Summary and Conclusions

Medicinal herbs have been in use in one form or another, under indigenous systems of medicine like Ayurveda, Siddha and Unani. India, with its traditional background, needs to increase its share in the world market. But India has not been able to capitalize on this herbal wealth by promoting its use in the developed world, despite their renewed interest in herbal medicines. This can be achieved by employing biotechnological approaches to ensure the supply of raw materials of superior quality from regular and viable source for commercial purpose. *Centella asiatica* (L.) Urban, a herbaceous plant belonging to the Apiaceae family, has great medicinal value; it has been used in traditional medicine as an antipyretic, diuretic and antibacterial drug and in the treatment of skin diseases, vein insufficiency and mental disorders (Mangas *et al*., 2008). The bioactive compounds of *C. asiatica* are triterpenoid saponins and sapogenins bearing an ursane skeleton among which the saponin Asiaticoside is of particular interest. Due to its medicinal properties, several efforts have been made to develop *in vitro* cultures for the biotechnological production of Asiaticoside (Omar *et al*., 2005; Nath and Buragohain 2005) but the results achieved until now have been far from the level required for economic exploitation. Therefore, in the present study efforts have been made for the development of efficient *in vitro* culture protocols to improve the biomass and Asiaticoside production in a medicinally important herb *Centella asiatica*.

In the present investigation entitled, "Improvement of a medicinally important herb Mandookaparni (*Centella asiatica* (L.) Urban) through *in vitro* culture", the experiments were organized in completely randomised design. The experiments had a minimum of 15 replicates and each experiment was repeated at least three times. The data was subjected to statistical analysis by using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at 5% probability level. The important findings and conclusions of this research work are as follows:

***In vitro* propagation and Asiaticoside in diploid plants**

1. Nodal, internodal and leaf explants were cultured on MS medium with and without growth regulators cytokinins (BA and Kinetin) alone and in combination with auxins (IAA, NAA and 2,4-D). Shoot formation was observed only in the case of nodal explants cultured on MS medium without

growth regulators. 98.4± 2.6% nodal explants exhibited shoot formation with an average number of 2.2±0.9 shoots per explant.

2. Nodal explants inoculated on medium supplemented with cytokinin alone, exhibited maximum shoot regeneration on MS medium containing 17.74 μM BA and 9.0±0.8 shoots per nodal explant were produced. This study also reveals that BA is more effective than Kinetin, for the induction and multiple shoot formation in *C. asiatica*.

3. The best response for multiple shoot proliferation was observed in the nodal explants cultured on 0.54 μM NAA along with 17.74 μM BA. From leaf explants, maximum shoot regeneration was observed on MS fortified with 8.87 μM BA and 0.26 μM NAA. However, both nodal and leaf explants responded for shoot formation but, the nodal explants were found to be most suitable for caulogenesis.

4. The results also reveal the capability of rhizogenesis in leaf and nodal explants of *C. asiatica* cultured on MS medium containing IAA, NAA and 2,4-D. Leaf and nodal explants inoculated on MS medium devoid of growth regulators failed to respond for rhizogenesis. The results of the present study on the effects of different auxins for rhizogenesis in *C. asiatica* varied depending upon the explant used and also on the type and concentration of the auxin employed for rhizogenesis. Consequently, it was found that low concentrations of auxins were suitable for induction and growth of the roots from the leaf and nodal explants.

5. Among the explants used, leaf explants were found to have more potential for root formation. NAA was found to be most effective and ideal for root induction. Maximum number of roots formed was 9.3±0.2 in leaf explant cultured on MS medium supplemented with 2.15 μM NAA.

6. Autoclaved sand and soil (1:1) mixture was found to be appropriate for *ex vitro* planting of *Centella asiatica* in the experiment. 64-88% plantlets were successfully acclimatized to natural conditions.

7. HPTLC analysis confirmed the presence of Asiaticoside in the plants. It was also found that the distribution of Asiaticoside throughout the plant was organ specific, with leaves containing the higher content of this compound. The results of the analysis of naturally grown plant suggest that, leaf of the plant possessed the maximum amount of Asiaticoside as compared to the other plant parts. The leaf was found to contain 0.969 ± 0.012 mg/g DW of Asiaticoside which was 5.80 times more as compared to the stem.

8. HPTLC analysis of *in vitro* grown *C. asiatica* biomass also confirmed the presence of Asiaticoside in these samples. The leaf was found to contain 0.297 ± 0.041 mg/g DW of Asiaticoside. The stem contained 0.086 ± 0.013 mg/g DW of Asiaticoside. Thus, the leaf contained 3.45 times more Asiaticoside content as compared to the stem in case of *in vitro* grown plant biomass.

9. Asiaticoside was not detected in roots of naturally grown and *in vitro* grown plants. This might be possible due to very low content or no formation of Asiaticoside in roots.

10. From the results, it is evident that *in vitro* raised biomass has the potential for biosynthesis of Asiaticoside. However, the Asiaticoside content of *in vitro* raised plants was quite low as compared to the Asiaticoside content of naturally grown plants.

Induction of Tetraploidy and Asiaticoside production

11. Induction of *in vitro* tetraploidy in *C.asiatica* by using antimitotic agents like Colchicine and Oryzalin was performed. Ploidy level analysis was done with the help of Flow Cytometry and the obtained tetraploids were acclimatized to natural conditions successfully.

12. The shoot-tips treated with 0.1% Colchicine solution showed a survival percentage of 9.14%. On increasing concentration of Colchicine to 0.2%, only 4.5% of the treated shoot-tips survived. The survival percentage further dropped to 2.8% on treatment with 0.4% and 0.6% Colchicine solution. On treatment with 0.8% and 1.0% Colchicine solution, only 1.7% and 0.6% of the

shoot-tips respectively were able to survive. Moreover, the treatment time of 2-8 hrs was found to be the most favourable for the endurance of the treated explants.

13. The survival and formation of shoot from the shoot-tips after colchicine treatment were found to be dependent on colchicine concentration and the treatment duration. Higher concentration and longer duration tended to reduce survival and shoot formation. The reason behind the high mortality rate might be the fact that, Colchicine being a spindle poison, acts in plant cell by destroying spindle fibres and modifies the differentiation process thus resulting into the retardation and inhibition of shoot-tip growth and survival.

14. 0.1% Colchicine treatment for 6 hrs was most favourable for the induction of polyploidy. The induction rate of polyploidy was 8.0% in this case. No mixoploid was obtained as a result of this treatment. Immersing the shoot-tips in 0.2%, 0.4% and 0.8% Colchicine solution for 4-6 hours was also found to be efficient in inducing tetraploidy.

15. The tetraploids were also obtained from shoot-tips inoculated on MS medium containing 15μM Oryzalin for a period of 7 days.

16. Maximum survival of the explant (11%) was observed on 5 μM Oryzalin but this concentration was not able to induce polyploidy in *C.asiatica*. Only 4% shoot-tips survived on MS medium containing 10μM Oryzalin. Further decline in the survival rate was observed on increasing Oryzalin concentration to 15μM. Only 3% of the inoculated explants survived, but this concentration of Oryzalin was found to be effective in the production of autotetraploids, on treatment for 7 days. Shoot-tips incubated on MS medium containing 20μM Oryzalin depicted only 1% survival rate.

17. Induction of polyploidy in *C. asiatica* plants brought about some morphological differences in tetraploid plants as compared to their diploid counterparts. The tetraploids were stouter and possessed less number of leaves. Growth of the confirmed tetraploid plants of *C.asiatica* appeared to be

normal. However, an increase in leaf index and a decrease in length of the petiole were recorded in tetraploid plants. The leaves were also thicker and darker green in appearance than those of the diploids. A decrease in stolonification was also observed in the tetraploid plants.

18. As compared to the diploids, the tetraploids were found to have higher fresh weight and dry weight of the leaves, these being, on an average, 1.40±0.6g and 0.44±2.9g respectively. In diploids, the fresh weight and dry weight of the leaves was found to be 1.37±1.2g and 0.31±0.7g. Thus, there was a 1.02 and 1.41 fold increase in the fresh weight and dry weight of the tetraploids.

19. Moreover, a significant difference was found in the moisture percentage of the tetraploid and the diploid plants, where a reduction in the case of tetraploid plants was observed. Moisture percentage was found to be 77.3±1.8 in case of diploid plants and it was 68.5±2.5 in case of tetraploids. Thus, an increase in the biomass in induced polyploidy plants was registered.

20. Microscopic studies revealed that the average density of stomata in tetraploid plants was significantly lower than that of diploid plants. The leaves of the diploid plants were found to possess 183.2 stomata/square mm and there were 164.6 stomata/square mm in case of tetraploid plants. Also, the stomata of the tetraploid plants were larger than that of the diploid control plants. In this study, the average size of stomata in diploid plants was 31.4±0.6 - 24.8±1.5 µm (length - width), and that of tetraploid plants was 37.8±3.1 - 26.3±0.2 µm respectively. Hence, there was 1.20 fold increment in the length and 1.06 fold increase in the width of the stomata of the tetraploid plants of *C.asiatica*.

21. The total Chlorophyll content of the diploid and the tetraploid plants of *C.asiatica* were estimated by portable chlorophyll content meter and the chlorophyll content in the case of tetraploid was found to be 1.09 times higher than the diploid plant. Fv/Fm was found to be slightly (1.01 times) more in the tetraploid plant. PI reveals that the tetraploid plants possess 1.04 times more vitality as compared to the diploid plants.

22. CO_2 assimilation was measured by using TPS-2 IRGA Portable Photosynthesis System. Net photosynthetic rate of the plants was found to be significantly more in obtained tetraploids as compared to the diploids. It was found to be 9.8±0.4 μmol $CO_2/m^2/s$ in the tetraploids as against 4.1±0.2 μmol $CO_2/m^2/s$ in case of diploid plants. Thus, it was found to be 2.39 times higher in case of tetraploid plants.

23. Results obtained point towards the superior photosynthetic efficiency of the tetraploid plants of *C. asiatica*. These superior physiological parameters might be accountable for an increase in the biomass of the tetraploid plants.

24. The influence of ploidy levels on Asiaticoside biosynthesis was tested by HPTLC analysis of diploid and the obtained tetraploid plants. The results reveal that diploid and tetraploid forms of *C.asiatica* have different abilities to produce alkaloids. Higher content of Asiaticoside was recorded in the tetraploid plants i.e., 0.66% (dry wt. of *C. asiatica*). The diploid plants were found to contain 0.48% (dry wt. of *C. asiatica*) Asiaticoside. Thus, the tetraploid plants demonstrated a 1.37 fold increase in Asiaticoside content over the diploid plants.

25. The present study proves that tetraploids possess a better biosynthetic ability leading towards higher Asiaticoside accumulation in their leaves. Thus, the diploid and tetraploid forms of *C.asiatica* have different abilities to produce alkaloids. These differences could be attributed to superior morphological and physiological characteristics of the obtained tetraploids.

26. The findings establish the fact that the production of tetraploid plants of *C. asiatica* inflicts positive trends in both biomass and triterpenoid production. These results prove that, polyplodization is a promising strategy to increase yields of secondary metabolites of interest.

Asiaticoside in Callus and Cell culture

27. Leaf, stem and fruit explants were tested for callogenesis on MS medium containing several concentrations of auxins (IAA, NAA and 2,4-D) and

cytokinins (BA and Kin) alone and in combination. The explants failed to produce callus in the absence of exogenously provided growth regulators in MS medium.

28. Inclusion of low levels of Auxins in culture medium resulted in direct root formation from the leaf explants but higher levels resulted in callogenesis. Addition of 2,4-D in the medium was found to be most effective for callus formation from root explants.

29. Inoculation of leaf explant on MS medium containing cytokinin Kinetin (9.2 µM) produced 137 ± 1.6 mg DW callus biomass. Incorporation of BA was found to be more favourable for callus formation from leaf explant and addition of 17.74 µM BA resulted in the formation of 193 ± 2.3 mg DW callus.

30. Incorporation of 2, 4-D along with BA yielded the best results for callus formation from the leaf explants of *C. asiatica*. The callus formed was compact, dry and green in colour. MS medium supplemented with 9.05µM 2, 4-D along with 17.74 µM BA was found to be the most effective medium for extensive callogenesis from the leaf explant. The callus biomass obtained in this case was 239 ± 1.1 mg DW. The leaf explants were found to be more responsive for callogenesis as compared to the stem explants.

31. Callus from fruit explant of *C. asiatica* was initiated on MS medium supplemented with 0.90 µM 2,4-D and 1.78 µM BA.

32. Among the tried Auxins, NAA was found to be most favourable for rhizogenesis. Conjugation of low levels of Cytokinins BA and Kinetin with NAA resulted into an increase in the number of roots formed from the callus. The effect of Auxins was more or less same in case of stem and the fruit derived callus.

33. Treatment of leaf, stem and fruit derived callus with Cytokinins did not result in the formation of shoot. Presence of 8.87-17.74 µM BA and 9.29-18.58 µM

Kinetin resulted in the formation of green, hard and compact callus. However, on using low concentrations of Auxins along with the above concentrations of BA and Kinetin, the callus became more compact with concentrated green areas at few places in leaf callus. Also, light brown coloured small outgrowths were observed in case of stem derived callus. These neither got converted into roots, nor into shoots, on sub culturing on different concentrations and combinations of Auxins and Cytokinins. These outgrowths turned brown and perished eventually. The fruit callus also did not show any sign for caulogenesis.

34. Results for the accumulation of Asiaticoside in leaf, stem and fruit derived callus of *C. asiatica* reveal that callus obtained from fruit did not show the presence of Asiaticoside. On the other hand, all the cultures derived from both leaf and stem explants revealed the presence of Asiaticoside. But, its quantity varied depending upon the type of explant used as well as the type and concentrations of plant growth regulators employed. Best results for Asiaticoside production were obtained on MS + 9.05 µM 2, 4-D + 17.74 µM BA where the leaf derived callus was found to possess 0.201 ± 0.015 mg/g DW Asiaticoside.

35. The leaf derived callus cultured on MS medium supplemented with 9.05 µM 2,4-D and 17.74 µM BA yielded the best results for Asiaticoside and biomass production. Therefore, this combination of growth regulators was selected for the establishment of cell culture. Analysis of cell culture for Asiaticoside content showed that, maximum Asiaticoside accumulation was on day 21st, where 0.426±0.016 mg/g DW Asiaticoside content was recorded to be present in the cell biomass harvested. This was 2.12 times more as compared to the Asiaticoside produced in the callus.

36. From the present investigation, it is clear that *C. asiatica* cell cultures are capable of producing secondary metabolite of interest. However, Asiaticoside accumulation in cell cultures was comparatively less than the content in the *in vitro* raised shoot biomass.

Elicitation of Asiaticoside in Callus and Cell culture

37. The callus and cell-suspension cultures were subjected to different treatments of nutritional components, organic supplements and elicitors to study their effect on biomass and Asiaticoside production. The influence of different concentrations of elicitors on the growth and Asiaticoside content of callus and cell cultures of *C.asiatica* has been revealed.

38. Amongst the fortification of culture medium with varying concentrations of nutritional constituents like sucrose, calcium chloride and ammonium nitrate, the cell suspension and the callus cultures enriched with 4% sucrose exhibited the best results for Asiaticoside accumulation. Moreover, 30-40 mM calcium chloride and 20-30 mM ammonium nitrate were also found suitable for increasing the biomass and Asiaticoside content of the callus and cell cultures of *C. asiatica*.

39. Addition of organic supplements, glycine and casein hydrolysate enhanced the production of Asiaticosides in *in vitro* cultures. The results demonstrate that casein hydrolysate is more relevant additive than glycine for increased Asiaticoside production. Treatment of cell cultures with 100 mg/l Casein hydrolysate exhibited a 1.24 and 1.14 fold increase in the biomass and Asiaticoside content respectively, as compared to the control. In this investigation, it was found that 50-75 mg/l glycine, added into the culture medium was also responsible for an increase in biomass as well as an increase in Asiaticoside content.

40. Abiotic elicitors, sodium sulphate and particularly sodium chloride did not yield encouraging results for the growth of callus and cell biomass. In callus cultures supplemented with 0.10% sodium chloride, Asiaticoside content was found to be 1.13 times more than that obtained in case of the control. But at high levels, Asiaticoside content declined. Whereas in case of cell cultures, supplementation of the medium with 0.10 % sodium sulphate gave the best results for Asiaticoside production. A 1.12 fold increase in the Asiaticoside content, as compared to control was recorded in this case.

41. Treatment with increased level of Yeast extract also exhibited an increase in biomass and Asiaticoside production. In case of cell culture, a 1.36 and 1.18 fold increase in the biomass and Asiaticoside content respectively, as compared to control was recorded at 150 mg/l concentration of Yeast extract.

42. As a result of the present investigation, it was found that cell cultures yielded better results as compared to the callus cultures towards Asiaticoside elicitation. Among all the treatments, sucrose, the nutritional component at 4% (w/v) concentration was found to be most effective in increasing the Asiaticoside content. This was followed by treatment with 150 mg/l yeast extract, which acted as a biotic elicitor and 100 mg/l casein hydrolysate, which was added as an organic supplement.

The main highlight of present research is the *in vitro* production of tetraploid plants. The study reveals that, it is possible to produce the tetraploid plants of *C.asiatica* through *in vitro* culture by using anti mitotic agents, Colchicine and Oryzalin. 0.1% Colchicine treatment for 6 hrs was most favourable for the induction of polyploidy. The tetraploids were also obtained on inoculating shoot-tips on MS medium containing 15µM Oryzalin for a period of 7 days. Although, the treatment with Oryzalin was not found to be as effective as Colchicine, but the health hazards were significantly minimized by its usage.

Analysis of the obtained tetraploid plants has revealed their upgradation over their diploid counterparts, in terms of biomass production and higher content of medicinally important Asiaticoside. Comparative study of the Chlorophyll content and the Photosynthetic parameters of the diploid and the tetraploid plants proved that the superior physiological parameters of tetraploids might be accountable for an increase in their biomass. This in turn is desirable in *C.asiatica*, in which the vegetative parts are a source of medicinally important active compounds. Moreover, the Asiaticoside content can also be enhanced by the treatment of callus and cell cultures with elicitors.

Thus, it is possible to improve *C.asiatica* with enhanced production of secondary metabolites for crude drug preparation. This can considerably facilitate crop improvement, large scale propagation and conservation of this medicinally important herb and can help in increased availability of material for Ayurvedic and Pharmaceutical companies.