6. DISCUSSION

*Withania somnifera* Dunal belonging to family Solanaceae, commonly known as Ashwagandha and *Andrographis paniculata* Nees belonging to family Acanthaceae, commonly known as Kalmegh are widely used medicinal herbs. Both the plants are indigenous and are widely grown throughout the country. Both have anti-cancer property and are thus required to be of utmost quality, since it will be consumed by immunocompromised patients. The intention of subjecting Ashwagandha and Kalmegh plant samples to gamma irradiation at doses of 5kGy and 10kGy was to reduce the microorganisms present in them and also to ensure that the sterile nature of the packed product is maintained for at least 12 months when stored at room temperature. Along with this main objective, it is also necessary to ascertain that the gamma irradiated medicinal plants does not show any change in pharmacognostical, physicochemical, phytochemical, toxicological and pharmacological parameters. The quality, safety and efficacy of these two medicinal plants are ascertained in this study. Further, the shelf-life extension studies are done upto 12 months of storing non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh.

Results obtained in this research, showed that there were no significant changes in pharmacognostical, physicochemical and phytochemical parameters during 0, 6 and 12th month of storage of samples of Ashwagandha and Kalmegh gamma irradiated at doses of 5kGy and 10kGy. Short term toxicological studies revealed, that gamma irradiation did not contribute to acute oral toxicity upto 12 months of storage after irradiation and thus found safe. Evaluation of pharmacological activity of both the plants showed that, the chosen reported activity of both the plants was further confirmed and also gamma irradiation at doses of 5kGy and 10kGy did not cause any
significant changes in the pharmacological activity, which shows that the bioactive molecules are intact.

Results of microbial analysis during 0, 6 and 12th month of storage at room temperature showed that gamma irradiation at a dose of 5kGy could reduce the level of microbes to acceptable limits and a dose of 10kGy could help in attaining commercial sterility.

Detailed discussion is given below:

**Standardization of phytomedicine**

In traditional system of medicine, the drugs are primarily used as such or as aqueous extract. The medicinal plants should be authentic and free from harmful materials like pesticides, heavy metals and microbes. A drug has to be thoroughly checked for its pharmacognostical, physicochemical, phytochemical, toxicological, pharmacological and microbiological parameters. The bioactive extract should be standardized on the basis of active compound. The bioactive extract should also undergo safety studies.

Relation to efficacy, wholesomeness and sensory attributes:

In this study, gamma irradiation at doses of 5kGy and 10kGy are chosen particularly for microbial decontamination of Ashwagandha and Kalmegh. Once the physical processes by which ionizing radiation loses energy to atom, it is the resultant formation and reaction of specific chemical entities that ultimately determines the destruction of contaminating microorganisms, the potential formation of a toxic compound, the retention of micronutrients, the retention of sensory attributes and even the retention of package functionality. Microorganisms are destroyed primarily because hydroxyl radicals formed within their cells react with the base and sugar moieties of DNA, which results in breakage of sugar phosphate bonds and loss of the replication function. Micronutrients, in particular vitamins, will be degraded to an
extent that will depend both upon their ability to compete against other major constituents for the primary radicals and upon the irradiation conditions, including dose. Sensory attributes, such as flavour, colour and texture, will similarly be affected if the constituents normally associated with these attributes can effectively compete for the primary radicals and then follow a reaction pathway that leads to a stable product with different sensory characteristics. Package functionality might be favourably or unfavourably affected by the competition between bond-breaking and bond-making reactions, which are influenced by the chemical structure of the material and irradiation conditions.

**Pharmacognostical, Physicochemical and Phytochemical analysis:**

The macroscopic and the microscopic evaluation of any plant drug are considered to be the primary steps for establishing its quality control profile and according to WHO\(^\text{10}\), botanical standards should be proposed as a protocol for the diagnosis of the herbal drug.

The histochemical studies give a preliminary idea about the type of compounds and their accumulation in the plant tissues. Thus, helps us in finding out if there are any changes in particular parts or tissue of that plant.

Role of World Health Organization (WHO) in phytomedicine medicine: In 1991 WHO developed guidelines for the assessment of herbal medicine and the 6th International Conference of Drug Regulatory Authorities held at Ottawa in the same year ratified the same. The salient features of WHO guidelines are:

1). Quality assessment: of crude plants or extract - plant preparation and finished product.

3). Safety assessment: Documentation of safety based on experience and toxicological studies.


➢ Results of pharmacognostical, physicochemical and phytochemical analysis showed that there were no significant changes in gamma irradiated samples of both Ashwagandha and Kalmegh upto 12th month of study. Morphological and powder microscopical analysis showed that non – irradiated and gamma irradiated samples (at doses of 5kGy and 10kGy) of both Ashwagandha and Kalmegh did not show any significant changes and the results were in concordance with Indian Herbal Pharmacopoeia.

➢ Results obtained from physicochemical analysis showed that, there were no changes among the non-irradiated and irradiated groups of both the plants upto 12th month of study. The values obtained for Ash value (total Ash, Acid insoluble ash) and Extractive values (water soluble extractive and alcohol soluble extractive) were in accordance with Indian Herbal pharmacopoeia. However, the results of moisture content showed that there was around 20% reduction in the moisture content of gamma irradiated samples of both the plants. Variation in moisture content as observed in present study could presumably be due to radiation sensitivity of these samples at the dose employed. Such type of change has also been reported by Gayawali et al.168.
Phytochemical:

Active principle identification and standardization

The identification of biologically active compounds is an essential requirement for quality control and dose determination of plant-based drugs. A medicinal herb can be viewed as a synthetic laboratory as it produces and contains a number of chemical compounds. Those compounds, responsible for medical activity of the herb, are secondary metabolites. Complete phytochemical investigations of most of the medicinally important herbs of India have not been carried out so far. This would be beneficial in standardization and dose determination of herbal drugs. Further, there should be quality control tests for the entire preparation to ensure the quality of the drug.

Results of phytochemical analysis showed that, there were no significant changes in the functional groups and chemical constituents present in both the plants upto 12th month of study. Qualitative analysis of Ashwagandha showed that the non-irradiated and gamma irradiated (dose of 5kGy and 10kGy) samples contained alkaloids, carbohydrates, glycosides, saponins and phytosterols. Amount of alkaloids, total withanolides and glycowithanolides present in all three samples, did not show any significant change and the readings were in concordance with standard books3,4,42. In all three samples of Kalmegh, the constituents present were glycosides, saponins, phytosterols, phenols, tannins, flavonoids and diterpenes, which is also reported earlier4,6,7,70. Further, quantitative analysis showed that, the amount of total bitters present in all samples of Kalmegh were almost similar, which again indicates that gamma irradiation at a dose of 5kGy and 10kGy does not cause any change in the actives present.
The fingerprint spectra’s of non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh showed that there were no changes in prominent functional groups in the IR spectra’s obtained during 0, 6 and 12 month analysis. The fingerprint spectra’s of A1, A2 and A3 shows the presence of OH and CH stretching, C=C – Alkenes and Alkynes and CH – Alkane groups. Similarly, the finger fingerprint spectra’s of K1, K2 and K3 shows the presence of OH stretching, CH – Alkanes, C=0 and C=C – Alkenes and Alkynes. As there are no significant changes in the samples, it clearly indicates that gamma radiation at a dose of 5kGy and 10kGy is not causing any changes in the functional groups of the chemical constituents.

➢ HPTLC analysis

Development of HPTLC method

➢ Herbal drugs are very complex and have a great deal of variation. The extracts are prepared in a variety of ways. But the routine quality control analysis is very difficult. Hence, there is a need to develop simple, accurate and sensitive techniques for analysis of crude drugs and formulations.

➢ Chromatography is a preferred technique, since it can resolve the formulation/extract into individual components for identification and quantification. High performance thin layer chromatography is the most widely used chromatographic method for analysis of herbal drugs. It is one of the most simple, reliable and sensitive method that allows detection and quantification of phytoconstituents present in crude drugs and single and poly herbal formulations. Analysis of herbal drugs by HPTLC is comparatively best as most of the plant extracts and formulations contain a number of constituents. It can be separated into distinct bands and quantified by scanning at different wavelengths. The HPTLC data include fingerprinting, identification of active constituents, determination of impurity and quantitative assays.
Withaferin A is one of the major active constituent in Ashwagandha. Pharmacopoeia and other monographs mention Withaferin A as the marker compound of Ashwagandha\textsuperscript{3,4,42}.

Andrographolide is reported as the marker compound of Kalmegh\textsuperscript{6,7,70}.

There are very few references for estimation of Withaferin A as standard by HPLC and HPTLC method\textsuperscript{45}. But, validations of these methods are not reported. Estimation of Andrographolide in Kalmegh is also reported in the literature\textsuperscript{169,170}. However, no thorough validation studies are documented. Hence in the present study an attempt was made to develop and validate HPTLC method for different applications.

Simple, sensitive and reliable methods were developed. Different ratios of mobile phases were tried and the one, which allowed good separation and gave compact dense spots, was chosen. Spectrum analysis was done to confirm \( \lambda \text{ max} \). Appropriate derivatizing reagent was selected for post chromatographic derivatization of the chromatograms. The developed methods gave good resolution.

**Validation of HPTLC method**

Suitability of any analytical procedure for its intended use must be based on objective validation data. Numerous papers have been published addressing analytical validation.

In the present study, the two HPTLC methods developed were validated using the parameters described in standard texts and ICH\textsuperscript{163,164} guidelines.

Prevalidation studies of Withaferin A and Andrographolide showed variation in peak areas with respect to time. In solution stability, peak areas increased as time increased, which may be due to evaporation of the solvent on the plate. In case of plate stability, peak area decreased with increase in time, which may be due to degradation of the
product on exposure to atmosphere. This indicated that there is variation in peak areas. Hence it is preferable to carry out the analysis without storing the spotted or developed chromatogram for longer periods.

- The HPTLC methods developed were further validated for Limit of Detection, Limit of Quantification, Linearity, Precision and Accuracy.

- LOD and LOQ values of Withaferin A and Andrographolide obtained were much lower (in nanogram concentration) compared to other literatures\textsuperscript{45,169}. This indicates that the HPTLC methods developed are highly sensitive.

- The method gave a good linearity curve in the range of 500-900ng for Withaferin A and 100-500ng for Andrographolide. Regression values of 0.99883 and 0.99998 respectively indicate good linearity between concentration and area.

- The methods appear to be precise and reproducible with good Coefficient of variation.

- The recovery values for both samples showed the reliability and suitability of the methods. Recovery values for Withaferin A and Andrographolide was found to be 100.20% and 99.82%. These values are in accordance with ICH\textsuperscript{163} limits, which is 80% - 120%.

**Application of validated HPTLC method**

The method was able to satisfactorily quantify the standards in non-irradiated and gamma irradiated (at doses of 5kGy and 10kGy) samples of Ashwagandha and Kalmegh. There were no significant differences among the non-irradiated and gamma irradiated samples at both doses in Ashwagandha and Kalmegh samples. The amount of the marker compound present in all three samples of both plants was almost similar. The Rf values obtained for Ashwagandha samples was about 0.49 and Kalmegh was 0.38. Further, the AUC of all samples did not significantly differ. This
clearly shows that gamma irradiation at doses of 5kGy and 10kGy is not causing any changes in chemical constituents present in both the plant samples upto 12 months of storage. As the biomolecules are intact, it can be ascertained that, there may be retention of therapeutic activity of the plants until this duration.

Thus gamma irradiation at a dose of 5kGy and 10kGy does not cause any significant change in pharmacognostical, physicochemical or phytochemical parameters upto 12 months of storage. This conclusion is in concordance with some literatures. Various physiochemical and sensory characters of the plants were observed after irradiation and the method was found to be suitable by many authors\textsuperscript{129-131}. Chemical, sensory and microbiological changes of gamma irradiated coconut cream powder was studied by Norimah Y et al\textsuperscript{139}. Samples were gamma irradiated between 0-15kGy. Sensory characters like taste, odour, and overall acceptance were studied. Microbiological studies were carried out and no colonies were detected after irradiation. Based on the results, gamma irradiation of 5kGy was found to be the optimum dose to decontaminate the sample. Al-Bachir\textsuperscript{140} reported that when seeds of *Pimpinella anisum* were gamma-irradiated at doses of 0-20kGy and evaluated after 0, 6 and 12 months of storage, their aerobic plate counts and sensory characteristics were improved. He has also studied the effects of gamma irradiation from 0-2.5kGy on fungal load, chemical and sensory characteristics of *Juglans regia* L\textsuperscript{141}. Fungal load, proximate composition, chemical changes and sensory properties were evaluated immediately after irradiation and after 12 months of storage. Results indicated that gamma irradiation reduced the fungal load and there was no significant difference in chemical and sensory characters. But after 12 months of storage, decreased total acidity and peroxide values and increased iodine value and volatile basic nitrogen were observed and also there was a negative effect on sensory characteristics. On
studying the effects of gamma-irradiation at doses of 0-20kGy on microbiological, chemical and sensory characteristics of licorice root powder by Al Bachir et al\textsuperscript{142} at 0 and 12 months of storage, it was observed that gamma-irradiation reduced the counts of micro-organisms in the samples. However, mineral ions from irradiated products were lower and glycyrrhizinic acid and maltose concentration were higher and there was no significant differences in sensory characters. Lycium fruit was exposed to several doses of gamma-irradiation (0-14kGy) by Hsiao-Wei et al\textsuperscript{144} to evaluate microbial decontamination efficiency, changes in chemical composition and sensory characteristics. After 10kGy of irradiation \textit{Bacillus cereus} was the only survivor. It was found that 14kGy is the optimal decontamination dose for Lycium fruit for retention of its sensory quality and extension of shelf-life.

- Some reports also showed that gamma irradiation is not causing any changes in major chemical constituents present\textsuperscript{172,173}. The same results are obtained in this research also. Gupta \textit{et al.}\textsuperscript{174} studied the effect of gamma irradiation (7.5-10kGy) on major fatty acids (Oleic, linoleic, linlenic, palmitic, stearic), saponins including Ginsenosides, (major effective components) in ginseng-red powder. No effect of irradiation on major fatty acid composition and no significant changes in saponins concentration was observed. Extensive research has shown that proteins, essential amino acids, minerals, trace elements and most vitamins do not represent significant losses during irradiation even at doses over 10kGy\textsuperscript{175,176}.

- Lots of researches are done to find out the efficacy of gamma irradiation on biologically active substances like flavonoids, anthocyanins, essential oils, glycosides, triterpenes, saponins, oleanosides and plants mucus. These bioactive compounds did not change significantly after irradiation. Pharmacological activity of medicinal herbs has been found satisfactory after microbiological decontamination by irradiation\textsuperscript{122}. 

\textit{Department of Pharmacognosy, \textit{KLE University’s College of Pharmacy, Bangalore}}
Koseiki et al\textsuperscript{156} reported that phytotherapy showed identical therapeutical action as non-irradiated preparations after exposure to a dose of 10, 20 and 30kGy of ionizing radiation. Irradiation of traditional medicines and herbal products did not result in any negative chemical changes or important losses of active components. It was shown that, after irradiation up to 17.8kGy, the content of the main biologically active substances of two medicinal herbs (ginkgo and guarana) was not modified\textsuperscript{157}.

**Results of short term acute oral toxicity** studies showed that, all test animals survived the entire duration of observation (14 days – post administration). The animals were found normal throughout the course of test and hence it can be concluded that test sample was safe up to 2000mg/kg body weight. Non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh did not show any signs of toxicity, which clearly indicates that the samples are safe. The same results were observed even during 6 and 12\textsuperscript{th} month analysis after irradiation of both Ashwagandha and Kalmegh samples. Thus, there is no short term toxicity of both plants at the radiation dose employed.

**Results of pharmacological activity showed that –**

a. **Ashwagandha** – Anti-stress activity of the plant was studied by Forced Swim Endurance Test.

It is commonly accepted that FST is currently a popular model, due to low cost of the experiments and because it is arguably the most reliable model available for acute drug treatments. In the present study, forced swim test in mice behavioral despair model was selected to evaluate the claimed antistress activity of Ashwagandha and also to check if gamma irradiation at the doses employed interferes with the activity. In this test, a mouse is forced to swim for 5 minutes in a cylinder of water from where
there is no escape and the animal’s behavior is measured. Initially, the animal displays escape oriented behaviors, however, their behavior changes eventually into movements that are just sufficient to keep their head above water – termed immobility. This was originally interpreted by Porsolt et al\textsuperscript{56} as “behavioral despair” such that the animal has lost the motivation to perform escape oriented behavior. Activity and immobility in the FST can be interpreted as representing alternately active and passive behavioral reactivity to stress. Antistress drugs, of all major classes reduce immobility and increase active behaviors like swimming in forced swim endurance test. Furthermore, the scoring behaviors of swimming gives additional information about the mechanism of the action that mediates the antistress like effects. Swimming is mediated by serotoninergic neurotransmission. In view of such opinion, the study employed all the above mentioned behavioral scores.

The effect continues to be statistically significant in non-irradiated aqueous extract of Ashwagandha when compared to vehicle treated animals. Reduction in immobility and increased time spent in swimming is a clear indication of antistress activity.

Since, swimming behavior was significantly influenced (increased), it is likely, that the antistress activity may also be involving serotoninergic neurotransmission. 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 5th hour of sample administration. Further there was no change in activity among the 3 groups A1, A2 and A3 indicating that gamma irradiation at doses of 5kGy and 10kGy is not interfering with the bioactive molecules responsible for the pharmacological activity of the samples. This is in accordance with some literatures. There was maximum pharmacological activity at
the 5\textsuperscript{th} hour of administration. The same results were also observed with Ashwagandha samples gamma irradiated at doses of 5kGy and 10kGy.

From all above mentioned observations taken together, it can be speculated that gamma irradiation at the dose employed is not interfering with the bioactive molecules present in the plant and hence can be used to deliver the primary motive of gamma irradiation being microbial decontamination of Ashwagandha and Kalmegh.

It is also previously investigated by Archana et al\textsuperscript{55} using adult Wistar strain Albino rats and cold water swimming stress test. The results indicated that the animals treated with Ashwagandha showed better stress tolerance. In another study, adaptogenic activity of an herbal formulation containing Ashwagandha was investigated in terms of Anti-stress activity. Sub-chronic administration of the formulation containing Ashwagandha for 7 days, in the doses of 5 and 10ml/kg, orally, showed increased swimming endurance under adverse ambient conditions, which clearly indicates that Ashwagandha has significant anti-stress activity\textsuperscript{57}. Salil Bhattacharya et al\textsuperscript{58} has also studied about anti-stress activity of few compounds including Withaferin A from Withania somnifera. It was observed that sitoindosides also produced anti-stress activity, which was potentiated by Withaferin-A. It was also observed from preliminary acute toxicity studies that the compounds have a low order of toxicity.

b. Kalmegh - Anti-inflammatory activity of the plant was studied by Carrageenan induced rat paw edema.

Carrageenann induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compound of natural products. More over the experimental model exhibits a high degree of reproducibility. Carrageenan induced edema is a biphasic event. The first phase is attributed to the release of
histamine, serotonin and kinins. The second phase of edema is due to release of prostaglandins (PG), protease and lysosome. The second phase is sensitive to most clinically effective anti-inflammatory drugs.

The results of the present study indicate the role of Kalmegh against carrageenan induced acute inflammation. Both standard and the test extracts suppress the biphasic response of carrageenan-induced inflammation.

The anti-inflammatory effect of Kalmegh may be due to suppression of prostaglandin, protease or lysosome synthesis or activity. Another reason could be that the Flavonoids present in Kalmegh may be responsible for this activity. Flavonoids are widely distributed in plant kingdom and possess various pharmacological activities like anticancer, anti-viral, anti-inflammatory, immunomodulatory, antithrombotic effects etc. Among these activities, anti-inflammatory capacity of flavonoids has long been utilized in Traditional Chinese medicine and cosmetic industries. Flavonoids possess antioxidative and free radical scavenging activities. They could regulate cellular activities of the inflammation related cells macrophages, mast cells, lymphocytes and neutrophils. Some flavonoids inhibit histamine release from mast cells and other T-cells proliferations. Certain flavonoids modulate the enzyme activities of arachidonic acid, metabolizing enzymes such as phospholipase A₂, Cyclooxygenase and Lipooxygenase and Nitric oxide producing enzyme - Nitric oxide Synthase. An inhibition of these enzymes by flavonoids reduces the production of the above enzymes.

Proposed possibility of anti-inflammatory property of flavonoids is their ability to inhibit neutrophil degradation. This is a direct way of diminishing release of arachidonic acid by neutrophils and other immune cells.
As a plant extract, flavonoids could improve symptoms of acute inflammatory as well as chronic inflammatory disorders. Since, flavonoids are found in Kalmegh, the anti-inflammatory effect of K1, K2 and K3 can be attributed to the presence of flavonoids. The reported anti-inflammatory activity was further confirmed by this research. There was a progressive increase in paw volume in the control group after injecting carrageenan and found to be maximum at the fourth hour. There was significant anti-inflammatory activity in all three samples (K1, K2 and K3). Maximum % inhibition was observed in all three Kalmegh samples at fourth hour and was significant. However, the activity among the three groups remained the same and did not show any change. Thus it was observed that the anti-inflammatory activity of K1=K2=K3. From this, it can also be concluded that gamma irradiation at doses of 5kGy and 10kGy is very low and does not interfere with the anti-inflammatory activity of the plant. Kalmegh is known to be a potent anti-inflammatory agent by several authors. Tajuddin et al\textsuperscript{79}, has studied anti-inflammatory activity of aqueous extract of \textit{Andrographis paniculata} by carrageenan induced oedema. The extract showed significant anti-inflammatory activity in the doses of 20 mg/100 gm body weight orally. Lin et al\textsuperscript{80} in his study observed that at a dose of 100 mg/kg, aqueous extract and andrographolide showed antioedema and analgesic activities in Kalmegh. Medicinal Plant Research Institute, Ministry of Public Health, Thailand\textsuperscript{81} found anti-inflammatory activity of \textit{Andrographis paniculata} in rats using method for testing carrageenan-induced hind paw edema. Dried powder, ethanolic and water extract of \textit{Andrographis paniculata} given orally to rats showed anti-inflammatory property when tested by carrageenan-induced hind paw edema and inhibition of white blood cells, infiltration and granuloma development were reported by actions of these extracts. Mechanism of anti-inflammatory actions is also reported. Anti-
inflammatory action of *Andrographis paniculata* is related to i) inhibition of nitric oxide (NO) production from inflammatory macrophages by neoandrographolide ii) inhibition of NO production by decreasing expression of inducible nitric oxide synthase (iNOS) in macrophages by andrographolide and iii) inhibition of neutrophil adhesion and transmigration and by prevention of reactive oxygen species.

#### Results of microbiological analysis during 0, 6 and 12th month of storage –

Herbs are naturally contaminated with high numbers of bacteria and fungi. Avoiding high levels of microbial contamination in herbs may be impossible. Herbs are relatively sensitive to treatment of any kind and are particularly damaged by ethylene dioxide treatment. Irradiation is an effective technology for resolving technical trade issues for many herbal products. As a disinfestation treatment, it offers the possibility of targeting different levels of quarantine security. As a microbial decontamination treatment, it offers good broad-spectrum control of many pathogenic organisms with minimal change to the herbs.

The biological effects of ionizing radiation on cells can be due both to direct interactions with critical cell components and to indirect actions on these targets by molecular entities formed as a result of the radiolysis of other molecules in the cell, particularly by radicals formed from water. As with other antimicrobial measures, the response of a microbial cell, and hence its resistance to ionizing radiation, depends on:

-- the nature and amount of direct damage produced.

-- the number, nature and lifetime of radiation-generated reactive chemical entities and the inherent ability of the cell either to tolerate radiation damage or to repair it accurately.
the influence of the intracellular and extracellular environments on the above factors.

Therefore, any attempt to categorize or compare the radiation resistance of microorganisms is only meaningful when all related conditions are precisely defined and understood.

Ionizing radiation is capable of causing a variety of chemical changes in microorganisms. It is generally assumed that DNA is the most critical target of ionizing radiation and that the inactivation of microorganisms by ionizing radiation is a result of damage to their DNA.

Ionizing radiation can affect DNA either directly, by energy deposition in this macromolecule, or indirectly, by energy deposition in the surrounding water leading to the formation of diffusive primary radicals, including hydrogen atoms (H.), hydroxyl radicals (OH.) and solvated electrons (es -- ). OH radicals formed in the hydration layer around the DNA molecule are responsible for 90% of the damage. Consequently, in living cells, the indirect effect is especially significant.

The principal effect induced in DNA is chemical alteration to the purine and pyrimidine bases and to the deoxyribose component, resulting in a break in the phosphodiester backbone in one strand of the molecule (single-strand break) and, to a lesser extent (5--10%) to breaks in both strands in close proximity (double-strand break). Both prokaryotes (bacteria) and eukaryotes (moulds and yeasts) are capable of repairing many of the different breaks. It is generally believed that microorganisms that are sensitive to radiation cannot repair double-strand breaks, whereas radiation-resistant species have some capacity to do so. Effects on the plasma membrane appear to play an additional role in radiation-induced damage to cells.
The primary target in the radiation sterilization is the DNA of foodborne bacteria. When this is damaged, the bacterium is eliminated within a few cell divisions. The molecular weight of DNA, far exceeds that of all other molecules in the living cell; hence, its energy absorption is the highest. Although this unique molecule is highly durable to gross radiation damage, owing to its aromatic groups, heterocyclic rings, hetero-atom rich backbone, and the double-helix structure bridged by a multitude of hydrogen bonds, certain base moieties can be affected, possibly leading to rupture of a sugar-phosphate linkage in a single strand. The radiation durability of DNA, particularly in the low-moisture environment within a spore, means that high doses of radiation are required to achieve sterilization, even after the heat pretreatment given to inactivate proteolytic enzymes. However, it is possible to attain the goal of damaging bacterial DNA without adversely affecting the product or the packaging.

On subjecting non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh to microbiological analysis, total aerobic count and total fungal count indicates that both plants are highly contaminated. On testing for specific pathogens, both showed positive for presence of *E*. *coli* and *Staphylococcus*. Values exceeded drastically on storing it upto 12 months, which were much beyond the standard acceptable limits. Such shelf-life studies were the microbial load is increased with time has also been reported.

The results of the bacterial count indicated that the non-irradiated samples of Ashwagandha and Kalmegh were highly contaminated with bacteria at the levels of $4.4 \times 10^4$, $4.9 \times 10^4$, $5.0 \times 10^4$ and $1.0 \times 10^3$, $1.4 \times 10^3$, $1.6 \times 10^3$ cfu/gm at 0, 6 and 12 months of storage respectively. Results of Total fungal count also indicated that the non-irradiated samples of Ashwagandha and Kalmegh were highly contaminated with
fungus at the levels of $3 \times 10^4$, $3.7 \times 10^4$ and $3.7 \times 10^4$ and $1.0 \times 10^2$, $1.2 \times 10^2$, $1.3 \times 10^2$ cfu/gm, respectively. These values exceeded the level allowed by WHO (1998) and European Pharmacopoeia as the maximum permissible total count level. The high contamination level could be attributed to the high natural micro flora of the herbs as well as the general conditions during their cultivation, harvesting, drying, handling, processing, storage, distribution and sales. However, it was reported that the microbial status of dried herbal material is not so much caused by secondary contamination during processing, but it is primarily due to the fact that plants have their own microbial flora.

The samples irradiated with 5 and 10kGy of gamma radiation had significantly lower bacterial counts than the non-irradiated (control). The Microorganisms are killed by direct or indirect damage to its DNA. The propagation of life is arrested due to DNA impairment by the ionizing radiation either by direct or indirect effect.

- Direct Effects: The damage to DNA may be as single breaks, double breaks, base damage, intra-or intermolecular crosslink formation.
- Indirect Effects: The killing effect of irradiation can be attributed to the ionization of water, which results in forming highly reactive radicals such as H, OH etc. These are due to the free radicals formed due to radiolysis of water: Hydroxyl, Hydrated electrons, Hydrogen atoms, Hydrogen molecule, Hydrogen peroxide and hydrated proton, which 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. The energy required depends on the number and radiation resistance of the micro-organisms in the mixed population.

The specific pathogens present in non-irradiated samples of both the plants were *E. coli* and *Staphylococcus aureus*. *Escherichia coli* in medicinal plants could also be
taken as indication of faecal contamination as well as the possible presence of enteric pathogens\textsuperscript{177,178}. \textit{Staphylococcus aureus} possess a public health hazard due to production of thermostable enterotoxin that is responsible for food poisoning\textsuperscript{177}. 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\textsuperscript{99}. However, a dose of 5kGy itself could completely reduce the specific pathogens to zero. This microbial quality was maintained upto 12 months of storage. The same was with a dose of 10kGy.

On the whole, the complete microbial quality in samples irradiated at 5kGy and 10kGy were significantly lower than the non-irradiated (control) even at 12\textsuperscript{th} month study\textsuperscript{131,179}. 5kGy could significantly lower the microbes to acceptable levels, however complete sterility could be attained at a dose of 10kGy.

The technique was best utilized for extension of shelf-life upto a period of 12 months, maintaining superior microbiological quality. Such decrease in microbial load of other plant materials following irradiation is also reported by several researchers. A dose of 5kGy was shown to reduce the aerobic populations of aniseeds to an acceptable level\textsuperscript{140}.

However, gamma radiation at a dose greater than 10kGy was required to achieve commercial sterility (i.e. a total aerobic plate counts of <10 per gram), according to IAEA (1992)\textsuperscript{180}. On testing for specific pathogens, non-irradiated samples of both Ashwagandha and Kalmegh were positive for \textit{E.coli} and \textit{Staphylococcus aureus}. According to literature data, these organisms are relatively sensitive to irradiation and in most cases, a dose of about 5kGy is sufficient for their elimination\textsuperscript{119}.

The results of the effect of gamma irradiation on the microbial and medicinal quality of Ashwagandha and Kalmegh upto 12 months of storage, support the concept that
gamma irradiation process is chemically inert, and could be used for the sterilization of these medicinal herbs. Thus, irradiation improves the microbial safety and maintains this up to 12 months of storage. It may emerge as one of the important techniques for preserving or improving the microbial and medicinal quality of Ashwagandha and Kalmegh.

**Limitations of the present research are:**

- Long term stability studies need to be done.
- Isolation of all possible microflora present in the plant samples has to be done.
- Gamma Irradiation of polyherbal formulations need to be studied in detail.
- Validation at the dose employed needs to be done and documented.

**Attitude towards gamma irradiation**

The main barrier that is identified for acceptance of gamma irradiation of medicinal plant is skepticism about its toxicity and reduction in activity of the plant. But the results obtained from the research helps to fade away such issues.

**Future scope**

- Commercial utilization of gamma irradiation technique in industries for microbial decontamination of medicinal plants and their phytopreparations.
- Global Competency of the product.
- This technique is widely accepted by FDA because of its dosimetric release of the products. (No post sterility test required).
- Economical (Single exposure is sufficient).