

- IUPAC Name: 5-methyl-2-propan-2-ylphenol

2.4.2.5 **Experimental properties**

- Physical Description: Pellets Large Crystals
- Color: Colorless, translucent crystals or plates from ethyl acetate, acetic acid or dimethyl carbonate
- Odor: Odor of thyme, Aromatic odor
- Taste: Pungent, caustic, sweet, medicinal, spicy
- Boiling point: 233 °C
- Melting point: 49.6 °C
• Flash point: 110 °C
• Solubility: In water, 900 mg/L at 20 °C
• Density: 0.9699 g/cu cm at 25 °C
• Vapor pressure: 0.016 mm Hg at 25 °C
• LogP: log Kow = 3.30
• Stability: Appreciably volatile at 100 °C; volatilizes in water vapors.
• Decomposition: When heated to decomposition it emits acrid smoke and irritating fumes.
• pH: Solution in alcohol is neutral to litmus.

2.4.2.6 Pharmacology and biochemistry

• Pharmacological activities: antimicrobial, antifungal, antioxidant and anti-inflammatory.
• Absorption, Distribution and Excretion: Thymol is readily absorbed from the gastrointestinal tract following oral administration. It is essentially excreted in the urine.
• Metabolism/Metabolites: Only small amounts of the absorbed substance undergo urinary excretion as hydroxylated compounds. Thymol is predominantly excreted unchanged and in the form of its glucuronide and sulfate conjugates.

2.4.2.7 Safety and hazards

• Flammable
• Corrosive to the skin and eye

2.4.2.8 Storage conditions

• Keep container tightly closed in a dry and well-ventilated place.

2.4.2.9 Toxicity: No human clinical trials have been performed

• In rats the toxic oral dose is 980 mg/kg.
• In mouse the toxic oral dose is 640 mg/kg.
2.4.3 Antioxidant activity of *Origanum vulgare* L. subsp *hirtum*

*Cervato et al., 2000:* stated that several peroxidative processes cause degenerative physiopathologic events such as aging, cancer, diabetes and atherosclerosis and a good pro-oxidant and antioxidant balance is essential. He tested the antioxidant properties of aqueous and methanolic extracts of *O vulgare* L. via the superoxide radical scavenging and DPPH method; he observed potent antioxidant activity of aqueous extract of *O vulgare* L. via the DPPH radical scavenging method when compared to the superoxide radical scavenging method. He attributed the demonstration of the antioxidant activity to the presence of high content of polyphenols in the herb.

*Bendini et al., 2002:* reported that ethanolic extracts of *O vulgare* L. showed potent radical scavenging activity via the DPPH assay. He attributed the antioxidant activity to the presence of phenolic compounds and flavonoids namely luteolin, herbacetin and quercetin.

*Kulisic et al., 2004:* examined the antioxidant properties of oregano essential oil by using three different methods, namely, the β-carotene bleaching (BCB) test, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and the thiobarbituric acid reactive species (TBARS) assay. The oregano essential oil (0.5 g) was fractionated on a silica gel [30-60 mm, column (length 20 cm)]. Pentane (50 mL) was used to obtain the fraction. These fractions were subjected to thin layer chromatography (TLC) on silica gel plates, in order to check results of the column; chromatography separation was used. The fraction consisted of thymol and carvacrol as phenolic constituents which exhibited potent radical scavenging activity via the three model systems due to their hydrogen donating ability. These results indicated...
that the oregano essential oil could be in use as a potential resource of natural antioxidants.

**Lee et al., 2005:** identified volatile components in Thyme vulgaris; a relative of the Orgenao genus Origanum using GC/MS and evaluated the antioxidant activity of the aromatic constituents using aldehyde/ carboxylic acid assay. The main components of the plant comprised of gamma - terpene (0.6 - 3.6 %), P-cymene (17.3 - 51.3 %), thymol (0.4 - 8%) and carvacrol (1.7 - 69.6%). The antioxidant activity of thymol, carvacrol, eugenol and 4-allylphenol in particular exhibited potent antioxidant activity which was comparable to BHT and α-tocopherol. The antioxidant activity of the volatile extract of thyme at 10 μg/mL was attributed to the most abundant component; thymol. He stated that the ingestion of the above aromatic compounds could prove to be beneficial in combating oxidative stress.

**Meizoso et al., 2008:** stated that the variation in the antioxidant activity of herbs can be attributed to various factors like temperature changes, seasonal collection of herbs, extraction procedure and solvent used for extraction. He tested the antioxidant activity of *O vulgare* L. by using subcritical water extraction technique at different temperatures via the DPPH radical scavenging activity. He observed that highest antioxidant activities were achieved at highest temperatures with maximum amount of phenolic constituents with increasing concentrations of the extracts.

**Ryszard et al., 2009:** performed a study comparing the antioxidant activity of the ethanolic extracts of thyme, oregano and marjoram via the reducing power and DPPH radical scavenging method. He observed that all the three herbs demonstrated potent radical scavenging activity in the order of oregano having the maximum activity followed by thyme and marjoram; he attributed the antioxidant activity to the...
presence of phenolic content in the herbs. The major phenolic constituents of oregano are namely thymol, carvacrol, benzyl alcohol and eugenol.

Khanum et al., 2011: stated that spices and herbs not only contribute to taste and aroma of foods but also contain a variety of bioactive molecules that have properties to quench free radicals. He also commented that the potent antioxidant activity of herbs and spices is due to the redox properties of the phenolic constituents which allow them to act as hydrogen donors, reducing agents and singlet oxygen quenchers. He tested and compared the antioxidant activity of ethanolic extracts of Origanum vulgare L., Ajowan and borage extracts via reducing power and DPPH radical scavenging method; he observed a dose dependent relationship with increase in concentration there was an increase in the antioxidant activity of both the aqueous and ethanolic extracts of O vulgare L., Ajowan and borage extracts. On comparison ethanolic extract of O vulgare L. had the maximum antioxidant activity via the DPPH radical scavenging method.

Kacaniova et al., 2012: stated that the antibacterial and antioxidant activities of herbs are essential for treating pathologies. Considering this aspect he tested the antioxidant activity of essential oil of O vulgare L. and Thyme vulgaris L. via the DPPH radical scavenging method. He observed that O vulgare L. had a higher antioxidant activity when compared to Thyme vulgaris L.; he attributed this variation to the presence of different phenolic constituents such as rosmarinic acid, thymol and carvacrol in O vulgare L.
2.4.4 Anti-inflammatory and antifibrotic activity of *Origanum vulgare* L. subsp *hirtum* and thymol

*Bodirlau et al., 2009:* enlisted the anti-inflammatory constituents from different plant species. His work states different phases of inflammation; an acute phase, subacute phase and chronic proliferative phase. Traditional medicinal plants have putative therapeutic effects which can be ascribed to compounds such as flavonoids; they are polyphenolic compounds distributed in the plant kingdom. Flavonoids inhibit key enzymes involved in inflammation and cell signalling pathways such as cyclooxygenase and lipooxygenase, protein kinase C and phosphoinositide 3-kinase (PI3Kinase). They exert a wide range of effects on the biological system; they are known to have antimicrobial, antiviral, antiulcerogenic, antineoplastic, hypolipidemic, antihepatotoxic, antiallergic and anti-inflammatory activities. Flavonoids are linked with sugar moieties and occur as glycosides. *O vulgare* L. belongs to the Lamiaceae family; it’s essential oil contains two important polyphenols carvacrol and Thymol which exhibit anti-inflammatory activity by their capacity to inhibit the production of cytokines (IL-6, IL-1β, TNFα) and prostaglandin at the inflammatory site.

*Mueller et al., 2010:* evaluated the anti-inflammatory activity of extracts from fruits, herbs and spices. Inflammation plays an important role in various diseases such as rheumatoid arthritis, asthma, atherosclerosis and several other fibrotic conditions. During an inflammatory response, mediators such as proinflammatory cytokines including IL-1, TNFα, IFNγ, IL-6, IL-12, and IL-18 and granulocyte-macrophage colony stimulating factor are released; this response is antagonised by anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IFN-α and transforming growth factor β. In the present study LPS-stimulated macrophage was used as a model for testing the various plant extracts for pro or anti inflammatory activities. The

*In-vitro* human buccal fibroblast cell line model for screening antifibrotic activity of plant compounds in Oral Submucous Fibrosis
various extracts used were basil, bay leaves, cardamom, cinnamon, ginger, oregano, sage and thyme. On ELISA testing it was observed that oregano at a dosage of 0.5 mg/mL reduced the secretion of proinflammatory cytokines IL-6 by 25%, at a dose of 0.5 mg/mL decreased the secretion of TNFα and increased the secretion of anti-inflammatory cytokine IL-10. Positive control used was cortisol; an anti-inflammatory hormone that reduced the secretion of IL-6 and TNFα; and increased the secretion of IL-10 at 50 or 100 nM concentration. These findings further the idea that a diet rich in fruits, vegetables, herbs and spices may contribute to the reduction of inflammation and prove to be preventive against related diseases.

Riella et al., 2012: investigated the anti-inflammatory activities of Thymol, a monoterpane of the essential oil from Lippia gracilis in rodents. Natural therapy or alternative medicine is an attractive approach for the treatment of several inflammatory disorders. Carrageenan-induced hind paw edema model and carrageenan-induced peritonitis was used to measure the anti-inflammatory effect of thymol; it was observed that thymol at 100 mg/kg reduced carrageenan induced paw edema at 2,3 and 4 hours, it inhibited 35.3% of edema response as compared to 47.1% of dexamethasone and also prevented leukocyte influx at the site of injury. In the second model it was observed that thymol at 10, 30 and 100 mg/kg evoked significant inhibition on carrageenan induced peritonitis. The study performed by Reilla et al. demonstrated that thymol exhibits anti-inflammatory effects and if incorporated in collagen based dressings would help in improving wound healing successfully. It was suggested that Thymol is a promising compound to be used in the treatment of inflammatory process and scar management.

Ershun et al., 2014: studied the effect of thymol on allergic airway inflammation on ovalbumin induced mouse asthma and compared its efficacy to dexamethasone.
Thymol has previously been reported as an antimicrobial and antiseptic agent against oral bacteria and also has wound healing properties. It was observed that pre-treatment with thymol in ovalbumin treated mice significantly reduced the number of inflammatory cells and TH2 cytokines (IL-4, IL-5 and IL-13) in a dose dependent manner at (4 mg, 8 mg and 16 mg/kg b.w) when compared to control group; dexamethasone showed an effective reduction at a concentration of 2 mg/kg body weight. Thymol pre-treatment also helped in inhibiting the activation of NF-κB that plays a very important role in activating proinflammatory cytokines in allergic inflammation.

**Liang et al., 2014:** studied the anti-inflammatory potential of thymol in LPS stimulated inflammatory response in mouse mammary epithelial cells. Thymol a natural monoterpene phenol is largely present in oregano, thyme and tangerine peel. To measure the cytokine levels ELISA was used; thymol pre-treatment (10, 20 & 40 µg/mL) for 24 hours significantly reduced TNF-α, IL-6 and IL-1β levels; on using western blotting it was observed that the expression of iNOS and COX-2 was also suppressed by thymol in a dose dependent manner. It also blocked the phosphorylation of IκBα, NF-κB, p65, ERK, JNK and p38 mitogen activated protein kinases (MAPK). Thereby proving the potential of thymol as a therapeutic anti-inflammatory agent and can be used in mastitis.

**Piva et al., 2015:** tested a microencapsulated mixture of citric acid and sorbic acid (OA) and thymol and vanillin (PB) on intestinal integrity and inflammation of weaned pigs. On treatment on OA+PB significantly reduced the mRNA for TNFα, IL-6, IL-1β and TGFβ1 simultaneously increasing the anti-inflammatory cytokine IL-10. OA+PB induced rapid mature proliferation of the intestinal mucosa by decreasing the local and systemic inflammatory responses.
Ku and Lin, 2013: investigated the effects of terpenoid fractions on cytokine profile secretion, terpenoids have earlier been reported as anti-oxidant, anti-inflammatory and antitumor agents. Among the 27 terpenoids selected, two were monoterpenoids namely linalool and thymol, the purity of which was above 95%. Earlier thymol from Lippia multiflora has been reported for its antioxidant, antimicrobial and anti-inflammatory activities, whereas linalool from Ocimum sanctum has also been reported for its potent anti-inflammatory activities. The effect of linalool and thymol was evaluated on cytokine secretion using murine primary spleenocytes, lipopolysacchride (LPS) an endotoxin was used as a positive control. The IC<sub>50</sub> determined was more than 500 µM. On treatment with the non-toxic concentrations of linalool and thymol for 48 hours, it was observed that both the mono terpenoids had an inclination towards Th2 cells. They significantly increased the anti-inflammatory cytokine (IL-10), thereby decreasing pro-inflammatory cytokines; IL-1 and TNFα produced from Th1 cells as a result of LPS treatment. The present study concluded that both linalool and thymol are potent anti-inflammatory agents in vitro.

Queiroz et al., 2012: evaluated the anti-inflammatory effects of Thyme vulgaris oil (TEO), its isolated constituents thymol and carvacrol (CVL) in a carrageenan induced pleurisy model, which produces a known mediator of inflammation; TNFα. On treatment with 400 mg/kg b.w both thymol and CVL significantly inhibited pleural inflammatory exudate by 47.3% via inhibiting leukocyte migration.

Javadian et al., 2015: reported and compared the anti-inflammatory activities of methanolic extract (ME) of oregano vulgare (OV) and it’s most important constituent thymol on activated microglial and mixed glial cells. On analysis, it was observed that both the ME of OV and solution of thymol exhibited strong anti-inflammatory activity through inhibition of iNOS and TNFα expression. Thymol was effective at 0.15
mg/mL and the leaves of ME of OV at 2.25 mg/mL effectively. The study concluded that extracts of plants exhibit activity at higher concentrations when compared to pure compounds.

**Ocana-Fuentes et al., 2010:** performed an anti-inflammatory study on two fractions (S1 and S2) of Oregano vulgare obtained by supercritical fluid extraction on activated human THP-1 macrophages (cellular model for atherogenesis). The supercritical oregano extracts at a concentration of 30 µg/mL significantly decreased the pro-inflammatory cytokine TNFα, IL-1β and IL-6 simultaneously increasing the production of anti-inflammatory cytokine IL-10.

**Saravanan and Leelevinothan, 2016:** investigated the effect of thymol against high fat diet induced diabetic nephropathy in C57BL/6 mice. Intragastric administration of thymol (40mg/kg b.w) subsequently for 5 weeks significantly decreased the urinary, blood parameters and body weight; it also inhibited the activation of TGFβ1 and vascular endothelial growth factor (VEGF) thereby preventing glomerulosclerosis, hyperlipidemia, oxidative stress and subsequent renal injuries. Histopathological study demonstrated a significant reduction in ECM mesangial matrix expansion.
**Figure 2.16: Proposed pathway: Inflammation and Fibrosis link in Oral Submucous fibrosis**

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

**Initial stages of OSMF:**
- Acute inflammation

**Keratinocytes**
- IL-1α
- KGF
- IL-6, IL-8, IL-1β
- TNFα
- PDGF
- TGFβ
- Collagenases

**Arecanut**

**Lymphocyte activation**

**Stimulation of fibroblasts**
- Smad dependent pathway
- Smad independent pathway
- Cross talk
- P38 pathways
- C-JUN Pathway
- Ras/ERK pathway

**Macrophage activation**
- Digestion of Arecanut components
- Phagocytosis → ROS

**Advanced stages:**
- Persistent chronic stimuli:
  - Chronic inflammation

**Upregulation of LOX**
- Excessive collagen crosslinking
- Resistant degradation
- Excessive accumulation

**COMBINED EFFECT OF ACTIVATED MECHANISMS**
1. Increased collagen I and III accumulation
2. Decreased Degradation of ECM
3. Hyalinization of Connective tissue
4. Compressed and engorged blood vessels

**FIBROSIS**