Chapter 7

Summary and Conclusion

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7.1. Summary
Evaluation of anti-aging efficacy of herbal drugs has become one of the major concerns with tremendous increase in the application of traditional medicine for the treatment of skin aging and related diseases worldwide. Lot of skin care products including anti-aging creams, ointments is available for protecting and restoring the skin damage. In spite of their potent antioxidant property or skin care property they have several disadvantages like side effects, allergic reactions, high cost, contact time during cleansing is too little to ensure any anti-wrinkle effect etc. Hence it is of great interest to search for new anti-aging skin care leads from natural resources so as to ensure the desire anti-aging effect of herbal products through quality control measures. Preclinical evaluation is important not only for establishing the therapeutic efficacy of the medicinal plants but also to validate their traditional claim in folklore system.

Chemo-profiling and standardization of herbals are used as potential cost-effective, simple and highly selective tool that can ensure both quality and batch-to-batch reproducibility of the products. In this thesis work three traditionally important Indian medicinal plants were standardized with respect to their major bioactive compounds through RP-HPLC analysis. All the analytical procedures were optimized in our laboratory. Among them some standard analytical methods for RP-HPLC were validated with special reference to ICH guidelines. Medicinal plants are therapeutically used for various diseases in different countries and mostly lack of proper validation on their quality control and assurance. Cosmetic formulations based on botanical ingredients have been used since ancient times, and botanical and natural extracts plays a major role in contemporary cosmetics. Scientific researches continue to corroborate traditional uses of many plants for skin benefits to elucidate biochemical mechanisms of action for a growing number of phytochemicals. Additional pre-clinical research is mandatory to optimize the application of natural ingredients for cosmetics. However, another aspect of this thesis work was to evaluate the anti-aging activity of some selected Indian medicinal plant. This thesis highlights anti-aging potential of different methanol extracts of botanical, and their ethyl acetate, n-butanol and aqueous fractions screened for in-vitro hyaluronidase, elastase (UV spectrophotometric) and matrix metalloproteinase-1 (MMP-1) (fluorescence) enzymes inhibition assays. The results can be useful for an initial assessment of anti-aging activity of selected plants for the potential risk of skin aging and related diseases.

Chapter 1 discuss with brief account of skin aging and bioactive compounds from botanicals. This chapter contains description about the etiology and symptoms of skin aging and wrinkle, factors effecting skin aging, role of dermal enzymes in skin aging such as hyaluronidase, elastase and MMP-1, regulation enzymatic activity over ECM, treatment of skin aging, antioxidant nutrients, different mechanisms of bioactive leads from natural resources to control
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skin aging. Furthermore, quality control and standardization of herbal medicine has been described. Reports of important herbs and their isolated molecules to control different signaling pathways involved in photo induced aging have been described in this section. One review article has been published:


Chapter 2 the objectives and scope of the work and the brief plan of the work has been described in this chapter.

Chapter 3 describes the hyaluronidase, elastase and MMP-1 inhibitory activity of standardized leaf extract of *Piper betel* Linn. Leaf of *P. betel* was collected and authenticated. *P. betel* was extracted and fractionated to perform the bioactivity guided isolation of marker compounds hydroxychavicol and chlorogenic acid. Ethyl acetate fraction produced a compound, which showed \( M^+ \) ion peak with m/z 150.1 at GC-MS spectra and molecular formula was calculated as \( \text{C}_9\text{H}_{10}\text{O}_2 \). While, in case of n-butanol fraction; sub-fraction obtained from the water/methanol (50:50) produced a compound, which was analogous to chlorogenic acid showed \( M^+ \) ion peak with m/z 353.9 and molecular formula was determined as \( \text{C}_{16}\text{H}_{16}\text{O}_9 \). All the extract, fractions and isolated marker compounds were screened for *in-vitro* hyaluronidase, elastase and MMP-1 assay. The hyaluronidase and MMP-1 inhibitory activity of extract was found to be more than oleanolic acid, but the elastase inhibition was comparatively parallel. This may be due to the presence of different compounds in the extract which have synergistic effect. The extract and fractions were standardized through RP-HPLC using hydroxychavicol and chlorogenic acid as marker. The developed RP-HPLC methods were validated followed by ICH guidelines. In RP-HPLC, presence of hydroxychavicol and chlorogenic acid in the plant extract was confirmed by comparing the peaks of individual marker with the extract and fractions for their corresponding retention time 16.14 min and 13.52 min respectively. Calibration curve was linear in the range of 10-1000 µg/mL. The quantity of hydroxychavicol and chlorogenic acid present in the test extract was found to be 28.56 % w/w and 0.40 % w/w respectively. The LOD and LOQ respectively and were found to be 1.6 µg/mL and 5.0 µg/mL for hydroxychavicol and 1.0 µg/mL and 3.0 µg/mL for chlorogenic acid, which suggested full capacity for quantification of each compound. The average intra-day recovery (n=3) of hydroxychavicol and chlorogenic acid were 106.65±0.85% and 98.33±2.16%, while the average inter-day recovery (n=9) was 106.73±0.66% and 98.03±1.56% respectively, with %RSD < 2%. Intra-day (n=3) and inter-day (n=9) precision of the method was represented as %RSD of peak area (response) and retention time. Retention time was highly repeatable, with %RSD < 2% both for standards and extract, even at high concentration. A high repeatability in the retention time and area response was obtained for both
standard and extract even at high concentration. The method was extremely adaptable because of the good precision and excellent repeatability. The method is rapid, simple, accurate, specific, precise and reproducible as well as has wide scope for separation and quality assessment of botanicals. Overall, the present studies revealed its high potency as an in-vitro enzyme inhibitory activity against skin aging-induced biological damages. Thus, this experiment establishes *P. betel* as anti-wrinkle agent and may further be explored in higher experimental model for the treatment of skin wrinkle. Based on this work following publication/presentation have been made:-


Chapter 4 deals with the methanol extract, ethyl acetate, n-butanol and aqueous fractions of flower of *T. erecta* were screened for in-vitro hyaluronidase, elastase (UV spectrophotometric) and MMP-1 (fluorescence) enzymes inhibition assay. *T. erecta* flower was collected and authenticated. β-amyrin and syringic acid were obtained and the activities were correlated with respect to individual phytoconstituents. Mass spectra of isolated compound β-amyrin showed the \( M^+ \) ion peak with \( m/z \) 426.71; molecular formula was calculated as \( C_{30}H_{50}O \) and melting range 197-198°C. Syringic acid showed \( M^+ \) ion peak with \( m/z \) 198.18 and molecular formula was calculated as \( C_{9}H_{10}O_{5} \). The hyaluronidase, elastase and MMP-1 inhibitory activity of methanol extract was found to be more than oleanolic acid. MMP-1 inhibitory activity of ethyl acetate fraction was more, but the n-butanol fraction showed similar activity comparing to oleanolic acid. Whereas the elastase inhibitory activity of n-butanol fraction was more compare to oleanolic acid. This may be due to the synergistic effect of different compounds in the extract, which have been enriched into fraction. Both the isolated compounds have similar inhibition potential on hyaluronidase and elastase, but β-amyrin has better inhibition potential on MMP-1 compared to oleanolic acid. The present study established that syringic acid and β-amyrin are the major constituents of *T. erecta* flower can heal photo-aging induced skin damage by its enzyme inhibitory action and ability to form extracellular matrix. Apparently, by minimizing the collagenolytic activity of MMP-1, *T. erecta* might be protecting the extracellular matrix, and in turn, the skin from the photo damage. Moreover, both the biomarkers are present in significant
amount in T. erecta as depicted from the HPLC chromatogram, which have been standardized and quantified through calibration curve with linearity range 200-1000 µg/mL. Retention time of β-amyrin and syringic acid were found to be 12.95 and 23.01 min. and the contents were found to be 0.06 % w/w and 2.30 % w/w respectively. LOD and LOQ were found to be 30.23 µg/mL and 94.75 µg/mL for β-amyrin and 44.13 µg/mL and 176.59 µg/mL for syringic acid suggested full capacity for quantification of the compounds. Mean % recovery (n=3) of β-amyrin and syringic acid from the T. erecta were calculated to be 99.88 ± 0.07 and 99.60 ± 0.20 respectively, which indicates good accuracy of the method. Retention times of both standards were highly repeatable, with %RSD < 2% even at high concentration. A better separation of the markers 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. This new RP-HPLC method has been developed for the identification and quantification of β-amyrin and syringic acid. The method is rapid, simple, accurate, specific, precise and reproducible as well as has wide scope for separation and quality assessment of botanicals. Thus, this experiment establishes T. erecta as anti-wrinkle agent and may further be explored for the treatment of skin wrinkle. Based on this work following publication/presentation have been made:


Chapter 5 deals with the anti-wrinkle potential of standardized leaf extract of Clitoria ternatea. In this chapter 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 structural characterization of the isolated taraxerol 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. 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). 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). 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. 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 CONB fraction. CONB 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.

The HPLC standardization of the extract and fractions were performed with respect to isolated taraxerol as marker through the calibration curve. 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 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. and the contents were found to be 5.32 % w/w. The taraxerol content in fractions was found to be 4.55 % w/w in ethyl acetate fraction and 0.43 % w/w in n-butanol fraction respectively. 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. The method is rapid, simple, accurate, specific, precise and reproducible as well as has wide scope for separation and quality assessment of botanicals. 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. Based on the study following article has been published.
Chapter 6 deals with the estimation of trace and heavy metals by Atomic Absorption Spectrophotometer. Herbal skin care ingredients/extracts enrich the skin with trace (nutrient) elements and other useful minerals to prevent infection and responsible for soothing effects over skin. But last few decades, it has been accounted that the absorption of toxic metals through skin is very significant and can cause deleterious effects over skin. The safety assessment of herbal ingredients becomes doubtful and need further attention of the scientific community and the regulatory agencies. It is essential to screen the level of trace and heavy metals contents in herbal ingredients used before preparing the cosmetic formulation. Thus, quantification of metals in plants especially medicinal herbs is part of quality control, which has been established their purity, safety and efficacy. In this chapter, five plants species including *Clitoria ternatea*, *Piper betel*, and *Tagetes erecta* were screened to determine the trace (Cu, Cr, Mn, Fe, Ni) and heavy (As, Pb, Hg) metals through atomic absorption spectrometry and thereby to assure the safer therapeutic application of these plants. The concentration of the metals obtained in plant material was expressed in terms of ppm. The levels of heavy metal quantified in all the plant samples were within prescribed limits (WHO, 1999). In the present study, different plant materials were found to contain variable amounts of trace (nutrient) elements and the content of heavy metals were found within permissible limit according to WHO. The concentration variation of these elements may be due to environmental condition and geographical origin, use of fertilizer, pesticides etc. In this study, a simple, reliable, sensitive and convenient AAS method has been developed for quantitative estimation of trace metals and heavy metals which can conveniently be utilized for the quality control of herbal cosmetic preparations at industrial level. It was observed that the plant materials collected from specific region are safe and may not produce any toxic effect up on their therapeutic application. Based on the study following article has been communicated

7.2. Conclusion
The thesis work deals with the “lead finding for matrix metalloproteinase inhibitors from Indian medicinal plants”. *Piper betel*, *Tagetes erecta*, and *Clitoria ternatea* were screened for *in-vitro* hyaluronidase, elastase (UV spectrophotometric) and matrix metalloproteinase-1 (fluorescence) enzymes inhibition assays. All the herbs were standardized with their respective marker compounds through RP-HPLC. Standardization parameter and marker profiling is important to maintain quality control and batch to batch reproducibility of the products. Possible anti-aging and anti-wrinkle potential of these medicinal plants was explored along with their bioactive compounds. This work established the rational approach to screen selected herbs used in Indian System of Medicine (ISM) for their MMP-1 inhibiting activity *in-vitro*. The developed procedure could be used for assessment of the therapeutic potential of selected herbs as cosmetic ingredients in anti-aging and anti-wrinkle therapy. Results indicated that test extract and phytocompounds have better IC50 values than respective positive inhibitors against hyaluronidase, elastase and MMP-1.

The results of HPLC standardization are highly repeatable for both marker compounds and plant extracts even at high concentration. The methods were extremely adaptable because of the good precision and excellent repeatability. The HPLC method were rapid, simple, accurate, specific, precise and reproducible as well as have wide scope for separation and quality assessment of these botanicals according to ICH guidelines. However, it can be concluded that therapeutic application of these selected herbal extract and their bioactive compounds would be very high; if they have been formulated singly or concomitantly with other ingredients having good efficacy against skin aging. In other word, the uses of these medicinal plants collected from local area for cosmetic application are safe, because the content of trace and heavy metals are within the prescribed limit. By this study we have been able to explore some leads from Indian medicinal plants in respect to their anti-aging and anti-wrinkle potential in one hand and on their quality control protocols to make standardized extracts of those herbs, which may be useful for their promotion and development in therapeutic application. Thus, this study based on combined approach on exploration and exploitation of chemo profiling of several plants leading to hyaluronidase, elastase and MMP-1 inhibition potential may help in developing some effective natural product based formulation.