7. SUMMARY AND CONCLUSION

- Samples of Ashwagandha roots and Kalmegh whole plant were collected from Natural Remedies and Pentacare Ayur Pharma, Bangalore.

- Samples of both the plants were authenticated and subjected to gamma irradiation at doses of 5kGy and 10kGy in a Co-60 irradiator.

- Aqueous extracts of non-irradiated and gamma irradiated samples were prepared.

- Non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh were identified as,
  
  A1 – Ashwagandha non-irradiated sample,
  
  A2 – Ashwagandha gamma irradiated at a dose of 5kGy,
  
  A3 – Ashwagandha gamma irradiated at a dose of 10kGy and

  K1 – Kalmegh non-irradiated sample,

  K2 – Kalmegh gamma irradiated at a dose of 5kGy,

  K3 – Kalmegh gamma irradiated at a dose of 10kGy.

- All 6 samples were stored at room temperature and analyzed during 0, 6 and 12 months of storage.

- Non-irradiated and gamma irradiated samples of both Ashwagandha and Kalmegh were subjected to Quadruple 4 ‘P’ (Pharmacognostical, Physicochemical, Phytochemical and Pharmacological, toxicological) and Microbiological analysis by standard methods.

- Pharmacognostical analysis – Morphological and powder microscopical analysis indicated that there were no significant variations between non-irradiated and gamma irradiated samples in both Ashwagandha and Kalmegh.
 Physicochemical analysis – Ash values and extractive values of non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh did not show any significant variation and all the values were in compliance with standard monographs. However, there was around 20% reduction in moisture content of gamma irradiated samples. This may be due to the radiation sensitivity of the samples.

 Phytochemical analysis of all samples of both the plants showed that qualitatively, there were no changes in the chemical constituents present in non-irradiated and gamma irradiated samples of both the plants. Qualitative analysis of Ashwagandha aqueous extracts, showed the presence of alkaloids, carbohydrates, glycosides, phytosterols and saponins. Kalmegh aqueous extracts showed the presence of glycosides, phytosterols, saponins, flavonoids and diterpene lactones. On subjecting non-irradiated and gamma irradiated samples of Ashwagandha to quantitative analysis - total alkaloidal content, glycowithanolide and total withanolides were within the limits of standard monograph and further there were no significant differences amongst the samples, which indicates that gamma irradiation did not cause any change. Similarly, all samples of Kalmegh showed the presence of Andrographolide which was within the specified limit. There were no significant differences among non-irradiated and gamma irradiated samples.

 On subjecting the samples to IR fingerprint analysis, all samples of Ashwagandha showed the presence of OH and CH stretching, C=C – Alkenes and Alkynes and CH – Alkane functional groups and all samples of Kalmegh showed the following functional groups - OH (Stretching), CH – Alkanes, C=0 and C=C – Alkenes and Alkynes. There were no significant differences amongst samples of both the plants.
HPTLC method was developed for estimation of Withaferin A in Ashwagandha and Andrographolide in Kalmegh. The method was specific as it allowed good separation and hence allowed good resolution of Withaferin A and Andrographolide.

The developed HPTLC methods were validated in terms of Prevalidation, LOD, LOQ, Linearity, Precision and Accuracy studies and were found to be specific, sensitive, precise and accurate.

Prevalidation studies of Withaferin A and Andrographolide showed variation in peak areas with respect to time. From this, it is clear that it is preferable to carry out the analysis without storing the spotted or developed chromatogram for longer periods. Results of LOD and LOQ obtained showed that the developed method is highly sensitive. A good linearity curve was obtained for both the samples. Regression values obtained indicates good linearity between concentration and area. The methods appear to be precise and reproducible with good Coefficients of variation. The recovery values for both samples showed reliability and suitability of the methods.

Respective standards in non- irradiated and gamma irradiated samples of Ashwagandha and Kalmegh could be satisfactorily quantified by the developed and validated HPTLC method. Both non- irradiated and gamma irradiated samples (at doses of 5kGy and 10kGy) of Ashwagandha and Kalmegh showed similar amount of Withaferin A and Andrographolide respectively, which clearly indicates that gamma irradiation at doses of 5kGy and 10kGy does not interfere with respect to the chemical constituent present and also that, the developed and validated HPTLC method could satisfactorily quantify Withaferin A and Andrographolide in samples of Ashwagandha and Kalmegh respectively.
Pharmacological and Toxicological analysis: IAEC clearance was taken for the animal studies.

Acute oral toxicity study for non-irradiated and gamma irradiated samples of both Ashwagandha and Kalmegh was carried out according to OECD 425 guidelines in order to arrive at maximum tolerable dose of sample under study and to check for short term toxicity. The test was restricted to limit test in view of no mortality being observed within 2000mg/kg of body weight. All test animals survived the entire duration of observation (14 days – post administration). The animals were found normal throughout the course of test. In the light of above observation, it was concluded that test sample was safe upto 2000mg/kg body weight. Selection of dosage as 100mg and 200mg/kg body weight was based on this study, which represented 1/20\(^{th}\) and 1/10\(^{th}\) of 2000mg/kg. This dose is further used to evaluate the anti-stress activity of Ashwagandha samples and anti inflammatory activity of Kalmegh samples.

Pharmacological activity of Ashwagandha by Forced Swim Endurance test: To evaluate the anti-stress effects of non-irradiated and gamma irradiated (5kGy and 10kGy doses) samples of Ashwagandha aqueous extracts, forced swim endurance test was employed. Duration of swimming, immobility and climbing in Swiss albino mice was recorded. Mice were grouped into 4 groups of six animals in each group. The first group served as Control. Second group A1 - received aqueous extract of Ashwagandha non-irradiated samples. Third group A2- received aqueous extract of Ashwagandha gamma irradiated with a dose of 5kGy and the fourth group A3- received aqueous extract of Ashwagandha gamma irradiated with a dose of 10kGy. All animals were administered with the respective samples at a dose of 100mg/kg body weight one hour prior to analysis.
orally. Then they were made to swim for 5 minutes and during this 5 minute test, duration of swimming, immobility and climbing was noted down. It was observed that swimming duration in non-irradiated and gamma irradiated samples were statistically significant. It was also observed that there was maximum activity in all three samples (A1, A2 and A3) at the 5\textsuperscript{th} hour of sample administration. Further there was no change in activity among the three groups A1, A2 and A3 indicating that gamma irradiation has not affected the pharmacological activity of the samples.

**Pharmacological activity of Kalmegh – Anti-inflammatory activity by Carrageenan induced rat paw edema** - To evaluate the anti-inflammatory activity by Carrageenan induced rat paw edema, aqueous extracts of non-irradiated and gamma irradiated (5kGy and 10kGy doses) samples of Kalmegh, Wistar Albino rats were grouped into 5 groups of six animals in each. The first group served as Normal Control. Second group was treated with standard Diclofenac sodium 15mg/kg p.o. The third group was treated with non-irradiated aqueous extract of Kalmegh – 200mg/kg p.o.– K1. Fourth group was treated with gamma irradiated (5kGy) aqueous extract of Kalmegh – 200 mg/kg p.o.– K2 and the fifth group was treated with gamma irradiated (10kGy) aqueous extract of Kalmegh – 200 mg/kg p.o.– K3. All animals were injected with 0.1ml of freshly prepared carrageenan suspension, into sub plantar region of left hind paw to induce inflammation. Paw volume was measured in plethysmograph. There was a progressive increase in paw volume in the control group after injecting carrageenan and found to be maximum at the fourth hour. After this hour there was gradual decrease in paw volume.
At the onset of first hour, there was a decrease in the paw volume in all test and standard group when compared to the control group. The same was observed in 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th} and 5\textsuperscript{th} hour, however, maximum decrease was observed at the fourth hour in all the groups. From first to fifth hour, a significant reduction in paw volume (p<0.001) was observed, when compared to control groups at respective hours. Maximum % inhibition was at fourth hour in all three samples of Kalmegh. There was significant anti-inflammatory activity in Groups II, III, IV and V. However, the activity in Groups III, IV and V remained the same and did not show any significant change amongst non-irradiated and gamma irradiated groups. Thus it was observed that the anti-inflammatory activity of K1=K2=K3.

- **Microbiological analysis:** Non-irradiated and gamma irradiated samples (at dose of 5kGY and 10kGy) of Ashwagandha and Kalmegh were subjected to microbiological analysis according to IP, to check for Total Microbial load (Total Bacterial load, Total Fungal load) and for specific pathogens (*E.coli*, *Salmonella*, *Pseudomonas* and *Staphylococcus*). Results of total aerobic count and total fungal count indicate that both plants are highly contaminated. On testing for specific pathogens, both the plants were positive for presence of *E.coli* and *Staphylococcus*. Values exceeded drastically on storing it upto 12 months. *Escherichia coli* in medicinal plants could also be taken as indication of faecal contamination as well as the possible presence of enteric pathogens. *Staphylococcus aureus* possess a public health hazard due to production of thermostable enterotoxin that is responsible for food poisoning. The high contamination level could be attributed to the natural microflora of the herb as well as the general conditions during cultivation, harvesting, drying, handling, processing, storage, distribution and sales. Samples irradiated at 5kGy and 10kGy
had significantly lower levels of microbes than the non-irradiated (control) upto 12th month study. 5kGy could significantly lower the microbes to acceptable levels, however complete sterility could be attained at a dose of 10kGy. The killing effect of irradiation can be attributed to breakage of DNA molecules by direct or indirect methods. It may be due to the ionization of water, which results in forming highly reactive radicals such as H, OH etc. These free radicals split carbon bonds of macromolecules such as DNA in living organisms, thereby killing them. They also destroy the chemical bonds by interacting with electrons of atomic constituents.

- Pharmacognostical, physicochemical, phytochemical, toxicological and microbiological analysis was carried out subsequently after 6 and 12 months of storage at room temperature. There were no significant changes in any of these parameters in comparison to 0 month analysis. Pharmacological analysis of non-irradiated and gamma irradiated samples of both Ashwagandha and Kalmegh showed that the reported activity was further confirmed and gamma irradiation at the dose employed did not cause any change in bio molecules, confirming that gamma irradiation at the doses employed is not affecting the chemical constituents present. Further the microbial safety also could be maintained till 12 months of storage which indicates, that the shelf-life of gamma irradiated samples of both the plants can be extended upto 12 months of storage.
To conclude, as the irradiated samples were not toxic and since there is no significant changes in pharmacognostical, physicochemical phytochemical and toxicological parameters among non-irradiated and gamma irradiated samples at doses of 5kGy and 10kGy with retention of pharmacological activity in both plants, gamma irradiation can be chosen as a suitable technique to microbially decontaminate Ashwagandha and Kalmegh.

A dose of 5kGy could significantly reduce the microbial load to acceptable limits.

Commercial sterility could be attained at a dose of 10kGy for both the plants.

Gamma irradiated samples could maintain their stability up to 12 months, which clearly indicates that the shelf-life of the products can be increased up to 12 months.

Thus, gamma irradiation at doses of 5kGy and 10kGy helps in:

2. Ensuring microbiological safety.
3. Overcoming quarantine barriers to international trade.
4. Dosimetric release.
5. Single exposure, thus economical benefits.
6. Products can compete in global market.