Chapter - 5

Anti-wrinkle potential of standardized leaf extract of *Clitoria ternatea*

5.1. *Clitoria ternatea* – A profile
5.2. Therapeutic uses of *Clitoria ternatea*
5.3. Phytochemical profiles of *Clitoria ternatea*
5.4. Inhibition potential of *Clitoria ternatea* on hyaluronidase, elastase and MMP-1
5.5. RP-HPLC standardization of *Clitoria ternatea* extract
5.6. Statistical analysis
5.7. Results and discussion
   5.7.1. Inhibition potential of *Clitoria ternatea* on hyaluronidase, elastase and MMP-1
   5.7.2. RP-HPLC standardization of *Clitoria ternatea* extract
5.8. Conclusion
5.9. Publications
5.1. *Clitoria ternatea* – A profile

**Scientific classification**

<table>
<thead>
<tr>
<th>Kingdom: Plantae</th>
<th>Vernacular names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division: Magnoliophyta</td>
<td>English: Butterfly pea</td>
</tr>
<tr>
<td>Class: Magnoliopsida</td>
<td>Sanskrit: Aparâjita, Shankapushpi</td>
</tr>
<tr>
<td>Order: Fabales</td>
<td>Hindi: Aparajit</td>
</tr>
<tr>
<td>Family: Fabaceae</td>
<td>Bengali: Aparajita</td>
</tr>
<tr>
<td>Genus: <em>Clitoria</em> L.</td>
<td>Tamil: Sangu pu</td>
</tr>
<tr>
<td>Species: <em>Clitoria ternatea</em> Linn</td>
<td>Telugu: Dintena</td>
</tr>
<tr>
<td></td>
<td>Marathi: Gokarna</td>
</tr>
</tbody>
</table>

5.1.1 Plant description

*Clitoria ternatea* plant (Figure 5.1A) is native to tropical equatorial Asia, but has also been introduced to Africa, Australia and America. It seems to be cultivated in Caribbean island, Central America and México and widely distributed to the Indian subcontinent. It is cultivated and naturalized throughout the humid tropics of the old and new world below 1600 m elevation. It is an ornamental perennial herbaceous plant, grows as a vine or creeper in moist neutral soil. Extremely well adapted to heavy clay alkaline soils, and especially on clay soils with pH 4.5-8.7. The most prominent feature about this plant is leaves are elliptic and obtuse and flowers are bright deep blue color, solitary, with light yellow or white markings. There are some varieties that yield white flowers. The fruits are 5-7 cm long, flat pods with 6 to 10 seeds in each pod (Figure 5.1B). There are more than 50 species of *Clitoria* is available, but due to economic potential, the mostly frequently cultivated species is *C. terneata*. In Ayurveda, the roots, seeds and leaves have long been widely used as a brain tonic and is believed to promote memory, intelligence and several other ailments (Mukherjee et al., 2008). *C. ternatea* stems fine twining, sparsely pubescent, sub-erect at base, 0.5-3 m long. Leaves pinnate with 5 or 7 leaflets; petioles 1.5-3 cm long; stipules persistent, narrowly triangular, 1-6 mm long, prominently 3-nerved; rachis 1-7 cm long; leaflets elliptic, ovate or nearly orbicular, 1.5-5 cm long, 0.3-3 cm wide, with apex acute or rounded, often notched, and rounded, both surfaces sparsely apprised pubescent. Flowers axillary, single or paired; colour ranges from white, mauve, light blue to dark blue; pedicles 4-9 mm long, twisted through 180° so that the standard is inverted. Calyx 1.7-2.2 cm long with a few fine hairs; tube campanulate, 0.8-1.2 cm long; lobes triangular or oblong, 0.7-1 cm long, acute or acuminate. Standard obovate, funnel-shaped, 2-5.5 cm long, 2-4 cm wide, notched or rounded at apex, blue with a pale yellow base, or entirely white, a few fine hairs at apex (Staples, 1992).
5.2. Therapeutic uses of \textit{C. ternatea}

According to the ancient texts on traditional and herbal medicines, especially in Ayurveda and other Indian and Asian systems of medicine, various parts of Clitoria have therapeutic value as a memory enhancer, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent. It has medicinal as well as food value. Decoction and the paste of whole plant are used in bleeding piles due to its haemostatic action and to prevent pus formation in external wounds. Leaf juice is used as nasal drops in headache, alleviates swelling and pain in rheumatoid arthritis. The paste of leaves externally applied over skin diseases and simultaneously consumed orally, powdered fried seeds in ghee with hot water. It has great benefit in combined with little salt applied in retro-auricular adenitis. The seeds powdered mashed with honey, applied topically, in tonsillitis render excellent relief. In Ayurveda, the juice of root used in migraine, and used for treatment of whooping coughs and the extracts from the white-flowered plants are used for the treatment of goiter. It is used in common cold, cough, asthma as it acts as an expectorant and reduces the irritation of respiratory organs. It is also used as febrifuge and refrigerant for cooling effect. The fresh juice of leaves combined with ginger juice, effectively controls the excessive sweating (Mukherjee et al., 2008; Kirtikar and Basu, 1935). It has antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anaesthetic, antidiabetic, insecticidal properties etc. Aqueous extract and alcoholic extract of roots and aerial parts have been reported as learning and memory enhancing, enhance acetylcholine content. Anthocyanins (Ternatin A1, A2, B1, B2, D1, and D2) isolated from flower was reported to inhibit platelet aggregation and vascular smooth muscle relaxant properties. Finotin, a flavonol glycoside from ethyl acetate soluble fraction of seed extract has been reported as antimicrobial agent (Mukherjee et al., 2008). Leaf extract has been reported to have hepatoprotective activity against paracetamol induced hepatotoxicity (Nithianantham et al., 2011).

5.3. Phytochemical profiles of \textit{C. ternatea}

Taraxerol [1] and taraxerone [2], two pentacyclic triterpenoids have been isolated and identified from the roots (Banerjee and Chakravarti, 1963; 1964). Content of Taraxerol in root was
determined through high performance thin layer chromatography (HPTLC) by Kumar et al. (2008). A flavon glycoside, 3,5,4′- trihydroxy-7-methoxyflavonol-3-O-β-d-xylopyranosyl-(1,3)-O-β-d-galactopyranosyl (1,6)-O-β-d-glucopyranoside isolated from the ethyl acetate soluble fraction of the defatted seed (Yadava and Verma, 2003). The seeds contain a water-soluble mucilage, delphinidin 3, 3′, 5′-triglucoside useful as a food dye, p-hydroxycinnamic acid, flavonol-3-glycoside, ethyl-α-d-galactopyranoside and 3, 5, 7, 4′-tetrahydroxyflavone. Leaves contain β-sitosterol, kaempferol-3-monoglicoside, kaempferol-3-rutinoside, kaempferol-3-neohesperidoside, Lactones aparajitin and clitorin. Ternatins are blue anthocyanins found in the petals. The six major anthocyanins ternatins A1, A2, B1, B2, D1, and D2, were isolated, and these structures were characterized as malonylated delphinidin 3, 3′, 5′-triglucosides having 3′, 5′-side chains with alternative d-glucose and p-coumaric acid units (Mukherjee et al., 2008).

Figure 5.2. Major phytoconstituents of C. ternatea

5.4. Inhibition potential of C. ternatea on hyaluronidase, elastase and MMP-1

ROS and free radicals are generated due to chronic UV irradiation to skin causes degenerative inflammatory processes largely mediated by the overproduction of dermal enzymes (hyaluronidase, elastase, matrix metalloproteinase) (Aquino et al., 2002). These dermal enzymes utilize the glycosaminoglycan; hyaluronan, dermatin sulfate, elastin, collagen fibers as substrate, which are the principal components of extracellular matrix [ECM] of skin. This ultimately leads to degradation ECM to form wrinkle over skin. There are several other
environmental factors such as pollution, chemicals, reagents, and genetic predisposition causes excessive generation of ROS leading to aged skin. Therefore, the deleterious effects of ROS in the skin have focused on the establishment and evaluation of antioxidants to enrich the endogenous cutaneous protection system to prevent UV irradiation induced skin damage. Hence, it is of interest to search new skin-care cosmeceuticals from natural resources which have inhibitory potential on these enzymes and could be used as anti-wrinkle agent in skin aging. *C. ternatea* leaf contains large number of bioactive compounds may possess antioxidants to prevent UV-induced skin damage. Traditionally the *C. ternatea* (CT) have been employed as anti-pyretic, anti-inflammatory, analgesic, local anesthetic, anti-diabetic, applied externally for skin diseases and several other activities (Mukherjee et al., 2008). This study investigated the potential use of *C. ternatea* leaf extract to prevent UV irradiation-induced dermal enzymes *in-vitro*. *C. ternatea* leaf has been selected to evaluate the anti-wrinkle activity depending upon previous pharmacological and phytochemical reports. In this experiment the leaf of *C. ternatea* was screened for anti-wrinkle activity in comparison to oleanolic acid, which is a pentacyclic triterpene found in several botanical extract to have anti-inflammatory, chemopreventive, skin protective property (Liu, 1995; Aguirre et al., 2006; Juan et al., 2008). It has been well established that plant extracts containing oleanolic acid are useful in skin care cosmetics. Oleanolic acid has regenerative properties in case of sun damaged skin. The damaged collagen fiber bundle can be restored by topical application of this pentacyclic triterpene, which has been used as anti-wrinkle ingredient in cosmetic formulations (Nishimori et al., 1997). These evidences suggested us to select oleanolic acid as standard reference compound in this experiment. In this experiment methanol extract (CTMeOH), ethyl acetate fraction (CTEA), n-butanol fraction (CTnB) and aqueous fraction (CTAQ) of leaf were screened for *in-vitro* hyaluronidase, elastase (UV spectrophotometric) and MMP-1 (fluorescence) enzymes inhibition assay and the activities were correlated with respect to the phytochemical standardization (RP-HPLC) using taraxerol as marker compound isolated from the leaf extract of *C. ternatea*. The MMP-1 fluorescence assay is more precise and sensitive compare to spectrophotometric assays, which uses a difference in the fluorescence intensity of fluorescein conjugated substrate from product to measure the enzymatic reaction.

### 5.4.1. Collection and authentication of plant materials

*C. ternatea* leaves were collected locally and authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants & Collection Unit, Ooty, Tamilnadu, India. A voucher specimen (specimen no. SNPS-JU/2010/1068) has been submitted to School of Natural Product Studies, Jadavpur University, Kolkata, India. (Figure 5.3)
5.4.2. Instruments and chemicals
All solvents and reagents used were of analytical grade. Human leucocyte elastase (HLE), hyaluronidase from bovine testes, hyaluronic acid (HA) potassium salt from human umbilical cord, N-succinyl-(Ala)₃-p-nitroanilide, oleanolic acid had been purchased from Sigma chemical (USA). Type-I collagen fluorescein conjugate (substrate) from bovine skin and MMP-1 were purchased from Invitrogen BioServices India Pvt. Ltd. Spectrophotometric measurements were performed with 96 well micro plate reader (BIO-RAD, Model: 680-XR) and fluorescence was measured by 96 well microplate reader (BioTek, FLx 800T, USA).

5.4.3. Extraction and fractionation
1 Kg of fresh leaves were crushed and kept for cold maceration with 95.5% methanol for 72 h. The extract was filtered through a nylon mesh and concentrated using rotary evaporator (EYELA, Tokyo, Japan) at a temperature not exceeding 45°C. The recovered solvent was again mixed with the same plant material and kept for 48 h. The process was repeated for another two times and the combined extract was lyophilized to obtain powder (yield 1.6 % w/w), which was stored in a vacuum desiccator (Table 5.1). The lyophilized extract was dissolved in water for successive fractionation with ethyl acetate and n-butanol and then all the fractions were again lyophilized. The C. ternatea methanol extracts (CTMeOH), ethyl acetate fractions (CTEA), n-butanol fractions (CTnB) and aqueous fractions (CTAQ) were used as test samples along with standard oleanolic acid for the enzyme assay.

<table>
<thead>
<tr>
<th>Extraction:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold maceration</td>
</tr>
<tr>
<td>Methanolic extract (CTMeOH)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate (CTEA)</td>
</tr>
<tr>
<td>n-Butanol (CTnB)</td>
</tr>
<tr>
<td>Aqueous (CTAQ)</td>
</tr>
</tbody>
</table>

Table 5.1. Plant material extraction and fractions

5.4.4. Hyaluronidase inhibition assay
Hyaluronidase inhibitory assay was performed by the method described previously using 96 well microplate (Kim et al., 1995). Hyaluronidase reacts with the substrate hyaluronic acid to release N-acetyl glucosamine. In presence of any inhibitor, the release of N-acetyl glucosamine is reduced and it is monitored by measuring the absorbance at 600 nm. The procedure has been explained in chapter 3.

For calculation of percentage of enzyme activity, the following formula was used:
% Enzyme activity = (100%) – {A600 nm of hyaluronic acid + hyaluronidase/A600 nm hyaluronic acid × 100}
5.4.5. Elastase inhibition assay
The elastase inhibition assay was performed by UV-Visible spectrophotometer according to the method of (Kraunsoe et al., 1996). The release of p-nitro aniline due to proteolysis of N-succinyl-(Ala)₃-p-nitroanilide by human leucocyte elastase in the presence or absence of inhibitor was monitored by measuring the absorbance at 410 nm. The procedure has been explained in chapter 3.

The percentage of inhibition was calculated as:
Inhibition (%) = (1 – B / A) × 100; where A is the enzyme activity without the sample and B is the activity in the presence of the sample.

5.4.6. Assay for MMP-1 inhibitory activity
The assay was performed according to the previous method with minor alteration using 96 well fluorescence microplate (Losso et al., 2004). When MMP-1 reacts with type-I collagen substrate, it causes collagenolysis but, in presence of inhibitor the reaction becomes slowed down due to inhibition of MMP-1. The procedure has been explained in chapter 3.

A high collagenolytic activity of MMP-1 is associated with high fluorescence reading and equally a low collagenolytic activity of MMP-1 is associated with low fluorescence reading.

5.4.7. Bio-assay guided isolation
Taraxerol (yield 5.27 % w/w) was obtained from ethyl acetate fraction [CTEA] by conventional column chromatography. Ethyl acetate fraction was subjected to column chromatography over silica gel (mesh size 230-400) using gradient elution technique. Approximately 5 g of ethyl acetate fraction was mixed with the mixture of solvent (chloroform/pet ether, 1:1) and the solution was mixed with 10 g of silica gel and kept for drying overnight. About 50 g of silica gel was mixed with solvent (pet ether/chloroform, 75:25) and the slurry was poured into the column and the column was packed with same solvent system. Then the overnight dried silica gel was mixed with solvent (pet ether/chloroform, 75:25) and the slurry was poured into the same column. Then the column was gradually eluted with pet ether/chloroform (75:25, 50:50, 45:55, 40:60, 30:70 and 100% Chloroform) solvent mixture. Fractions obtained from the pet ether/chloroform (45:55 and 40:60) collected together and this fraction gave single spot over TLC (mobile phase was optimized as pet ether/chloroform, 70:30) & single peak upon HPLC (mobile phase acetonitrile:water, 86:14 v/v). This fraction was further purified with activated charcoal and re-crystallized with methanol at 60°C. Further the compound was treated with activated charcoal and dissolved in methanol and boiled at water bath. Then the charcoal was filtered out and the clear solution was evaporated to produce a white crystalline powder, which has been characterized by Mass spectroscopy (Figure 5.4).
Figure 5.4. Mass spectra of isolated taraxerol

The structural characterization of the crystal was performed by LC-MS spectra showed $M^+$ ion peak with $m/z$ 449.59 and molecular formula was calculated as $C_{30}H_{50}O$, which was very much reassembled when compared with spectral data of taraxerol isolated earlier from *C. ternatea* root extract in our laboratory. Mass fragmentation analysis of the isolated taraxerol was performed by using TOF MS-ES$^+$ mass spectroscopy instrument. Electrospray ionization, (ESI) was used for analysis, mass-to-charge ratio [$m/z$; relative intensity]: 449 [M+23]$^+$ [100%], 301 [45%], 413[M-CH$_3$][35%], 185[40%], 233[8%], 384[10%] are indistinguishable from authentic taraxerol (Kumar et al., 2008).

5.5. RP-HPLC standardization of *C. ternatea*

Therapeutically potential bioactive compounds from plants depend on availability from wild sources and are present with greatest challenges for ensuring consistent quality product upon efficient extraction, herb quality, less decomposition of analytes during the extraction process. Since last few decades an increasing number of evidences have been found to adopt the quality control tool to secure clinically useful and safe herbal medicine is of great interest and challenge to the scientific community. The consistency of techniques in extraction, drying, evaporation and processing steps, results in commercial production of consistent product with less quality variation (Mukherjee, 2002a; Mukherjee and Verpoorte, 2003). Since a plant extract contains complex mixture of bioactive moieties, it is always reliably cannot be quantified. Therefore, marker analysis and standardization of the medicinal plant is of great importance to maintain quality, safety and efficacy (Houghton and Mukherjee, 2009). Thus, schedule quality control
analysis has recently become very important to control and assure the quality of the herbal products for their pharmacological importance and application in the food and pharmaceutical industry. The challenge for herbal drug manufacturers is to exemplify the herbal ingredients in such a way that enables consistent production, efficacious finished products and reduces batch to batch variation. Standardization with analytical techniques such as high-performance liquid chromatography assures consistent quality of the final product (Mukherjee et al., 2006b). Therefore, RP-HPLC method was used to standardization and quantification of bioactive marker in C. ternatea leaf methanol extract. This experiment was designed to develop a RP-HPLC method for the estimation of taraxerol in C. ternatea leaf. According to the method of estimation, this is a suitable method for the quality control of major content of C. ternatea leaf that can be used by pharmaceutical and food industries.

5.5.1. Chemicals and reagents
Solvents used for chromatography were HPLC grade. Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. Taraxerol standard marker compound, which has been used here, was isolated by the bioactivity guided isolation technique from the C. ternatea leaf extract in our laboratory and characterized and confirmed by spectroscopy study and described above.

5.5.2. HPLC instrument and chromatographic conditions
HPLC system consisted of a Waters (Milford, MA, USA) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (USA) with a sample loop of 20 µL, a Waters 2489 UV-Vis dual wavelength detector and the max-plot containing the peaks were obtained using Empower™2 software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) acetonitrile: (channel B) water (86:14, v/v) by means of isocratic elution (1 mL/min) and detection at 210 nm. Analysis of the taraxerol, methanol extract and fractions were performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

5.5.3. Standard & sample solution
A primary stock solution (1 mg/mL) containing taraxerol was prepared by dissolved in mobile phase and subsequently diluted to 10-1000 µg/mL and was used for mobile phase optimization. Methanol extract of C. ternatea was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of taraxerol present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.
5.5.4. Calibration curve (linearity)
The linear calibration plots of taraxerol was constructed by means of linear regression analysis between peak areas and concentrations after triplicate injection \((n=3)\) of the prepared standard solutions \((10-1000 \mu\text{g/mL})\) to distinguish the reproducibility of the detector response. Peak areas of the extract obtained from the chromatography were plotted against the concentration of the extract. The amount of each standard compound in the solution was obtained by linear regression analysis of peak areas in chromatograms within the linear range of the detector.

5.5.5. Specificity
Chromatograms of taraxerol and methanol extract and fractions were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

5.5.6. Robustness
The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

5.6. Statistical analysis
The IC50 values were expressed as mean ± SEM. Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Dunnett test and \(^*P < 0.05\) was considered as indicative of significance difference, as compared to the standard (oleanolic acid). All calculations were performed using Graph Pad Prism (version 5.0).

5.7. Results and discussion
It has been reported that repetitive exposure to UV radiation accelerates the synthesis of proteolytic enzymes present in dermis such as elastase, hyaluronidase and MMP-1. Skin aging is associated with such incidence where ECM of skin degraded by these enzymes. ECM is consists of elastin, hyaluronan and collagen fibers, which builds fiber network to hold the tensile strength of the skin. Degradation of ECM leading to loss of skin elasticity and decreasing the capacity of the skin to hold water, which are implicated in formation of the most obvious symptom of aging skin wrinkle \((\text{Gilchrest, 1990})\). Herbal extract, vitamins and antioxidant food supplements have created the most promising and widely accepted treatment of skin aging. Botanicals are able to scavenge free radicals from skin cells and reduces the level of dermal enzymes to restore skin elasticity and thereby slowdown the process of wrinkle \((\text{Kim et al., }\)
2007). Indeed, herbal anti-wrinkle or anti-aging formulations reverse the effects of skin aging and help people to live longer, healthier, happier lives. Topical herbal formulation can be used as a solo treatment to improve upon and maintain the desired anti-aging effects of skin. Naturally occurring polyphenols have been reported to have good anti-hyaluronidase and anti-elastase activity such as plants from Jeju Island (Kim et al., 2009; Kim et al., 2007; Kim et al., 1995). Chemo-profiling and standardization of herbals are being used as potential cost-effective, simple and highly selective tool that can ensure both reproducible quality and batch-to-batch variation of the products (Mukherjee, et al., 2008a). In this experiment methanol extract (CTMeOH), ethyl acetate fraction (CTEA), n-butanol fraction (CTnB) and aqueous fraction (CTAQ) of leaf were screened for in vitro hyaluronidase, elastase (UV spectrophotometric) and MMP-1 (fluorescence) enzymes inhibition assay and the activity was validated through RP-HPLC standardization using taraxerol as biomarker. The extract and fractions were prepared at different concentrations (1.56-50 µg/mL) and examined in triplicate (n = 3) for their inhibitory activities by monitoring the absorbance and fluorescence intensity. The percentage inhibition of the enzyme activity due to the presence of increasing test sample concentration was calculated and inhibition curves were obtained for each compound by plotting the percent inhibition versus the inhibitor concentration. The linear regression parameters were determined for each curve and the IC50 values were extrapolated.

5.7.1. Inhibition potential of C. ternatea on hyaluronidase, elastase and MMP-1

Photosensitization is a widely occurring phenomenon in biological systems due to the ubiquitous nature of solar ultraviolet light and a number of endogenous and exogenous factors. The photosensitization generates several ROS, which triggers the synthesis of dermal enzymes hyaluronidases, elastase, MMP-1 etc. These enzymes play a pivotal role in the degradation of ECM of a skin. The inflammatory possess is initiated by depolymerization of hyaluronan, hydrolyze elastin fiber and MMP-1 particularly breakdown the type I collagen (Seo and Chung, 2006). These are responsible for increased tissue permeability, inflammation progress and delayed wound healing. Therefore, there is a need to develop suitable formulations that can prevent photo-induced biological damages and inhibit the enzymatic factors in the process of photo-aging. Several experimental studies have supported that the daily external application of C. ternatea extract alleviate the skin diseases (Mukherjee et al., 2008). From the result obtained in this study, we can positively conclude that C. ternatea leaf will enhance the collagen production significantly and will contribute to the repair mechanism of skin wrinkle of sunscreen formulation.
Figure 5.5. Hyaluronidase inhibition potential of *C. ternatea*

*C. ternatea* extract and fractions were diluted at different concentrations and screened in triplicate (n = 3) for the inhibitory activities on elastase, hyaluronidase and MMP-1 compared to standard oleanolic acid, which is a pentacyclic triterpene found in several botanical extract and is also responsible to inhibit the dermal enzymatic process during skin wound (Wei et al., 2011; Facino et al., 1995).

Figure 5.6. Elastase inhibition activity of *C. ternatea*

The % of inhibition of each enzyme due to the presence of test samples concentrations was calculated. The inhibition curve of each enzyme was obtained by plotting the % inhibition (Y-axis) versus the test sample concentration (X-axis). The linear regression parameters were determined for each curve and the IC$_{50}$ values were extrapolated.

Figure 5.7. MMP-1 inhibition study of *C. ternatea*
The CTMeOH extract, CTEA and CTnB fractions showed significant ($^{a}P < 0.001, \ ^{b}P < 0.01$) hyaluronidase (IC$_{50}$ 18.08 ± 0.46, 28.01 ± 0.48 and 38.84 ± 0.41µg/ml) inhibition respectively compared to oleanolic acid (IC$_{50}$ 41.51 ± 0.50 µg/ml) (Figure 5.5). Lesser the IC$_{50}$ value of the test sample compare to the standard signifies the higher inhibitory activity of the test samples. Whereas, the elastase inhibition of all the test samples were insignificant compared to oleanolic acid (IC$_{50}$ 9.61 ± 0.36 µg/ml) (Figure 5.6). The inhibition of MMP-1 is the most an effective therapy to improve the structure of type I collagen in ECM and to alleviate active inflammation in the process of photo-aging. Therefore, regulation of MMP-1 activities may help regulate ECM turnover and reduces the level of dermal enzymes to restore skin function and thereby slowdown the process of wrinkle formation. The fluorescence MMP-1 inhibition was expressed in terms of total MMP-1 activity in Y-axis and concentrations of inhibitors are in X-axis. A high collagenolytic activity was associated with high fluorescence reading and equally a low collagenolytic activity is associated with low fluorescence reading (Losso et al., 2004). The CTMeOH extract and CTEA fraction showed significantly ($^{a}P < 0.05, \ ^{b}P < 0.01$) higher MMP-1 inhibition with less fluorescence reading compared to oleanolic acid (Figure 5.7). The hyaluronidase, elastase and MMP-1 inhibitory activity of C. ternatea extract and ethyl acetate fraction were found to be more than oleanolic acid. It may be due to the presence of different compounds, which may have synergistic effect. These enzymes are the prime targets in screening of new leads, which could inhibit the process of skin aging through the modulation of wide variety of signaling pathways and pathological processes by these enzymes. The hyaluronidase, elastase and MMP-1 inhibitory activity of CTMeOH extract was found to be more than oleanolic acid. MMP-1 inhibitory activity of CTEA fraction was more compare to CTMeOH extract and CTnB fraction. CTnB fraction showed almost similar activity like oleanolic acid. Whereas the elastase inhibitory activity of all the test samples was insignificant compare to oleanolic acid. This may be due to the presence of different array of compounds are present in the extract which have synergistic effect and the compounds have been enriched into fractions.

5.7.2. **RP-HPLC standardization of C. ternatea**

The HPLC standardization of the extract and fractions were performed with respect to isolated taraxerol as biomarker through the calibration curve. The content of taraxerol in C. ternatea was determined using calibration curve plotted between mean peak area (Y-axis) and concentration (X-axis). Linearity was evaluated by regression analysis using 5 different concentrations of the standards (100-800 µg/mL).
The coefficient of determinants ($r^2$) was 0.9958 and $y = 1.01e + 004x + 1.14e + 005$ for taraxerol (Figure 5.8), which represents that the data is closest to the line of best fit. Chromatogram was found to be directly proportional to concentrations of the calibration solutions. Retention time of taraxerol was found to be 14.00 min. (Figure 5.9) and the contents were found to be 5.32 % w/w.

The standardization method provides good resolution and separation of taraxerol from other constituents of *C. ternatea* methanol extract (Figure 6.0).
The taraxerol content in fractions were found to be 4.55 % w/w in ethyl acetate fraction and 0.43 % w/w in n-butanol fraction respectively (Figure 6.1 and 6.2), whereas aqueous fraction did not show any taraxerol peak.

Figure 6.2. HPLC chromatogram of n-butanol fraction of *C. ternatea*; the separated taraxerol showed specific peaks.

Retention times of standard was highly repeatable, with %RSD < 2% even at high concentration. A better separation of the taraxerol marker in the extract was noted by the peak purity analysis. Robustness of the experimental procedure was found to be in the range of acceptability as there was not much deviation. It can be concluded that the method of qualitative and quantitative standardization of *C. ternatea* using taraxerol was properly validated in respect of linearity, specificity, peak purity, and robustness. The specificity test of the proposed method demonstrated that other constituent presents in the methanol extract of the *C. ternatea* do not interfere with peak of interest identified as taraxerol. Furthermore, well shaped peaks indicate the specificity of the method. This RP-HPLC standardization protocol has been developed for the identification and quantification of taraxerol. The method is rapid, simple, accurate, specific, precise and reproducible as well as has wide scope for separation and quality assessment of botanicals. Also cost effective, environment friendly and satisfactory precision and accuracy are the main features of this method. The method was successfully developed as per ICH guidelines and statistical analysis proves that the method is sensitive, specific, and repeatable. It can be conveniently employed for routine quality control analysis of taraxerol as bulk drug in marketed formulations without any interference from excipients. The method will also be applicable for the estimation of equilibrium solubility of taraxerol in various botanicals. Taraxerol from other plant has already been reported to have plenty of pharmacological activities like anti-tumor, anti-inflammatory, anti-proliferative, antioxidant etc. (Tan et al., 2011; Csupor-Löffler et al., 2011; Khiev et al., 2011; Correia et al., 2010). These reports suggested that *C. ternatea* may also have potential therapeutic application over wound skin due to the presence of taraxerol. Thus, this experiment revealed that *C. ternatea* has effective inhibitory potential against hyaluronidase, elastase, MMP-1 and establishes *C. ternatea* as wound healing agent, which may further be explored in higher experimental model for the treatment of skin wound.
5.8. Conclusion
The present studies revealed high potential of *C. ternatea* leaf as an in vitro enzyme inhibitory activity against skin aging-induced biological damages. All these results suggested the possible use of *C. ternatea* leaf as a natural, non-toxic protector against photosensitization-induced biological damages. Currently, there is a growing interest of the plant derived medicines Worldwide; but lack of proper quality control and variation in the marker profile often leads to spurious drugs based formulations. It has been well established that factors such as habitation, time of collection, maturity of the plants, etc., affect the concentrations of their biomarkers. Hence, proper quality control of the herbal drugs is essential to identify the bioactive constituents.

From this perspective, the identification of taraxerol as the active principles for the preventive property of *C. ternatea* leaf extract against skin aging is extremely important. In conclusion, the extract and fractions of *C. ternatea* have inhibitory activity against hyaluronidase, elastase and MMP-1 and taraxerol has been identified as potential anti-wrinkle agents present in *C. ternatea* leaf. Thus, this experiment establishes *C. ternatea* as anti-wrinkle agent and may further be explored in higher experimental model for the treatment of skin wrinkle.

5.9. Publications