Chapter - 4

4.0. Matrix Metalloproteinase, Hyaluronidase and Elastase Inhibitory Potential of Standardized extract of *Centella asiatica* (L.) Urban.

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4.1. *Centella asiatica* (L.) Urban. - A profile

### 4.1.1. Botanical description

#### Scientific classification

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<th>Kingdom</th>
<th>Plantae</th>
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<td>Genus</td>
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<td><em>asiatica</em> L.</td>
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#### Vernacular names

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#### 4.1.2. Plant description

*Centella asiatica* L. belongs to the family Apiaceae is an ancient Ayurvedic plant (Figure 4.1A and 4.1.B), traditionally well-regarded for its nutritional and medicinal values. It is found in damp and shady banks of water bodies and other moist places of tropical and subtropical India upto 1800 m in hills.

![Figure 4.1A. Centella asiatica](image1.png)  
![Figure 4.1B. Centella asiatica leaf](image2.png)

This plant is a small perennial trailing herb, which grows horizontally upto 3 or 6 inches long. The stems of the plant are glabrous and reddish in color. It has slender branches, faintly aromatic, and small internodes. The creeper has abundant leaf growth and new plants shoot out of various nodes. A number of leaves shoot out at each node on the upper side and numerous roots grow into soil on down side. They are appeared as long, prostate, filliform and rooting at nodes. Schizogenous oil ducts present in the stems and roots. Leaves are orbicular – crenate, reniform, long stalked, 1.3 to 6.3 cm in diameter. They are glabrous on the upper surface and sparsely hairy on the lower with numerous slender nerves from a deeply cordate base with parrot green in color. Flowers are minute, pinkish and red, 3-6 in a cluster. These are characteristic of bisexual, regular and epigynous. Fruit is small and compressed like a grain of barley with 7-9 ridges, 8 mm
long, woody, ovoid, oblong, dull brown, hard with a thick pericarp. Seeds are dull brown and oblong in shape.

4.2. Therapeutic uses of C. asiatica

C. asiatica has been used widely in folk medicine to treat a wide range of illness. All the parts of plant or extracts have been utilized in different purpose of indigenous and folk medicines (Nadkarni, 1976; Warrier, 1994). This is a very valuable herb described by Charaka Samhita and Ayurveda used in the indigenous system of medicine as an anti-aging plant (Datta and Paramesh, 2010) or as a tonic to treat skin diseases. It is useful in vitiated conditions of pitta, insomnia, cardiac debility, epilepsy, hoarseness, asthma, bronchitis, hiccough, amentia, abdominal disorders, leprosy and fever. The plant has also been used as cardio tonic, nerve tonic, stomachic, carminative, anti-leprotic, diuretic and febrifuge. The leaves are useful in abdominal disorders due to dysentery in children (Nadkarni, 1976). Juice of the plant is used to cure inflammation and as a tonic for improvement of memory. Infusion of the plant is applied on the forehead to get rid of headache. This plant is included in Chinese herbal medicines also used root, stem, leaf as traditional food dishes (Chau and Wu, 2006). C. asiatica plant is widely consumed in the form of soup by local people of Malay and javanese populations in Malaysia and Indonesia (Hashim, 2011). This plant is used by Tibetan for promoting their memory, voice and for rejuvenating in general (Dash, 1994). Leaf decoction of oldenlandia umbellate and C. asiatica is used to expel the phlegm from the respiratory tract (Jeeva et al., 2005). Female of Western Ghats region of Karnataka are used leaf extract of C. asiatica along with milk 3 times a day for about 3days to cure menstrual disorder (Parinitha et al., 2004). People from Baganda tribes of southern Uganda, are used crushed form of whole plant to get relief form joint pain (Hamill et al., 2000). Ethnic communities of Dibru-Saikhowa in Northeast India is used aerial part of C. asiatica in the form of infusion to treat abdominal pain and applied topically in form of paste to treat skin diseases (Purkayastha, 2005). Some parts of India, the plant are used as a nervine tonic for memory disorders or as memory enhancer and for blood purifier (Ahuja, 1965).

4.3. Phytochemical profiles of C. asiatica

Centella asiatica has been constituted a wide range of phytoconstuents presented in figure 4.2. Triterpenes, one of the classified groups of terpenes are the major constituent and are widely distributed in all parts of plants. C. asiatica contains mainly pentacyclic triterpenes and their respective glycosides. It contains triterpene compound of asiatic acid (1) and madecassic acid (2) along with derivative of triterpene namely as asiaticoside (a trisaccharide moiety is linked to the aglycone unit of asiatic acid) (3) and madecassoside (a trisaccharide moiety is linked to the aglycone unit of madecassic acid) (4) (WHO). These glycosides are the major biomarker
constituent in terms of quality control (Brinkhaus et al., 2000; Inamdar, 1996). The variation of phytoconstituents of *C. asiatica* reveals that contents of principal compounds vary according with its plant origin. Pentacyclic triterpenes mainly, asiaticoside found more amounts in *C. asiatica* population grows at high altitude than a lower altitude (Das and Mallick, 1991). Asiaticoside A and asiaticoside B are same group of pentacyclic triterpenes have been isolated from *C. asiatica* plant also (Anonymous, 2010). Brahmic acid, isobrahmic acid, cenic acid and centoic acid are the triterpenoid acids while brahmoside, brahminoside, centelloside are the glycosides derivatives of acid. Indcentelloside, thankuniside and isothankuniside are other prime phyto constituents (Mukherjee, 2002). Other terpenes are present in monomer form namely as monoterpenes (α-Pinene, β-Pinene, myrcene, α-Terpinene, Limonene, γ-Terpinene and Terpinolene in fresh and processed juices of *C. asiatica* and 1,8-Cineole, Borneol, Camphene, Carveol I, Carveol I, Citronellol acetate, Para-cymene, Geraniol acetate, Myrcene, Sabines in plant part of the Rhizome) (5), dimer form known as diterpenes (galanalin A, galanalin B and galanolactone) (6) or as form of sesquiterpen (α-hemulene, β-carophyllene, bicyclogermacrene, myrcene, trans-β-farnesene, germacrene D, α-Cubebene, α-Ylangene, α-Copaene, β-Elemene, β-Copaene, Alloaromadendrene, δ-Cadinene, γ-2-Cadinene, γ –Curcumene, Valencene, β-Selinene, α-Murolene, α-Selinene, Cuparene, γ –Cadinene, δ-Cadinene and Calamenene) (7) (Wongfphun, 2010; Inamdar, 1996) Its volatile oil constituted as α-hemulene, β-carophyllene, germacrene B and bicyclogermacrene are the sesquiterpenes classified compounds (Evans, 2009). 3-glucosylkaempferol (8), 3-glycosylquercetin (9) and 7-glucosylkaempferol are polyphenolic compounds have been isolated from leaves of *C. asiatica* (Anonymous, 1992). It has been reported that root, leaf and petiole of *C. asiatica* containing varying proportion of total phenolic compounds (Zainol et al., 2003). Castilliferol and castillicetin are two flavonoid compound were isolated from the whole plant of *C asiatica* (Subban et al., 2008) where as hydrocotyl was isolated from the leaves of the plant. Phytosterols: campesterol (10), sitosterol, and stigmasterol (11) are the steroidal compound obtained from *C. asiatica* plant. 21 compounds of mono and sesquiterpenoids have been isolated from diethylether extract of the whole plant of *C. asiatica* (Asakawa et al., 1982). Two new flavonoids compound castilliferol and castillicetin have also been reported from the whole plant of *C. asiatica* (Subban et al., 2008). A new triterpene compound named as 2α, 3β, 23-trihydroxyurs-20-en-28-oic acid and a saponin compound 2α, 3β, 23-trihydroxyurs-20-en-28-oic acid O-α-L- rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl ester have been isolated from the aerial part of *C. asiatica* (Yu et al., 2007). Rastogi et al., (1960) have been reported the isolation of the triterpene acids betulic acid, brahmic acid and isobrahmic acid along with two saponins, brahmoside and brahminoside from the whole plant (Rastogi et al., 1960). Palmitic acid, stearic acid, lignoceric acid, oleic acid, linoleic acid and linolenic acid are the fatty acid present in the plant. Centellose,
arabinose, rhamnose, galactose, xylose, galacturonic acid, pectin and arabinogalactan are the carbohydrate constituents present in C asiatica plants (Singh et al., 1969; Wang et al., 2005 a, b). Its leaves are rich source of carotenoids, vitamins B and vitamins C. 1-penten-3-ol, 3-methyl- 1-butanol, 2-methyl- 1-butanol and hexanol are alchol containing, 2-methyl-propanal, 3-methyl-butanal, 2-methyl-butanal, Hexanal are aldehydes containing and 2-butanon, 2-nonanone, 5-methyl-2-hexanone ketone containing constituents were identified in fresh and high pressure processing juice of C. asiatica. (Wongfhun, 2010)

Polyacetylene: Cadiyenol, is an organic polymer compound have been isolated from aerial parts of C. asiatica (Govindan et al., 2007). It contains important amino acids also such as alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and tyrosine (Brinkhaus et al., 2000).
Figure 4.2. Major phyto-constituents of *C. asiatica*
The nutritive analysis shows that 100 g of the leaves of *C. asiatica* contain 319.3 Kcal energy, 86.6% water, 12.6 g ash, 12.7 g protein, 6.2 g fat, 53.1 g carbohydrate, 15.3 g dietary fiber, 902.0 g calcium, 28.6 g iron, 107.0 g vitamin C (Maisuthisakul et al., 2008). Further analyses of *C. asiatica* leaves reveals variable amount of mineral as 2.90 mg/g of phosphorus, 31.82 mg/g of potassium, 12.83 mg/g of calcium, 3.45 mg/g of magnesium, 4.35 mg/g of sulphur, 1.76 mg/g of sodium, 0.06 mg/g of iron, 0.017 mg/g of manganese, 0.07 mg/g of zinc, 0.01 mg/g of copper, 0.055 mg/g of aluminum (Zhang et al., 2011).

### 4.4. Pharmacological activities of *C. asiatica*

*C. asiatica* has been reported to posses considerable pharmacological activities like as memory enhancing, sedative, anti-convulsant, anti-depressant, analgesic, anti-diabetic, anti-fertility, anti-filarial, anti-psoriatic, anti-inflammatory, anti-oxidant, anti-leptotic, anti-microbial, anti-spasmodic, anti-tubercular, anti-tumor, anti-ulcer, anxiolytic, immunomodulatory and wound healing effect (Chaitanya et al., 2011). The pharmacological activity of *C. asiatica* is thought to be due to several saponin constituents, including asiaticoside, asiatic acid, madecassoside and madecassic acid (Kartnig, 1988).

*C. asiatica* possesses various CNS effects especially memory improvement and intelligence promoting effects. Some related properties like stimulatory, nervine tonic, rejuvenant, sedative and tranquilizing effect are also associated with this plant species. Scientific findings exhibited that the aqueous extract of *C. asiatica* leaf extract improves spatial learning performance and enhances memory retention in neonatal rats. It was also found that extract enhances hippocampal CA3 neuronal dendritic arborization in rats. Treatment with the extract increased the acetylcholine esterase activity and results shown that treatment during the postnatal developmental stage can influence the neuronal morphology and promote the higher brain function of juvenile and young adult mice (Rao et al., 2005a, b, c). The supporting evidence suggests the usefulness of plant extract over neuronal disorders. Cognitive enhancing effect of *C. asiatica* whole plant was studied on memory impairment on male wistar rats with the subject matter of brain and simultaneous different paradigms of memory behavior by monitoring the parameters like malondialdehyde, glutathione, catalase, superoxide dismutase enzyme label. Study reveals that extract improves the memory and restore the endogenous anti-oxidant enzymes in the brain. Out of aqueous, methanolic and chloroform extract of *C. asiatica* aqueous extract (200 and 300 mg/kg) significant increased in the step down and transfer latency in elevated plus maze model (Kumar and Gupta, 2002). A double blind clinical study with the plant powder for 12 week in 30 mentally retarded children produced significant improvement in general mental ability (Rao, 1977). The age-related defense system of *C. asiatica* extract at the dose of 300 mg/kg bodyweight/day was proved with the result that extract reduces brain regional
lipid peroxidation, protein carbonyl content levels and increases anti-oxidant status (Subathra et al., 2005). C. asiatica plant extract has promising anti-depressant activity in variety of animal models of depression. Bioactive constituent, asiaticoside was isolated from isopropyl alcohol extracts of C. asiatica leaves and were evaluated for their anti-depressant activity in behavioral depression of rat model. The test drug asiaticoside, at dose of (3, 10, or 30 mg/kg) orally in olfactory bulbectomy induced behavioral depression of rat (Kalshetty et al., 2012). Aqueous extract improved cognitive functions and decreased oxidative stress in rats (Nalini et al., 1992). Ethanol extract of the plant as well as brahmoside and brahminoside isolated from the extract have been reported to have tranquilizing activity in rats (Ramaswamy et al., 1970).

Immunomodulatory activity of the ethanol extract was proved against cell-mediated and humoral immune responses at the dose of 100 mg/kg body weight (Punturee et al., 2005). Methanol extract of C. asiatica at dose label of 500 mg/kg increased WBC count and showed maximal phagocytic index to support immunomodulatory activity (Jayathirtha and Mishra, 2004) and strong anti-oxidant activity in lymphoma bearing mice (Jayasree et al., 2003). Aqueous suspension of the plant, given orally, had strong immunostimulant activity (Patil et al., 1998) and its isolated constituent pectin showed immunostimulating activity (Wang et al., 2005). Methanol extract was reported to possess cytotoxic and tumor reducing properties against Ehrlich ascites tumour cells and Daltons lymphoma ascites cells (Babu and Padikkala, 1993). Aqueous leaf and fruit extracts had anti-mutagenic activity (Edwin et al., 2000). Anti-bacterial activity against gram +ve and gram –ve bacteria has been demonstrated in methanol extract (Valsaraj et al., 1997) and in essential oil (Oyedeji and Afolayan, 2005). Anti-fungal activity was performed on ethanol and aqueous extract including of essential oil (Minija and Thoppil, 2003). Aqueous extract of the plant showed radio protective activity against gamma radiation and increased survival time of mice (Sharma and Sharma, 2002). Cold and restraint stressed gastric ulceration was prevented by ethanol extract of the fresh plant (Chatterjee et al., 1992). Fresh juice of the plant also prevented ethanol, aspirin, cold resistant and pyloric ligation induced gastric ulceration (Sairam et al., 2001). Cheng and Koo prevented ethanol induced gastric mucosal lesion (Cheng and Koo, 2000). Further aqueous extract and isolated compound asiaticoside was found to reduce the size of the ulcers in a dose dependent manner with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues (Cheng et al., 2004). Various extracts such as ethanol, water and light petroleum obtained from different parts of C. asiatica (leaves, petioles and roots) showed the anti-oxidant activity against the linoleic acid model and thiobarbituric acid test.

Local application of aqueous extract showed wound healing property. Aqueous extract of C. asiatica with 5% propylene glycol showed healing property in experimentally induced open
wounds in albino rats (Rao et al., 1996). Isolated compound asiaticoside also showed the healing activity when it was given orally to guinea pig or applied topically on wound of diabetic rats (Shukla et al., 1999). Plant extracts accelerate the healing process against chronic postsurgical and post-trauma wounds and prevent second- and third-degree burns. Oral and topical administration of the aqueous extract on rat dermal wound also proved its efficacy and healing property (Suguna et al., 1996) whereas formulations of the same extract, increased cellular proliferation and collagen synthesis at the wound site and improved the tensile strength when applied topically, thrice daily for 24 days on the open wounds (Kumar et al., 1998).

Saponin constituents, including asiaticoside, asiatic acid, and madecassic acid stimulated the production of human collagen I, a protein involved in wound healing (Bonte et al., 1994). Asiaticoside promoted the healing of superficial postsurgical wounds by accelerating cicatricial action. Topical application of asiaticoside is significantly decrease fibrosis in wounds and increases the tensile strength of newly formed skin and, thus preventing new scar formation. Increasing collagen synthesis and acidic mucopolysaccharides, and by inhibiting the inflammatory phase against hypertrophic scars and keloids may be the possible mechanism behind this prevention. It has further been proposed that asiaticoside interferes with scar formation by increasing the activity of myofibroblasts and immature collagen (Morisset, 1987; Rosen et al., 1972). Plant extract stimulate collagen synthesis in foreskin fibroblast monolayer cultures (Maquart, 1990). Whereas triterpene compound asiaticoside stimulates the cells of malpighian layer and activate the epidermis and keratinization (May, 1968). Wang also found similar kind of results and reported that C. asiatica can increase the cellular hyperplasia and type-1 collagen production at the site of injury. Cross-linking of collagen fiber helps to elevate its stability and increase the tensile strength (Wang et al., 2006). C. asiatica is useful in herbal cosmetic for improvement of skin viscoelastic and hydration properties (Reed et al, 1998). Asiaticoside, isolated from C. asiatica, has been shown to induce type I collagen synthesis in human dermal fibroblast cells (Kartnig, 1998) Isolated irbic acid from cell cultures of C.asiatica shown significant inhibitory effect on collagenase activity at dose label range of 50-250 μg/mL. additionally anti-oxidant (free radical scavenging) activity against DPPH assay support the possible utilization of this substance as a topical agent to reduce the skin ageing process (Antognoni et al., 2011).

4.5. Inhibitory potential of C. asiatica extract and isolated compound

4.5.1. Collection and authentication of plant materials

Centella asiatica L. plant (whole) materials were collected from fields’ near to Kolkata, India and authenticated by Dr S Rajan, Field Botanist.
The Medicinal Plants Collection Unit, Emerald, Govt. of India. A voucher specimen (specimen no. SNPS-JU/2010/1069) has been submitted to School of Natural Product Studies, Jadavpur University, Kolkata, India for future references (Figure 4.3).

4.5.2. Reagents and instruments

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, ursolic acid and oleanolic acid have been purchased from Sigma chemical (USA) for the study. 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). Standard asiaticoside was isolated and used for HPLC standardization.

4.5.3. Extraction and fractions of plant materials

Crude extract was obtained by cold maceration method. Whole plant material was grinded and extracted with CHCl$_3$. The dried marc was further extracted with methanol for 72 h with intermittent shaking. This macerated product was decanted and entire process was repeated for another two times. The combined extract was filtered through a nylon mesh and concentrated under reduced temperature (not exceeding 45 °C) by using rotary evaporator (EYELA, Tokyo, Japan) and lyophilized. The yield of dry methanolic extract of *C. asiatica* (CAMeOH) was 37.34% w/w. The lyophilized methanolic extract was suspended in water and successively fractionated with n-butanol (yield 9.73% w/w), ethyl acetate (yield 8.47% w/w) and aqueous solvent (yield 5.67% w/w) based on polarity index. All sub-fractions were dried under the vacuum below 45 °C using rotary evaporator. The test samples of *C. asiatica* methanolic extract (CAMeOH), n-butanol fraction (CAnB), ethyl acetate fraction (CAEA), aqueous fraction (CAAQ and standard ursolic acid were used for the *in vitro* study. Various concentrations were prepared from the stock solution and stored at 2-8 °C until use.

Table 4.1. Extraction and fractions of *C. asiatica*

<table>
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<td>Methanolic extract (CAMeOH)</td>
<td>37.34% w/w</td>
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<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield</th>
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<tr>
<td>Ethyl acetate (CAEA)</td>
<td>08.47% w/w</td>
</tr>
<tr>
<td>n-butanol (CAnB)</td>
<td>09.73% w/w</td>
</tr>
<tr>
<td>Aqueous (CAAQ)</td>
<td>05.67% w/w</td>
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</table>
4.5.4. Hyaluronidase inhibition assay

Hyaluronidase inhibitory assay was performed by the method described previously using 96 well microplate (Hayati et al., 2007; 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 principle and details of assay have been discussed in chapter 3. Test sample 5 µL of C. asiatica extract and fractions (diluted to 1.56, 3.12, 6.25, 12.50, 25 and 50 µg/mL concentrations) in DMSO (dimethyl sulphoxide) was used to perform this assay. Ursolic acid was used as a standard (Lee et al., 2001). Percentage of enzyme activity, was calculated using the following formula:

\[
\text{% Enzyme activity} = (100\%) - \left\{\frac{A_{600 \text{ nm of hyaluronic acid + hyaluronidase}}}{A_{600 \text{ nm hyaluronic acid}}} \times 100\right\}
\]

4.5.5. Elastase inhibition assay

The elastase inhibition assay was performed by UV-Visible spectrophotometer according to the previous reported method (Kim et al., 2009; 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 principle and assay details have been discussed in chapter 3. The test solutions (100 mL) made by dissolving the C. asiatica extract and fraction in DMSO and subsequently it was diluted to different concentrations 1.56, 3.12, 6.25, 12.50, 25 and 50 µg/mL to perform this assay. Ursolic acid was used as a standard (Lee et al., 2001). The percentage of inhibition was calculated using following formula:

\[
\text{Inhibition} (%) = (1 - \frac{B}{A}) \times 100; \text{ where A is the enzyme activity without the sample and B is the activity in the presence of the sample.}
\]

4.5.6. Matrix Metalloproteinase-1 (MMP-1) inhibition assay

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 principle and details of assay have been discussed in chapter 3. A reaction buffer containing 0.5 mol Tris-HCL, 1.5 mol NaCl, 50 mM CaCl2 and 2 mM sodium azide at pH 7.6 was prepared for all types of dilution. 20 µL of type-I collagen fluorescein conjugate was mixed with 80 µL of each of diluted (1.56, 3.12, 6.25, 12.50, 25 and 50 µg/mL) inhibitor C. asiatica extract and fractions along with standard oleanolic acid as reference.
standard (Maity et al., 2011). 100 µL of diluted (0.2 U/mL) MMP-1 was added to each well and the plate was incubated at room temperature for 1-2 h protected from light. After that fluorescence was measured at excitation maxima at 495 nm and emission maxima at 515 nm.

4.5.7. 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 (ursolic acid). All calculations were performed using Graph Pad Prism (version 5.0).

4.5.8. Isolation of Asiaticoside

Asiaticoside was isolated from n-butanol fraction of C. asiatica extract by column chromatography as described method previously (Sahu et al., 1989) with slight modification. Approximately 20 g of extract was subjected to column chromatography over silica gel (100-120 mesh size) using gradient elution technique with increasing polarity of solvents. The column initially eluted with pet ether/chloroform (100:0, 80:20, 50:50 and 20:80) followed by 100% of chloroform (Fr-1 to Fr-20). Further column was eluted with chloroform/methanol (95:5, 90:10, 85:15, 80:20, 75:25) and Fr-21 to Fr-35 (each 100 mL) were collected. The fraction, collected from chloroform/methanol 80:20 (Fr-31-33) gave single spot over TLC (chloroform/ glacial acetic acid/ methanol/ water 60:32:12:8) Rf = 0.4 (Mukherjee 2002) with yield 3.8% w/w and identified as asiaticoside by GC-MS and 1H NMR spectroscopy (Du et al., 2004). Mass spectra showed (M+Na ion) + peak with m/z 981; molecular formula was calculated as C48H78O19 with melting point range 235-238 °C. The isolated asiaticoside were evaluated for their enzyme inhibitory potential also by methods described earlier.

4.6. RP-HPLC standardization of C. asiatica extract and fractions

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 asiaticoside as the active principles for the preventive property of C. asiatica extract against skin aging is extremely important.
4.6.1. 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. Asiaticoside standard marker compound, which has been used here, was isolated by the bioactivity guided isolation technique from the C. asiatica plant extract in our laboratory, characterized, confirmed by spectroscopy study, and described in previous chapter.

4.6.2. 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) methanol: (channel B) water acidified with 1% glacial acetic acid (70:29:1 v/v) by means of isocratic elution (1 mL/min) and detection at 280 nm. Analysis of the asiaticoside, methanol extracts 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.

4.6.3. Standard and sample solution

A primary stock solution (1 mg/mL) containing asiaticoside was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of C. asiatica 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 asiaticoside 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.

4.6.4. Calibration curve (linearity)

The linear calibration plot of asiaticoside was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.
4.6.5. **Specificity**

Chromatograms of asiaticoside 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.

4.6.6. **Limits of detection and quantification (LOD and LOQ)**

The LOD and LOQ were calculated by the method based on the standard deviation (\(\sigma\)) and the slope (S) of the calibration plot, using the formula LOD = 3:1\(\sigma\)/S and LOQ = 10:1\(\sigma\)/S (ICH, 2005 and FDA, 1994); where, \(\sigma\) = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.6.7. **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.

4.7. **Results and discussion**

In this experiment methanol extract (CAMeOH), ethyl acetate fraction (CAEA), n-butanol fraction (CAaB) and aqueous fraction (CAaQ) 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 ursolic acid as biomarker. The extract, fractions and isolated compounds 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 IC\(_{50}\) values were extrapolated.

4.7.1. **Inhibition potential of C. asiatica on hyaluronidase, elastase and MMP-1**

In hyaluronidase assay experiment, CAMeOH extract and CAaB fractions were showed significant (*P* < 0.05) concentration dependent inhibition of hyaluronidase with IC\(_{50}\) value of
19.27 ± 0.37 µg/mL and 27.00 ± 0.43 respectively (Figure 4.4) compared to ursolic acid. CAEA and CAAQ fractions (IC<sub>50</sub> 58.55 ± 0.40 39.81 ± 0.34 µg/mL respectively) did not show any significant results. The CAMeOH and CANb were showed significant (<sup>a</sup>P < 0.05) elastase inhibitory activity respectively (IC<sub>50</sub> at 14.54 ± 0.39 and 29.15±0.31 µg/mL) (Figure 4.5), compared to ursolic acid but CAEA and CAAQ fractions did not show significant results (IC<sub>50</sub> 47.21 ± 0.30 36.57 ± 0.33 µg/mL). The isolated compounds asiaticoside was showed higher anti-hyaluronidase and anti-elastase activity with IC<sub>50</sub> of 18.63 ± 0.33 and 19.45 ± 0.25 µg/mL respectively compared to ursolic acid.

![Image](image1.png)

**Figure 4.4. Hyaluronidase Inhibition assay**
All values are expressed as mean ± SEM (n=6). <sup>a</sup>P < 0.05 and <sup>c</sup>P < 0.001 compared with standard.

![Image](image2.png)

**Figure 4.5. Elastase Inhibition assay**
All values are expressed as mean ± SEM (n=6). <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 and <sup>c</sup>P < 0.001 compared with standard.

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).

![Image](image3.png)

**Figure 4.6. MMP-1 Inhibition Assay**
All values are expressed as mean ± SEM (n=6). <sup>c</sup>P < 0.05, <sup>b</sup>P < 0.01 compared with standard.
MMP-1 inhibition was found to be significant (aP < 0.001) in case CAMeOH (Figure 4.6) by lower fluorescence reading, which is due to less amount of substrate hydrolyzed by MMP-1 compared to oleanolic acid. Among the fractions, CAnB was showed significant (aP < 0.001) inhibition of MMP-1 activity with low fluorescence readings. 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 and elastase inhibitory activity of CAMeOH extract was found to be more than ursolic acid. This may be due to the presence of different array of compounds present in the extract together shown synergistic effect and the compounds have been enriched into fractions. MMP-1 inhibitory activity of CAnB fraction was more compared to CAEA and CAAQ.

4.7.2. Mass and NMR spectra of isolated asiaticoside

Asiaticoside molecules were obtained from column chromatography. The sub-fraction obtained from chloroform/methanol (80:20) produced a compound, which showed (M+Na ion)^+ peak with m/z 981 at LC-MS spectra and molecular formula was calculated as C_{48}H_{78}O_{19} with melting point range 235-238 °C. Structural and functional groups were confirmed by ^1H NMR spectral data comparable to formerly reported literature of asiaticoside (Du et al., 2004).

Figure 4.7 shows the results of gas chromatography-mass spectrometry (GC-MS-SHIMADZU-QP5050A, Japan) analysis of isolated compound asiaticoside. Figure 4.8 shows the results of ^1H-NMR measured in CDCl3 (300 MHz) with trimethylsilylate as an internal standard.
4.7.3. RP-HPLC standardization and method development of *C. asiatica*

The content of asiaticoside in *C. asiatica* was determined using calibration curve plotted between mean peak area (Y-axis) and concentration (X-axis). Linearity was evaluated by regression analysis using 6 different concentrations of the standards (10-500 µg/mL). The coefficient of determinants ($r^2$) was 0.929 and $Y = 2.68e + 003X + 1.25e + 004$ for asiaticoside (Figure 4.9), 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 in the range of 10-500 µg/mL. Retention time of asiaticoside was found to be 4.277 min. (Figure 4.10).
Figure 4.10. HPLC chromatogram of standard asiaticoside showed specific peak with retention time of 4.277 min.

The standardization method provides good resolution and separation of asiaticoside from other constituents of *C. asiatica* methanol extract (Figure 4.11). Retention time of asiaticoside was found to be 4.234 min. The content of asiaticoside in CAMeOH extract was found to be 3.3% w/w.

Figure 4.11. HPLC chromatogram of *C. asiatica* methanol extract; the separated asiaticoside showed specific peak at 4.234 min.

Retention time of asiaticoside was found to be 4.358 min. in n-butanol fraction. The content of asiaticoside was found to be 4.27% w/w in n-butanol fraction (Figure 4.12), whereas ethyl acetate and aqueous fraction did not show any asiaticoside peak.

Figure 4.12. HPLC chromatogram of n-butanol fraction of *C. asiatica*; the separated asiaticoside showed specific peaks at 4.358 min.
The signal to noise ratios 3:1 and 10:1 were considered as LOD and LOQ respectively and were found to be 35.75 μg/mL and 170.55 μg/mL for asiaticoside suggested full capacity for quantification of the compounds. Retention times of standard was highly repeatable, with %RSD < 2% even at high concentration. A better separation of the asiaticoside 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. asiatica* with respect to asiaticoside was properly validated in respect of linearity, specificity, peak purity, LOD, LOQ and robustness. The specificity test of the proposed method demonstrated that other constituent presents in the methanol extract of the *C. asiatica* did not interfere with peak of interest identified as asiaticoside. Furthermore, well shaped peaks indicate the specificity of the method. 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 asiaticoside as bulk drug in marketed formulations without any interference from excipients. The method can also be applicable for the estimation of equilibrium solubility of asiaticoside in various botanicals.

4.8. Conclusion

The present studies revealed high potential of *C. asiatica* as an *in vitro* enzyme inhibitory activity against skin aging-induced biological damages. In conclusion, the extract and fractions of *C. asiatica* were found to be inhibitory activity against hyaluronidase, elastase and MMP-1 and asiaticoside was identified as potential anti-aging agents present in *C. asiatica*. Thus, these experiments established *C. asiatica* as anti-aging and anti-wrinkle agent and may further be explored in higher experimental model for the treatment of skin wrinkle. The effects were comparable to that of ursolic acid and oleanolic acid, which were already reported for their skin protective activity. Thus, the screening and HPLC standardization were validated the traditional claim of *C. asiatica* as anti-wrinkle agent where asiaticoside may be a responsible biomarker.

4.9. Publications