9. ANNEXURES

ANNEXURE – I – MARKET SURVEY

QUESTIONNAIRE – PART I

Topic: Efficacy of gamma-irradiation on microbial safety and medicinal quality of Ashwagandha and Kalmegh.

Respected Sir/Madam,

I AM A RESEARCH SCHOLAR, DOING A PROJECT AT KLE COLLEGE OF PHARMACY, ON “EFFICACY OF GAMMA-IRRADIATION ON MICROBIAL SAFETY AND MEDICINAL QUALITY OF ASHWAGANDHA AND KALMEGH”. THIS IS A SMALL SURVEY CONDUCTED TO IMPROVE SHELF LIFE OF MEDICINAL PLANT PRODUCTS AND TO BRING MORE CONFIDENCE IN PATIENTS USING HERBAL MEDICINES. FOR THIS PURPOSE, IT WOULD BE GRATEFUL IF YOU KINDLY SPARE SOME OF YOUR VALUABLE TIME AND MARKET EXPERIENCE TO ANSWER THE QUERIES. THE INFORMATION PROVIDED TO US IS FOR ACADEMIC PURPOSE AND WOULD BE KEPT CONFIDENT WITH UTMOST CARE.

THANKING YOU,

MAMATHA.A,
RESEARCH SCHOLAR (PhD)

ABOUT THE RESPONDENT:

NAME OF THE PHARMACIST:

DESIGNATION:

EXPERIENCE:

NAME AND ADDRESS OF PHARMACY:

SIGNATURE:
ANNEXURE – I

QUESTIONNAIRE – PART II

1. Are Ashwagandha products sold in your outlet?
   Yes ☐ No ☐

2. Are Kalmegh products sold in your outlet?
   Yes ☐ No ☐

3. What is the percentage of sale of Ashwagandha products?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

4. What is the percentage of sale of Kalmegh products?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

5. What is the percentage of sale of Ashwagandha – single formulation products?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

6. What is the percentage of sale of Kalmegh – single formulation products?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

7. As per your experience - what is the demand for Ashwagandha products in the market?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

8. As per your experience - what is the demand for Kalmegh products in the market?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

9. Is there anytime, out of stock of Ashwagandha products?
   a. Yes ☐
   b. No ☐
   c. Seldom ☐
   d. Often ☐

10. Is there anytime, out of stock of Kalmegh products?
    a. Yes ☐
    b. No ☐
    c. Seldom ☐
    d. Often ☐

11. Does any product of Ashwagandha remain after its expiry period?
    a. Yes ☐
    b. No ☐
    c. Rarely ☐
    d. Often ☐
12. Does any product of Kalmegh remain after its expiry period?
   a. Yes [ ]
   b. No [ ]
   c. Rarely [ ]
   d. Often [ ]

13. If so, what is usually done to the expiry products?
   a. Discarded [ ]
   b. Kept as such [ ]
   c. Sent back to suppliers [ ]
   d. Sold to customers [ ]

14. Has any product changed physically before their expiry periods?
   a. Sometimes [ ]
   b. Many times [ ]
   c. Not observed [ ]
   d. Not at all [ ]

15. Has any product changed physically after their expiry periods?
   a. Sometimes [ ]
   b. Many times [ ]
   c. Not observed [ ]
   d. Not at all [ ]

16. Has any product shown microbial growth before their expiry periods?
   a. Sometimes [ ]
   b. Many times [ ]
   c. Not observed [ ]
   d. Not at all [ ]

17. Has any product shown microbial growth after their expiry periods?
   a. Sometimes [ ]
   b. Many times [ ]
   c. Not observed [ ]
   d. Not at all [ ]

18. Do you use any special method for storage of Ashwagandha products?
   a. Storing at room temperature [ ]
   b. Storing in dark [ ]
   c. Refrigeration [ ]
   d. No specific methods [ ]

19. Have you received any market complaint with respect to Ashwagandha product?
   Yes [ ]
   No [ ]

20. Have you received any market complaint with respect to Kalmegh product?
   Yes [ ]
   No [ ]

21. What type of complaint have you received?
   a. Complaint related to microbial contamination [ ]
   b. Complaint related to inefficiency of product [ ]
   c. Complaint related to quality [ ]
   d. Complaint related to appearance [ ]

22. Would it be better if the drug is microbially decontaminated and has increased Shelf life?
   a. Yes [ ]
   b. No [ ]
   c. Don’t know [ ]
   d. Other comments ---------------------------
TO WHOM SO EVER IT MAY CONCERN

This is to certify that the samples procured from Natural Remedies Pvt. Ltd, Bangalore by Smt. Mamatha A, Lecturer, Department of Pharmacognosy, K.L.E.S’s College of Pharmacy, Rajajinagar, Bangalore for the purpose of her Ph.D research work are the dry samples of Ashwagandha roots - Withania somnifera(L.) Dunal and Kalmegh dry herb - Andrographis paniculata (N. Burman) Wall. ex Nees as per my knowledge and experience in this field.

(M. VASUNDHARA)

Dr. Vasundhara M, Ph.D (Hort)
Professor
Division of Horticulture
UAS, GKVK, Bangalore - 560 065
ANNEXURE – III

Ms. Mamatha,
Research Scholar
K.L.E. University
Department of Pharmacognosy,
K.L.E. Society’s College of Pharmacy,
2nd Block, Rajajinagar
Bangalore-560010

Dear Madam,

Sub: Experimental Irradiation of Ashwagandha and Kalmegh samples

Please refers to your request dated 06/01/2009 on the subject. As desired, the above samples have been exposed to radiation doses of 5 kGy & 10 kGy. The pertinent details of irradiation process are given below:

1. Source of Gamma Radiation : Cobalt-60
2. Present source strength : 398 Kilo Curies
3. Date of Irradiation : 09/01/2009
4. Average dose rate : 0.8 KiloGray per hour
5. Dosimetry system used : Chemical; Ceric-Ceros
6. Method of measurement : Potentiometric

This is for your information and records please. The processed samples are returned herewith through courier. Kindly acknowledge receipt. It will be our pleasure to be of service to you.

Wishing you all success in your Research Endeavours

Yours Sincerely,

P. Madhusoodanan
Chief Officer
Gamma Operations

Encl: As above
ANNEXURE-IV

K.L.E. Society's
COLLEGE OF PHARMACY
P. B. No. 1062, 2nd Block, Rajajinagar, BANGALORE - 560 010, Karnataka, (INDIA).

[Accredited by NBA - AICTE for 5 years]

Recognised by PCI, AICTE, Govt. of Karnataka and Affiliated to RGUHS, Karnataka, Bangalore.

Website: www.kleblrpharm.org  e-mail: kleblr@bgl.vsnl.net.in

Ref. No. KLE/B.Ph./IAEC/KLECP/BNG/08/2007  Date: ...17th-Dec-2007...

CLEARANCE CERTIFICATE FOR ANIMAL EXPERIMENTATION

TO WHOMSOEVER IT MAY CONCERN

This is to certify that proposal no: Ph.D/03/2007 dated 11th Oct 2007 submitted to IAEC of this institution for clearance was scrutinized and cleared in the IAEC meeting held on 15th Dec 2007.

The details of the proposal are as follows:

Title: EFFICACY OF GAMMA IRRADIATION ON MICROBIAL SAFETY AND MEDICINAL QUALITY OF ASHWAGANDHA AND KALMEGH

Chief Investigator: Dr. Kalpana Patil

Investigator: Mrs. Mamatha A

Mr. Vinay N
CPCSEA nominee for
Institutional animal ethics committee

Dr. Purnima Ashok
Chairperson
Institutional animal ethics committee

Mr. Prasanna G S
Member Secretary
Institutional animal ethics committee
Annexures

ANNEXURE – V
POST MARKET SURVEY - QUESTIONNAIRE – PART I

Topic: Efficacy of gamma-irradiation on microbial safety and medicinal quality of Ashwagandha and Kalmegh.

Respected Sir/Madam,

I am a research scholar, doing a project at KLE University’s College of Pharmacy, Bangalore, on “Efficacy of gamma-irradiation on microbial safety and medicinal quality of Ashwagandha and Kalmegh”. This is a small survey conducted to know the practical issues of gamma irradiation and also to know whether the products of Ashwagandha and Kalmegh, will be prescribed if their shelf life is increased by gamma irradiation. For this purpose, it would be grateful if you kindly spare some of your valuable time to answer the queries. The information provided to us is for academic purpose only and would be kept confident with utmost care.

THANKING YOU,

MAMATHA.A,
RESEARCH SCHOLAR (PhD)

BACKGROUND

1. NAME:

2. DESIGNATION:

3. PROFESSIONAL DEGREE:

4. GENDER: ☐ MALE ☐ FEMALE

5. AGE: (ACTUAL IN YEARS) _____________

6. ADDRESS CONTACT DETAILS:
   TELEPHONE (WORK): _____________________
   (CELL PHONE): _____________________
   EMAIL ID: _____________________

7. ARE YOU (PLEASE FILL THE BOX WITH THE CORRECT CATEGORY)?
   a. MANUFACTURER ☐
   b. TRADER ☐
   c. PRACTITIONER ☐
   d. RESEARCHER ☐
   e. HEALTH ADMINISTRATOR ☐

8. WHAT ORGANISATION DO YOU WORK IN OR FOR?
   01. INDIVIDUAL PRACTICE ☐
   02. GROUP PRACTICE ☐
   03. MANUFACTURING COMPANY ☐
   04. TRADING COMPANY ☐
   05. HOSPITAL/ PUBLIC HEALTH UNIT ☐
   06. MEDICAL RESEARCH ☐
   07. NGO ☐
   08. OTHER ☐

9. NAME AND ADDRESS OF PLACE OF WORK:

   SIGNATURE :

Department of Pharmacognosy, KLE University’s College of Pharmacy, Bangalore
QUESTIONNAIRE – PART II

PLEASE ANSWER ALL THE QUESTIONS THAT ARE RELEVANT TO YOU. IF YOU NEED EXTRA SPACE, PLEASE ATTACH ADDITIONAL INFORMATION ON A SEPARATE SHEET OF PAPER. PLEASE ALSO PROVIDE ANY INFORMATION THAT YOU THINK IS RELEVANT WHERE WE HAVE NOT ASKED FOR IT.

1. Do you prescribe Ashwagandha products for your patients?
   Yes ☐ No ☐

2. Do you prescribe Kalmegh products for your patients?
   Yes ☐ No ☐

3. What is the percentage of consumption of Ashwagandha products in a year?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

4. What is the percentage of consumption of Kalmegh products in a year?
   a. 0 – 25% ☐
   b. 25 – 50% ☐
   c. 50 – 75% ☐
   d. 75 – 100% ☐

5. For, what diseases do you prescribe Ashwagandha products?
   a. Antistress ☐
   b. Aphrodisiac ☐
   c. To strengthen immune system ☐
   d. Any other ☐
       Mention ____________________________________________________________

6. For, what diseases do you prescribe Kalmegh products?
   a. Hepatoprotective ☐
   b. Febrifuge ☐
   c. Anti-inflammatory ☐
   d. Any other ☐
       Mention ____________________________________________________________

7. Do you agree that microbial contamination is a major issue which decreases the shelf life of the medicinal plants and their preparations?
   Yes ☐ No ☐

8. What is the expected shelf life of Ashwagandha products you have come across?
   a. Churna _________________
   b. Extract _________________
   c. Preparations made with milk _________________
   d. Liquid preparations _________________
   e. Leha _________________
   f. Any others, Please specify ___________________________________________
9. What is the expected shelf life of Kalmegh products you have come across?
   a. Churna __________________
   b. Extract __________________
   c. Preparations made with milk __________
   d. Liquid preparations ____________
   e. Leha ______________________
   f. Any others, Please specify ______________________

10. Do you use any special method for storage of Ashwagandha and Kalmegh products?
    a. Storing at room temperature [ ]
    b. Storing in dark [ ]
    c. Refrigeration [ ]
    d. No specific methods [ ]

11. Are you aware of any techniques, which can increase the shelf life of these products?
    Yes [ ] No [ ]

12. If yes, which methods.
    ______________________________________________________
    ______________________________________________________

13. Are you aware of gamma irradiation as a technique for microbial decontamination of medicinal plants and their preparations?
    Yes [ ] No [ ]

14. From my research, I have found that gamma irradiation at a dose less than the acceptable limits can reduce the microbial load and increase the shelf life of Ashwagandha and Kalmegh products. If such products are given to you, will you,
    a. Prescribe [ ]
    b. Manufacture [ ]
    c. Market [ ]
    d. Use for further research [ ]

15. If not, what do you think may be the practical issues of gamma irradiation?
    a. Toxicity [ ]
    b. Reduction in pharmacological activity [ ]
    c. Any others, Please mention ______________________

16. Any suggestions for overcoming the practical issues of gamma irradiation.
    ______________________________________________________
    ______________________________________________________
    ______________________________________________________

17. If we provide you with documented evidence confirming that there is no toxicity and that the efficacy of Ashwagandha and Kalmegh products is retained, would you prescribe gamma irradiated products.
    Yes [ ] No [ ]
Effect of gamma irradiation on pharmacological activity of *Andrographis paniculata*

Mamatha A*,1, Kalpana S Patil2, Purnima Ashok3, Kushal1, Soujanya1, Gokul1

1Department of Pharmacognosy, KLE University’s College of Pharmacy, Rajajinagar II Block, Bangalore 560 010. 2Department of Pharmacognosy, KLE University’s College of Pharmacy, Nehru Nagar, Belgaum 590 010. 3Department of Pharmacology, KLE University’s College of Pharmacy, Rajajinagar II Block, Bangalore 560 010.

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ABSTRACT

The study was intended to evaluate the anti-inflammatory activity of non-irradiated and gamma-irradiated (dose 5KGy and 10KGy) samples of *Andrographis paniculata* in carrageenan induced rat paw oedema in Wistar rats at a dose level of 200mg / kg body weight administered orally. Significant anti-inflammatory activity was observed in aqueous extracts of all the groups in comparison to control and further there were no significant differences among non irradiated and gamma irradiated samples (5KGy and 10KGy). Thus, gamma irradiation at doses of 5 and 10KGy may have no influence on the pharmacological activity of *Andrographis paniculata*. As it does not interfere with the above, gamma irradiation can be considered as an important technique to microbially decontaminate the medicinal herbs and thus increase their shelf life and stability of the commercial products of *Andrographis paniculata*.

Key words: Gamma irradiation, *Andrographis paniculata*, carrageenan, rat paw oedema, flavonoids.

1. INTRODUCTION

*Andrographis paniculata* (Kalmegh) is a popular traditional medicinal plant in India [1] mainly used as antipyretic, hypoglycemic, antithrombotic and hepatoprotective. Andrographis contains, as its primary chemical constituents, diterpenoid lactones (andrographolides), paniculides, farnesols and flavonoids[2,3]. In this study an attempt was made to evaluate the pharmacological effect of gamma irradiation at doses of 5 KGy and 10 KGy on pharmacological activity of *Andrographis paniculata*. The anti-inflammatory activity of *Andrographis paniculata*, both non irradiated and gamma-irradiated samples were investigated in this study using Wistar albino rats by carrageenan induced rat paw oedema and if found safe, gamma irradiation will be applied for microbially decontamination of the product. This study may help in increasing the sability and thus the shelf life of the products of *Andrographis paniculata*.

2. MATERIALS AND METHODS

2.1 Plant materials:

*Andrographis paniculata* herb was procured from Natural Remedies, Bangalore. The herb was identified and authenticated by Dr. Vasundhara, Professor, Department of horticulture, GKV, Bangalore and voucher specimen deposited. They were further ground to powder and kept at ambient temperatures.

2.2 Preparation of extracts:

100g of coarsely powdered samples were packed in Soxhlet apparatus and extracted with water for 12h at 95±1°c. The extract was concentrated over water bath, labelled and stored. Similarly another portion was prepared and two portions of the above samples were packed in sterile polyethylene bags. Gamma irradiation was carried out at a commercial scale Cobalt 60 irradiation service facility, loaded with Cobalt-60 source with strength of 90 kilo curies (KCi), owned and operated by Microcontrol Sterilisation services Pvt. Ltd. at Bangalore. The applied dose levels were 5 and 10 KGy and this absorbed dose was monitored with ceric/cerous dosimeters.

2.2.1 Preparation of the drug for experimental study:

Aqueous extracts of *Andrographis paniculata* was administered orally; and control animals received the corresponding quantities of vehicle one hour prior to the carrageenan injection. Non-irradiated and gamma irradiated (5 and 10KGy) samples of *Andrographis paniculata* were dissolved in water and filtered through a filter paper. The supernatant aliquots were used for pharmacological experiment.

2.3 Pharmacological activity:

2.3.1 Animals

Male Wistar albino rats weighing 150–180g were purchased from Indian Institute of Science, Bangalore for experimental purpose. These were used in the investigation of pharmacological effects of *Andrographis paniculata*. Animals were housed in animal house at 22±2°C, relative humidity 50±20%, light (10 h): dark (14 h) cycle. They were given standard granular diet and water ad libitum. The experimental protocol was approved by IAEC (Institutional Animal Ethical Committee), Bangalore.

2.3.2 Acute toxicity studies:

Acute oral toxicity study was performed according to OECD 423 guidelines [4]. Male Wistar rats (n=6) selected by random sampling technique were used for the study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose level of 200mg/kg b.w by intragastric tube and observed for 14 days. If mortality was observed in 2-3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for a further high dose of 2000 mg/kg b.w.

2.3.3 Anti-inflammatory activity by carrageenan induced rat paw edema:

The animals were divided into 5 groups each composed of six animals. Group I : Control animals received the vehicle (water ) p.o. Group II : Animals received standard Diclofenac sodium, 15 mg/kg. Group III: Animals received non irradiated aqueous extract of *Andrographis paniculata* (K1) at a dose of 200 mg/kg p.o. Group IV: Animals received 5 KGy gamma irradiated aqueous extract of *Andrographis paniculata* (K2) at a dose of 200 mg/kg p.o. Group V – Animals received 10 KGy gamma irradiated aqueous extract of *Andrographis paniculata* (K3) at a dose of 200 mg/kg p.o. Percentage inhibition of oedema = Vc-Vt x 100/Vc

Where, Vc is the inflammatory increase in paw volume in control group of animals and Vt is the inflammatory increase in paw volume in drug treated animals.

Paw oedema was induced injecting 0.1ml of 1% carrageenan in physiological saline into the subplantar tissues of the left hind paw of each rat [5].Standard and sample drugs (K1, K2 and K3) were administered orally 1 hour prior to carrageenan administration. The paw volume was measured at intervals of 1,2,3,4 5 and 24 hours by mercury displacement method using a plethysmograph. The percentage inhibition of paw volume in drug treated group was compared with the carrageenan control group (Group I). Diclofenac sodium (15mg/kg po) was used as standard drug.

2.4 Statistical Analysis

Data obtained from the pharmacological experiments are expressed as mean±SD. Statistical analysis was performed using One way ANOVA followed by Tukey’s multiple comparison test with p<0.001 considered to be statistically significant.
Apart from the medicinal and therapeutic characters, the safety of a drug is also very important. All three samples of */Andrographis paniculata* did not elicit any side effect even when administered at doses of 2000 mg/kg.

### 4. CONCLUSION

In conclusion, the results of the present study support the traditional use of */Andrographis paniculata* in inflammation. Aqueous extract of */Andrographis paniculata* possess significant anti-inflammatory activity, which may be due to the presence of flavonoids and deserves further studies to establish its therapeutic value as well as its mechanism of action.

Further, it was also proved that gamma irradiation used for microbial decontamination of the sample, at doses of 5 KGy and 10 KGy does not interfere with the pharmacological activity (anti-inflammatory) of the plant. Hence, this treatment can be considered as a safe tool for microbial decontamination and thus increase the quality of the commercial products, and also to increase the shelf life of the products.

### 5. ACKNOWLEDGEMENTS

We thank KLE University’s College of Pharmacy, Bangalore – 10, for providing experimental facilities.

### 6. REFERENCES

Effect of Gamma Irradiation on Pharmacological Activity of Ashwagandha

Mamatha A1, Kalpana S Patil2, Purnima Ashok1, Kushal1, Soujanya1, Gokul K3, Anusuya Patil1

Abstracts: The effect of gamma irradiation on pharmacological activity of non-irradiated and gamma-irradiated (at doses of 5 KGY and 10 KGY) samples of Ashwagandha were examined. Investigations were performed to study the anti-stress activity by forced swim endurance test in Albino Swiss mice at a dose level of 100mg / kg b.w. administered orally. Significant anti-stress activity (p<0.001) was observed in aqueous extracts of all the groups in comparison to control. Further there were no significant differences among non irradiated and gamma irradiated samples (5KGY and 10KGY) indicating that gamma irradiation at doses of 5 and 10Kgy may have no influence on the pharmacological activity of Ashwagandha. As it does not interfere with the pharmacological activity, gamma irradiation can be considered as an effective method in reducing microbial contamination and thus increase the shelf life and stability of the commercial products of Ashwagandha.

Key Words: Gamma irradiation, Ashwagandha, Forced swim endurance test, Anti-stress, Microbial decontamination

INTRODUCTION

In this world, where health and longevity has become a major concern, many people show enhanced interest in medicinal plants. Unfortunately, they are often contaminated with high levels of bacteria, molds, and yeasts. If untreated, the herbs will result in rapid spoilage and do not serve the purpose they are supposed to augment. Microbiological contamination of medicinal herbs is a serious problem in the production of therapeutic preparations. A good quality of the product according to pharmaceutical requirements may be achieved by different methods of decontamination. Decontamination treatments should be fast and effective against all microorganisms. The conventional methods of decontamination were fumigation with gaseous ethylene oxide or methyl bromide, which are now prohibited or being increasingly restricted in most advanced countries for health, environmental or occupational safety reasons. Treatment by ionising radiation is already a well known method, but it received less attention for medicinal plants, especially on fresh herbs. Irradiation is technically feasible, very effective and eco friendly.

Gamma irradiation is one of the most effective means of microbial decontamination of dried herbs and their phytopreparations. The concentration of the main biologically active components is a measure of therapeutic quality in any herbal materials. Therefore said that the most appropriate way to evaluate the therapeutic quality of medicinal plant is to determine its pharmacological activity after irradiation treatment. However, it is not well known whether the pharmacological activity is or is not influenced by the action of gamma irradiation on Ashwagandha.

Ashwagandha (Withania somnifera) is an important herb in Ayurvedic and indigenous system of medicine. The plant has been used as an anti stress, adaptogen, antioxidant, aphrodisiac, anti-inflammatory and more recently to treat ulcers, venom toxins, bacterial infection and senile dementia. More recent research supports the use of Ashwagandha in anxiety, neurological disorders, hyperlipidemia and cancer. It is found that it inhibits lipid peroxidation in stress induced animals.

Studies on its antistress activity in mice when subjected to swimming stress showed an increase in swimming time and reduction in gastric ulcers. In this study an attempt is made to evaluate the effect of gamma irradiation at doses of 5 KGY and 10 KGY on pharmacological activity of Ashwagandha. Its anti stressor property will be investigated using albino Swiss mice by Forced swim endurance test and if found safe and efficient, gamma irradiation technique will be successfully applied for microbial decontamination of the raw material, phytopreparations and commercial products of Ashwagandha, which will in turn help in increasing the stability and thus the shelf life of the products of Ashwagandha.

MATERIALS AND METHODS

Plant Materials
Ashwagandha herb was procured from Natural Remedies, Bangalore. The herb was identified and authenticated by Dr. Vasundhara, Professor, Department of horticulture, GKVK, Bangalore and voucher specimen deposited. They were further ground to powder and kept at ambient temperatures.

Acute Toxicity Studies
Albino Swiss mice were maintained in animal house (12:12h dark: light cycle), with adequate ventilation, hygienic conditions, maintained on normal pelleted diet (M/s Lipton India, Bangalore) and water ad libitum. A group of animals were housed in polypropylene cage of 42X 27 X13 cm paddy husk bed covered with stainless steel wire mesh 28 X 20.5 cm with provision for water and feed. Experimental animals were purchased from IISc, Bangalore. The animals were subjected to Toxicological study as per OECD / OCED guideline 425 (modified, adopted 23rd march 2006)

Male albino Swiss mice (n=6) selected by random sampling technique were used for the study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose level of 100mg/kg b.w and observed for 14 days. If mortality was observed in 2-3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for a further high dose of 2000 mg/kg b.w.

Preparation of Extracts for Study of Pharmacological Activity
100g of coarsely powdered samples were packed in Soxlet apparatus and extracted with water for 12h at 95±1°C. The extract was concentrated over water bath, labelled and stored. Similarly another portion was prepared, and two portions of the above samples were packed in sterile polyethylene bags. Gamma irradiation was carried out at a commercial scale Cobalt 60 irradiation service facility, loaded with Cobalt-60 source with strength of 90 kilo curies (KCI), owned and operated by Microtrast Sterilisation services Pvt Ltd. at Bangalore. The applied dose levels were 5 and 10 KGY and this absorbed dose was monitored with cenic/cerous dosimeters.

Preparation of the Drug for Experimental Study
Aqueous extracts of Ashwagandha was administered orally, and control animals received the corresponding quantities of vehicle.

Non-irradiated and gamma irradiated (5 and 10Kgy) samples of Ashwagandha were dissolved in water and filtered through a filter paper. The supernatant aliquots were used for pharmacological experiment.
Table 1. Effects on duration of swimming in Forced Swim Endurance test

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean duration of swimming (in sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hour</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>163.7 ± 5.584</td>
</tr>
<tr>
<td>2.</td>
<td>A1</td>
<td>210.8 ± 1.939**</td>
</tr>
<tr>
<td>3.</td>
<td>A2</td>
<td>206.5 ± 3.704**</td>
</tr>
<tr>
<td>4.</td>
<td>A3</td>
<td>215.5 ± 3.990**</td>
</tr>
</tbody>
</table>

All values are mean ± SEM in sec (n=6).
One-way ANOVA followed by Newman-Keul’s test. **p<0.01, ns- not significant, when compared to vehicle treated animals.

Table 2. Effects on duration of immobility in Forced Swim Endurance test

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean duration of immobility (in sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hour</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>66.67 ± 2.376</td>
</tr>
<tr>
<td>2.</td>
<td>A1</td>
<td>82.17 ± 2.344**</td>
</tr>
<tr>
<td>3.</td>
<td>A2</td>
<td>77.67 ± 2.741**</td>
</tr>
<tr>
<td>4.</td>
<td>A3</td>
<td>73.83 ± 1.922**</td>
</tr>
</tbody>
</table>

All values are mean ± SEM in sec (n=6).
One-way ANOVA followed by Newman-Keul’s test. *P<0.05, **P<0.01, when compare to vehicle treated animals.

Table 3. Effects on duration of climbing in Forced Swim Endurance test

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean duration of climbing (in sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hour</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>69.67 ± 3.938</td>
</tr>
</tbody>
</table>

All values are mean ± SEM in sec (n=6).
One-way ANOVA followed by Newman-Keul’s test. **P<0.01, when compare to vehicle treated animals.

Table 4. Comparative changes in various behavioral profiles at 5h of acute treatment. Maximum effect was observed in the 5h after drug administration. *Values are statistically significant - p<0.001

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Gp</th>
<th>Mean time duration (in sec) spent in various behavior during test.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Swimming</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>163.7 ± 5.584</td>
</tr>
<tr>
<td>2.</td>
<td>A1</td>
<td>266.0 ± 6.995*</td>
</tr>
<tr>
<td>3.</td>
<td>A2</td>
<td>264.5 ± 5.749*</td>
</tr>
</tbody>
</table>

All values are mean ± SEM in sec (n=6).
One-way ANOVA followed by Newman-Keul’s test.

Animals
Naive, healthy, albino Swiss mice weighing between 18-30gm were employed for study. The chosen experimental animals were kept in our animal house (12:12h dark: light cycle), with adequate ventilation, hygienic conditions maintained on normal pelleted diet (M/s Lipton India, Bangalore) and water ad libitum. A group of six animals were housed in polypropylene cage of 26 X 19 X 13cm on paddy husk bed and covered with stainless steel wire mesh 28 X 20.5 cm with provision for water and feed. Experimental animals were purchased from IISc, Bangalore. All experiments were performed in Psychopharmacology laboratory illuminated with 11W CFC lamp (dim light), free from acoustic stress and after acclimatization of animals for 4-5 days in this laboratory. All experiments were performed during 10:00 am to 4:00 pm to reduce influence of circadian rhythm on the observation.
All parameters were observed and recorded in person. For all experiments, group refers to group of six animals (n=6).

Data generated from various experimental procedures were analyzed for statistical significance by ANOVA followed by Newman-Keul’s multiple comparison tests. Values P < 0.001 & P<0.05 were considered significant.

Experimental protocols of animal experiments were duly approved by Institutional Animal ethics Committee, IAEC of KLE College of Pharmacy, Bangalore.

Treatment
Aqueous extracts of Ashwagandha was administered orally; and control animals received the corresponding quantities of vehicle one hour prior to the experiment.

Experimental Procedure
Antistress property of Ashwagandha (100 mg/kg) was studied by Forced Swim Endurance test.

Forced Swim Endurance Test
Forced swim endurance test (FST)[31], was employed with modification to suit out our laboratory conditions. Briefly, mice were individually placed in acrylic cylinder 24 x 12 cm filled 10cm high with water at 25±2°C. All animals were forced to swim for 5 minutes and duration of swimming, immobility and climbing were recorded and interpreted. In the acute study, four groups of naive, healthy, albino Swiss mice of either sex, weighing between 18-30gm were grouped and treated with saline 0.2ml/animal.

Group 1 - Control animals received the vehicle p.o.
Group 2 - Animals received non-irradiated

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Effect of duration of swimming in FST at 5th hour

Figure 1. Duration of swimming in secs at 5th hour of drug administration in FST

Group III - Animals received 5 KGY gamma irradiated aqueous extract of Ashwagandha (A2) at a dose of 100 mg/kg p.o.

Group IV - Animals received 10 KGY gamma irradiated aqueous extract of Ashwagandha (A3) at a dose of 100 mg/kg p.o.

The extracts were administered orally through gavage tube at a dose of 100mg/kg b.w. After an hour of drug administration animals were forced to swim in the cylinder and the duration of swimming, immobility and climbing were observed and recorded. Swimming was defined as animal making active swimming motions, immobility as animal making only the those movements necessary to keep its head above the water and climbing as animal making vigorous movements with its forepaws in and out of the water, usually directed against the walls. The same was carried out at 5th and 24th hour of drug administration. Mean duration of swimming, immobility and climbing of various groups of animals are tabulated in Table 1, 2, 3 and 4. The activities observed were compared with the control group and also among treated groups.

Statistical Analysis
Data was analyzed using Graphpad Prism 5.0 version. The results were expressed as the mean±SEM. The significance of the mean difference between the control group and each treatment group was determined by Newmankeul’s comparison test. The level of p<0.001 and p<0.05 were used as the criterion of statistical significance.

RESULTS AND DISCUSSION

Toxicological Analysis

Acute oral toxicity study for Ashwagandha was carried out in order to arrive at maximum tolerable dose of sample under study, according to modified OECD test guidelines 425[10]. The test was restricted to limit test in view of no mortality being observed within 2000mg/kg of b.w. 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 b.w. Selection of dosage as 100mg/kg b.w. was based on this study, which represented 1/20th of 2000mg/kg. This dose is further used to evaluate the anti stress activity of the test formulation.

Anti-Stress Activity

The main objective of the present study was to evaluate the anti stress activity of non-irradiated and gamma-irradiated (5KGY and 10KGY) samples of Ashwagandha by Forced swim endurance test. The mice was made to swim for 5 minutes and during this 5 minute test, duration of swimming, immobility and climbing attempts were noted down. (Table 1, 2, 3 and 4). It was observed that swimming duration in all 3 samples – A1, A2, A3 were statistically significant. The increase in the total swimming time in both non-irradiated and gamma irradiated samples indicates better stress tolerance in these mice. It was also observed that there was maximum activity in all three samples (A1, A2 and A3) at the 5h of sample administration. (Table 1, Graph 1).

The results of the current study support the traditional use of Ashwagandha as an anti-stress agent. Aqueous extract of Ashwagandha possess significant anti-stress activity. Further, it was also proved that gamma irradiation used for microbial decontamination of the sample, at doses of 5KGY and 10KGY does not interfere with the pharmacological activity (anti-stress) of the plant. Hence, gamma irradiation can be considered as an efficient method for microbial decontamination of Ashwagandha and thus increase the quality and shelf life of commercial products of Ashwagandha.

CONCLUSION

In conclusion, the results of the present study support the traditional use of Ashwagandha as an anti-stress agent. Aqueous extract of Ashwagandha possess significant anti-stress activity. Further, it was also proved that gamma irradiation used for microbial decontamination of the sample, at doses of 5KGY and 10KGY does not interfere with the pharmacological activity (anti-stress) of the plant. Hence, gamma irradiation can be considered as an efficient method for microbial decontamination of Ashwagandha and thus increase the quality and shelf life of commercial products of Ashwagandha.

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7. P GorenkLB Kedzia, W Migdal, HB Owczarczyk, Irradiation as an alternative environment friendly method for microbiological decontamination of herbal raw material, in
32. I Lucki, The forced swimming test as a model for core and component behavioral effects of antidepressant drugs, Behav Pharmacol. 523-532, 8 (1997).
Effect of Gamma Irradiation on Microbial Load of Ashwagandha (Withania somnifera)

Mamatha A1, Kalpana S Patil2

Abstract: Roots of Ashwagandha (Withania somnifera) were exposed to gamma irradiation at doses of 5 and 10 kGy in a Co 60 irradiator. Irradiated and unirradiated samples were stored at room temperature. Microbial load of root powder were evaluated at 0 and 6 months of storage. Results indicated that gamma irradiation reduced the total aerobic count and total fungal count in comparison to the non-irradiated sample. Further, the non-irradiated samples showed the presence of E. coli and Staphylococcus aureus. On irradiation there was a drastic reduction in E. coli and Staphylococcus aureus. Microbial load was found to be increased after 6 months of storage in the non-irradiated samples, where as there were no significant differences in the irradiated samples. Thus, gamma irradiation can be used as a suitable technique for microbial decontamination of phytopreparations and products of Ashwagandha and thus increase their shelf life and stability.

Key Words: Ashwagandha, Gamma irradiation, Microbial load, Shelf life, Decontamination.

INTRODUCTION

Ashwagandha (Withania somnifera) is widely used in Indian system of medicine. Ashwagandha holds a prominent place in Ayurveda and Unani. The plant finds its use as a rejuvenative herb. It is used as health care food supplement1. The fresh berries are used as emetic, sedative and anti-asthmatic. Dried fruits and roots are sedative, carminative, diuretic, anti-inflammatory and used for curing general and sexual weakness in human beings, goiter, fainting, blood disorders, leucoderma, chronic liver complications, skin diseases, bronchitis and ulcers. In Rajasthan, roots are used for curing rheumatism and dyspepsia; in Punjab they are used to relieve loin pain and in Sind for abortion. Leaves are used as anthelmintic, insecticide, febrifuge and tonic. It is used to cure inflammation of tubercular glands, piles, sore eyes, boils and swelling of hand and foot. In some areas, warm leaves are used to provide comfort during eye diseases. The plant is used as abortifacient, anodyne, anti-asthmatic, bacticide, contraceptive, diuretic, sedative, tonic and anti-inflammatory and in treatment of cold, dropsy, head aches, convulsions, sleeplessness, insufficient breast milk, anaemia, fever, hypertension and lumbago2-4.

Reports have shown that the plant possess memory enhancing5, adaptogenic and antistress6, immunomodulatory7, antihypertensive8-10, anti-inflammatory, antiviral11, hepatoprotective12, antioxidant13 and other properties.

Non-standardised herbal preparation does not get accepted in the global market. It becomes wrong to assume that the biological agents are safe because they are natural. The microbial content of herb is one of the most important parameters as they make the heel potentially dangerous for sensitive population. One of the major problems associated with herbs is its microbial contamination resulting in quality deteriorations19-27. Inspite of substantial efforts to avoid microbial contamination, an upward trend in non-acceptance of herals are reported. Several decontamination methods exist, but the most versatile treatment among them is the processing of herbs with gamma irradiation. Microbial decontamination of herbal products by gamma irradiation is a safe, efficient, environmentally clean and energy efficient process28-30.

Gamma irradiation at doses of 2-10 kGy has shown to be effective in eliminating pathogenic non-spore forming bacteria including other pathogens like salmonella, staphylococcus, Pseudomonas E.coli and others31-41. With today's demand for high quality herbs, gamma irradiation holds a promise for enhancing its safety and quality.

While exposure to gamma rays offers an effective alternative means of reducing microbial contamination, the dose increased to the extent of microbial kill has to be checked and also the method should be suitable to effectively reduce the microorganism and maintain this for a longer period42. Although gamma irradiation has been found as a suitable technique for microbial decontamination, there are no studies related to microbial decontamination of gamma irradiated Ashwagandha. Accordingly, the objective of this study was to examine the effect on microbial contamination of irradiated samples of Ashwagandha at 0 and 6 months of storage.

MATERIALS AND METHODS

Plant Materials

Ashwagandha roots were procured from Natural remedies, Bangalore. The herb was identified and authenticated by Dr. Vasundhara, Professor, Department of horticulture GKV, Bangalore and voucher specimen deposited. They were further ground to powder and kept at ambient temperature.

Preparation of Extracts

100 g of coarsely powdered samples were packed in 6 different sterile polythene bags at ambient temperature. Gamma irradiation was carried out at a commercial scale Cobalt 60 irradiation service facility loaded with Cobalt 60 source with strength of 90 kilo curies (KCi), owned and operated by Microtrol sterilization services Pvt.Ltd. at Bangalore. The applied dose levels were 5 and 10 kGy and this absorbed dose was monitored with ceric/cerous dosimeters. Two packets were kept as such (A1- non-irradiated) two packs were gamma irradiated at a dose of 5 kGy (A2) and remaining two packets were gamma irradiated at a dose of 10 kGy (A3). 1st set of sample (A1, A2 & A3) were analysed soon after irradiation and the second set was analysed after 6 months of storage.

Media for Microbial Studies

Total aerobic count was enumerated on nutrients agar (HI media) and total fungal count on Sabourud’s dextrose agar. E.coli was tested on Macconkey agar and EMB agar, Selenite F broth and Brilliant green agar were the media’s for Salmonella, bismuth sulphite agar and certrimide broth were the selective media used for determination of Pseudomonas and Staphylococcus aureus was tested on Mannitol salt agar and Vogel-Johnson medium. All the individual organisms were analysed as per the procedure given in IP.

SAMPLING AND ENUMERATION

Total Microbial Load

1 gm of each sample was suspended in 10 ml of respective medias. To determine total aerobic count the sample was incubated at 370 ± 1°C for 24-48 hrs and for total fungal count – samples were incubated at 28±1°C. Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gm (cfu/gm). Duplicate plates of appropriate dilutions were plated for specific organisms with there respective medias and incubated at 370 ±1°Cfor 24-48 hrs.

Identification Test for Escherichia Coli

1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 18 to 24 hours.
Table 1. Details of total microbial load in the Ashwagandha samples

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the sample</th>
<th>Total bacterial count (cfu/gm)</th>
<th>Total fungal count (cfu/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>4.4x10^4</td>
<td>3x10^4</td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>1.3x10^2</td>
<td>1x10^2</td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Compiled results for pathogen detection in the samples

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the sample</th>
<th>E. coli</th>
<th>Salmonella</th>
<th>Pseudomonas</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*+ = Present  *- = Absent

Table 3. Results of E. coli detection test of the samples

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the sample</th>
<th>Visual Observation</th>
<th>Preliminary test</th>
<th>Secondary test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>1) Extensive growth of bacterial colonies and few colonies were surrounded with yellow zones were observed in Macconkey agar plates 3.1x10^4</td>
<td>1) Colonies with metallic sheen were observed in EMB agar plates. 2) Cherry red ring was observed in lactose broth on addition of Kovac's reagent 2.3x10^5</td>
<td>Confirms the presence of E. coli</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>1) Few bacterial colonies were observed.</td>
<td>1) No colonies were observed with metallic sheen in EMB agar plates. 2) Indole formation was not observed.</td>
<td>Confirms the absence of E. coli</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>1) No bacterial colonies were observed.</td>
<td>Not performed</td>
<td>Confirms the absence of E. coli</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Results of Staphylococcus aureus detection test of the samples

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the sample</th>
<th>Visual Observation</th>
<th>Preliminary test</th>
<th>Secondary test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>Yellow colonies were observed in Mannitol salt agar medium 5.4x10^4</td>
<td>Observed black colonies surrounded by yellow zones in Vogel-Johnson agar medium 4.7x10^4</td>
<td>Confirms the presence of Staphylococcus aureus</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>No bacterial colonies were observed on Mannitol salt agar medium</td>
<td>No bacterial colonies were observed on Vogel-Johnson agar medium</td>
<td>Confirms the absence of Staphylococcus aureus</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>No bacterial colonies were observed on Mannitol salt agar medium</td>
<td>No bacterial colonies were observed on Vogel-Johnson agar medium</td>
<td>Confirms the absence of Staphylococcus aureus</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Details of total microbial load in the samples after 6 months of storage

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the sample</th>
<th>Total bacterial count (cfu/gm)</th>
<th>Total fungal count (cfu/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>4.9x10^4</td>
<td>3.7x10^4</td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>1.2x10^2</td>
<td>1x10^2</td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6. Results of E. coli detection test of the samples after 6 months of storage

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the sample</th>
<th>Visual Observation</th>
<th>Preliminary test</th>
<th>Secondary test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>1) Extensive growth of bacterial colonies and few colonies were surrounded with yellow zones were observed in Macconkey agar plates 3.9x10^4</td>
<td>1) Colonies with metallic sheen were observed in EMB agar plates. 2) Cherry red ring was observed in lactose broth on addition of Kovac's reagent 3.0x10^6</td>
<td>Confirms the presence of E. coli</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>1) Few bacterial colonies were observed.</td>
<td>1) No colonies were observed with metallic sheen in EMB agar plates. 2) Indole formation was not observed.</td>
<td>Confirms the absence of E. coli</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>1) No bacterial colonies were observed.</td>
<td>Not performed</td>
<td>Confirms the absence of E. coli</td>
<td></td>
</tr>
</tbody>
</table>
Primary Test
0.1 ml of the enrichment culture was spread on MacConkey agar plate using sterile autoclaved spreader uniformly maintaining aseptic conditions, in laminar airflow cabinet. Plates were incubated at 37°C ± 1°C.

Secondary Tests
0.1 ml of the enrichment culture was spread on EMB agar plates using sterile autoclaved spreader uniformly maintaining aseptic conditions, in laminar air flow cabinet. Simultaneously 0.1 ml of enrichment culture was incorporated in tubes containing 5 ml of peptone water. Plates and tubes were incubated at 37°C±1°C, examined for the formation of metallic sheen in the agar plates and formation of indole (To test for indole add 0.5 ml of Kovac’s reagent, shake well, and allow to stand for one minute; if a red colour is produced in the reagent layer - indole is present). The formation of metallic sheen and indole in the secondary test indicates the presence of Escherichia coli.

Identification Test for Salmonella
1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 1 hr (4 hours for gelatin).

Primary Test
Added 1.0 ml of the enrichment culture to tubes containing 10 ml of Selenite F broth and incubated at 37°C for 24 hours. Tubes were observed for the microbial growth and for the color of the broth. Microbial growth with no color in tubes shows presence of salmonella.

Secondary Test
Cultures from the preliminary test tubes were inculcated on to the bright green agar and bismuth sulphite agar plates. Plates were incubated at 37°C for 24 hr. Small, transparent and colourless, or opaque, pinkish or white (frequently surrounded by a pink or red zone) colonies on brilliant green agar and black or green colonies on bismuth sulphite agar confirms the presence of Salmonella typhi.

Identification Test for Pseudomonas
1 gm of the sample was placed in a sterile screw capped jar containing 10 ml of Cetrimide broth and incubated at 37°C for 24 hours. Subcultured on a plate containing a layer of Cetrimide agar and incubated at 37°C for 24–48 hours, examined for the growth by Gram’s stain.

RESULTS AND DISCUSSION

Microbial load and the effect of gamma irradiation on non-irradiated and gamma irradiated Ashwagandha samples were evaluated and are shown in Table 1.

Before irradiation levels, total aerobic bacteria (4.4 x 10⁴ cfu/gm) and total fungal count (3 x 10⁴ cfu/gm) were quite high. On subjecting to specific pathogen tests as per IP, non-irradiated samples showed the presence of E.coli (2.3 x 10⁶ cfu/gm) and Staphylococcus aureus (4.7 x 10⁴ cfu/gm). Salmonella and Pseudomonas were totally absent in all the samples (Table 2). On gamma irradiating the samples at 5 kGy and 10 kGy, there was a drastic reduction in microorganism. In samples irradiated at a dose of 5 kGy initially there was reduction in total bacterial count from 4.93 x 10⁶ cfu/gm to 1.2 x 10⁵ cfu/gm and the total fungal count from 3.7 x 10⁴ cfu/gm to 1 x 10³ cfu/gm to acceptable levels. However, the specific microorganisms - E.coli and Staphylococcus aureus were totally absent in samples A₂ and A₃ (Table 3 and 4). Pictures of E.coli on Macconkey and EMB agar are shown in Photo 1 and 2. Pictures of Staphylococcus on Mannitol and Vogel Johnson media are shown in Photo 3 and 4.

Further after 6 months storage the total bacterial count (4.9 x 10⁶ cfu/gm) and total fungal count (3.7 x 10⁴ cfu/gm) were increased compared to 0 month analysis (Table 5). Pathogenic organism E.coli (3.0 x 10⁶ cfu/gm) and Staphylococcus aureus (5.2 x 10⁴ cfu/gm) were also increased, where as gamma irradiated samples maintained their quality by showing no microbial contamination. In samples irradiated at a dose of 5 kGy initially there was reduction in total bacterial count from 4.93 x 10⁶ cfu/gm to 1.2 x 10⁵ cfu/gm and the total fungal count from 3.7 x 10⁴ cfu/gm to 1 x 10³ cfu/gm to acceptable levels. However, the specific microorganisms - E.coli and Staphylococcus aureus were totally absent in samples A₂ and A₃ (Table 6 and 7).

This research clearly indicates that the irradiation at doses of 5 kGy and 10 kGy improved the stability and shelf life of the product.

E.coli and Staphylococcus aureus species seemed to be the major bacteria’s in Ashwagandha samples. The herbs could be contaminated during cultivation, harvesting or processing. Gamma irradiation was found to be an effective technology for resolving technical trade issue (WHO 1994, FDA 2001) and thus increase the quality of Ashwagandha. This concept of gamma irradiation can be commercially used while exporting Ashwagandha phytopreparations and formulations.

As a disinfection treatment it offers good control of many pathogenic and spoilage organisms with minimal change to the herbs.

CONCLUSION
Thus gamma irradiation at doses of 5 kGy and 10 kGy is definitely safe for the treatment of microbial contamination of Ashwagandha up to 6 months of storage. The effect of gamma irradiation on the elimination or control of microorganisms on Ashwagandha root powder is in agreement with

Table 7. Results of Staphylococcus aureus detection test of the samples after 6 months of storage

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the sample</th>
<th>Visual Observation</th>
<th>Secondary test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1 d/w gm</td>
<td>Yellow colonies were observed in Mannitol salt agar medium. 6.1x10⁴</td>
<td>Observed black colonies surrounded by yellow zones in Vogel-Johnson agar medium 5.2x10⁴</td>
<td>Confirms the presence of Staphylococcus aureus.</td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>No bacterial colonies were observed on Mannitol salt agar medium</td>
<td>No bacterial colonies were observed on Vogel-Johnson agar medium</td>
<td>Confirms the absence of Staphylococcus aureus</td>
</tr>
<tr>
<td>3</td>
<td>A-3</td>
<td>No bacterial colonies were observed on Mannitol salt agar medium</td>
<td>No bacterial colonies were observed on Vogel-Johnson agar medium</td>
<td>Confirms the absence of Staphylococcus aureus</td>
</tr>
</tbody>
</table>

Staphylococcus aureus were totally absent in samples A₂ and A₃ (Table 3 and 4). Pictures of E.coli on Macconkey and EMB agar are shown in Photo 1 and 2. Pictures of Staphylococcus on Mannitol and Vogel Johnson media are shown in Photo 3 and 4.

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As a disinfection treatment it offers good control of many pathogenic and spoilage organisms with minimal change to the herbs.

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Thus gamma irradiation at doses of 5 kGy and 10 kGy is definitely safe for the treatment of microbial contamination of Ashwagandha up to 6 months of storage. The effect of gamma irradiation on the elimination or control of microorganisms on Ashwagandha root powder is in agreement with
Figure 1. *E. coli* on EMB agar in samples of Ashwagandha

Figure 2. *E. coli* on Macconkey agar in samples of Ashwagandha.

Figure 3. *Staphylococcus aureus* on Mannitol

Figure 4. *Staphylococcus aureus* on Vogel Johnson
that of Migdal et al. who showed that using irradiation (10kGy) satisfactory results pertaining to microbiological decontamination of medicinal herbs could be obtained. Also, after 6 months of storage, the quality and stability of Ashwagandha products were maintained.

REFERENCES AND NOTES

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ABSTRACT:
Whole plant powder of Kalmegh (Andrographis paniculata) was exposed to gamma irradiation at doses of 5 and 10 kGy in a Co 60 irradiator. Irradiated and unirradiated samples were stored at room temperature. Microbial load of whole plant powder were evaluated at 0, 6 and 12 months of storage. Results indicated that gamma irradiation reduced the total aerobic count and total fungal count in comparison to the non-irradiated sample. Further, the non-irradiated samples showed the presence of *E.coli* and *Staphylococcus aureus*. On irradiation there was a drastic reduction in *E.coli* and *Staphylococcus aureus*. Microbial load was found to be increased after 6 and 12 months of storage in the non-irradiated samples, where as there were no significant differences in the irradiated samples. A dose of 5 kGy could reduce the microbial load to acceptable levels, where as commercial sterility could be obtained at a dose of 10kGy. Thus, gamma irradiation can be used as a suitable technique for microbial decontamination of phytopreparations and products of Kalmegh and thus increase their shelf life and stability.

KEYWORDS: Kalmegh, Gamma irradiation, microbial load, shelf life, stability, microbial decontamination.

1. INTRODUCTION:
Kalmegh (Andrographis paniculata) is widely used in Indian and Chinese system of medicine. The plant is mainly used for liver diseases. Leaves are used in general debility, dyspepsia, stomach ailments in infants, griping. The roots are used as a tonic, stimulant and aperient. Whole plant finds its use as antispasmodic, febrifuge, stomachic, alternative, anthelmintic, anodyne, antiseptic, choleric, chologogue, diphoretic, expectorant, depurative, immunostimulant, laxative, astringent and antipyretic. They are used in the treatment of jaundice, diabetes, malaria, cholera, dysentery, enteritis, gastritis, pneumonia, pylonephritis, hyperdipsia, flatulence, colic, diarrhea, haemorrhoids and oedema.

Reports have shown that the plant possess hepatoprotective anthelmintic, antidiarrhoeal, antimalarial, antiviral, antipyretic, antiatherosclerotic, hypotensive, immunomodulator, antifertility and other activities.

Non-standardised herbal preparation does not get accepted in the global market. It becomes wrong to assume that the biological agents are safe because they are natural.

The microbial content of herb is one of the most important parameters as they make the heel potentially dangerous for sensitive population. One of the major problems associated with herbs is its microbial contamination resulting in quality deteriorations. Inspite of substantial efforts to avoid microbial contamination, an upward trend in non-acceptance of herbals are reported. Several decontamination methods exist, but the most versatile treatment among them is the processing of herbs with gamma irradiation. Microbial decontamination of herbal products by gamma irradiation is a safe, efficient, environmentally clean and energy efficient process.

Gamma irradiation at doses of 2-10 kGy has shown to be effective in eliminating pathogenic non-spore forming bacteria including other pathogens like *Salmonella*, *Staphylococcus*, *Pseudomonas E.coli* and others. With today’s demand for high quality herbs, gamma irradiation holds a promise for enhancing its safety and quality.

While exposure to gamma rays offers an effective alternative means of reducing microbial contamination, the dose increased to the extent of microbial kill has to be checked and also the method should be suitable to effectively reduce the microorganism and maintain this for a longer period.
Although gamma irradiation has been found as a suitable technique for microbial decontamination, there are no studies related to microbial decontamination of gamma irradiated Kalmegh. Accordingly, the objective of this study was to examine the effect on microbial contamination of irradiated samples of Kalmegh at 0, 6 and 12 months of storage.

2. MATERIALS AND METHODS:

2.1. Plant materials:
Whole plant of Kalmegh was procured from Natural remedies, Bangalore. The herb was identified and authenticated by Dr. Vasundhara, Professor. Department of horticulture GKV, Bangalore and voucher specimen deposited. They were further ground to powder and kept at ambient temperature.

2.2 Preparation of extracts:
100 g of coarsely powdered samples were packed in 9 different sterile polythene bags at ambient temperature. Gamma irradiation was carried out at a commercial scale Cobalt 60 irradiation service facility loaded with Cobalt 60 source with strength of 398 kilocuries (KCi), owned and operated by Microtrol sterilization services Pvt. Ltd. at Bangalore. The applied dose levels were 5 and 10 kGy and this absorbed dose was monitored with ceric/cerous dosimeters. Three packets were kept as such (K1- non-irradiated), three packs were gamma irradiated at a dose of 5 kGy (K2) and remaining three packets were gamma irradiated at a dose of 10 kGy (K3). 1st set of sample (K1, K2 & K3) were analysed soon after irradiation, second set was analysed after 6 months of storage and the third set was analysed after 12 months of storage.

2.3 Media for microbial studies: Total aerobic count was enumerated on nutrients agar (Hi media) and total fungal count on Sabourud’s dextrose agar. *E. coli* was tested on MacConkey agar and EMB agar, Selenite F broth and Brilliant green agar were the media’s for *Salmonella*, bismuth sulphite agar and cettrimide broth were the selective media used for determination of *Pseudomonas* and *Staphylococcus aureus* was tested on Mannitol salt agar and Vogel-Johnson medium. All the individual organisms were analysed as per the procedure given in IP26.

2.4 Sampling and enumeration:
I. Total microbial load: 1 gm of each sample was suspended in 10 ml of respective media. To determine total aerobic count the sample was incubated at 37°C ±1°C for 24-48 hrs and for total fungal count – samples were incubated at 28°C±1°C. Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gm (cfu/gm). Duplicate plates of appropriate dilutions were plated for specific organisms with there respective media and incubated at 37°C ±1°C for 24-48 hrs.

II. Identification test for *Escherichia Coli*:
1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 18 to 24 hours.

a) Primary test: 0.1 ml of the enrichment culture was spread on MacConkey agar plate using sterile autoclaved spreader uniformly maintaining aseptic conditions, in laminar airflow cabinet. Plates were incubated at 37°C ±1°C.

b) Secondary tests: 0.1 ml of the enrichment culture was spread on EMB agar plates using sterile autoclaved spreader uniformly maintaining aseptic conditions, in laminar air flow cabinet. Simultaneously 0.1 ml of enrichment culture was incorporated in tubes containing 5 ml of peptone water. Plates and tubes were incubated at 37°C±1°C, examined for the formation of metallic sheen in the agar plates and formation of indole (To test for indole add 0.5 ml of Kovac’s reagent, shake well, and allow to stand for one minute; if a red colour is produced in the reagent layer - indole is present). The formation of metallic sheen and indole in the secondary test indicates the presence of *Escherichia coli*.

III. Identification test for *Salmonella*:
1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 1 hr (4 hours for gelatin).

a) Primary test: Added 1.0 ml of the enrichment culture to tubes containing 10 ml of Selenite F broth and incubated at 37°C for 24 hours. Tubes were observed for the microbial growth and for the colour of the broth. Microbial growth with no colour in tubes shows presence of *salmonella*

b) Secondary test: Cultures from the preliminary test tubes were inoculated on to the brilliant green agar and bismuth sulphite agar plates. Plates were incubated at 37°C for 24 hr. Small, transparent and colourless, or opaque, pinkish or white (frequently surrounded by a pink or red zone) colonies on brilliant green agar and black or green colonies on bismuth sulphite agar confirms the presence of *Salmonella typhi*.

IV. Identification test for *Pseudomonas*:

a) Preliminary test: 1 gm of the sample was placed in a sterile screw capped jar containing 10 ml of Cetrimide broth and incubated at 37°C for 24 hours. Subcultured on a plate containing a layer of Cetrimide agar and incubated at 37°C for 24-48 hours, examined for the growth by Gram’s stain.

b) Secondary test: Since the preliminary test for *Pseudomonas* was negative, the confirmatory secondary tests were not performed.

V. Identification test for *Staphylococcus aureus*:
1 gm of sample placed in a sterile screw capped jar containing 10 ml of nutrient broth and incubated at 37°C for 24 hours. Subcultured on a plate containing a layer of mannitol salt agar medium and Vogel-Johnson agar medium and incubated at 37°C for 24 hours. Yellow colonies in mannitol salt agar medium and black colonies
surrounded by yellow zones in Vogel-Johnson agar medium indicate the presence of *Staphylococcus aureus*.

3. RESULTS AND DISCUSSION:
Total microbial load and the effect of gamma irradiation on non-irradiated and gamma irradiated Kalmegh samples were evaluated and are shown in Graph 1.

Before irradiation, total aerobic bacteria (1x10^3 cfu/gm) and total fungal count (1x10^2 cfu/gm) were quite high. On subjecting to specific pathogen tests as per IP, non-irradiated samples showed the presence of *E.coli* (3.4 x 10^3 cfu/gm) and *Staphylococcus aureus* (2.9x10^5 cfu/gm). *Salmonella* and *Pseudomonas* were totally absent in all the samples (Table 1). On gamma irradiating the samples at 5 kGy and 10 kGy, there was a drastic reduction in microorganism. In samples irradiated at a dose of 5 kGy initially there was reduction in total bacterial count from 1x10^7 cfu/gm to 1.3x10^5 cfu/gm and the total fungal count from 1x10^6 cfu/gm to acceptable levels. However, the specific microorganisms (*E.coli* and *Staphylococcus aureus*) were totally absent in samples K2 and K3. (Graph 2 and 3. Pictures of *E.coli* on Macconkey and EMB agar are shown in Photo 1 and 2 and pictures of *Staphylococcus* on Mannitol and Vogel Johnson media are shown in Photo 3 and 4.)
Further after 6 and 12 months of storage, the total bacterial count (1.4x10⁴ cfu/gm and 1.6x10⁵ cfu/gm respectively) and total fungal count (1.2x10⁴ cfu/gm and 1.3x10⁵ cfu/gm respectively) were increased compared to 0 month analysis (Graph 1). Pathogenic organism during 6 and 12 months storage also drastically increased to - E.coli (3.4 x 10⁴ cfu/gm and 3.5 x 10⁵ cfu/gm respectively) and Staphylococcus aureus (2.9x10⁴ cfu/gm and 3.0x10⁵ cfu/gm respectively), where as gamma irradiated samples maintained their quality by showing no microbial contamination (Graph 2 and 3).

In samples irradiated at a dose of 5 kGy initially there was reduction in total bacterial count from and total fungal count to acceptable levels. However, the specific microorganisms -E.coli and Staphylococcus aureus were totally absent in samples K2 and K3. This research clearly indicates that Kalmegh plant sample is highly contaminated. On testing for specific pathogens, Kalmegh samples were positive for presence of E.coli and Staphylococcus. Values exceeded drastically on storing it upto 12 months. 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.

Gamma irradiation was found to be an effective technology for resolving technical trade issue (WHO 1994, FDA 2001) and thus increase the quality of Kalmegh. 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 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. Samples irradiated at 5kGy and 10kGy had significantly lower levels of microbes than the non-irradiated (control) even at 12th month study. 5kGy could significantly lower the microbes to acceptable levels, however complete sterility could be attained at a dose of 10kGy.

4. CONCLUSION:

A dose of 5kGy could significantly reduce the microbial load to acceptable limits. However, commercial sterility could be attained at a dose of 10kGy.

Gamma irradiated samples could maintain their stability upto 12 months from the time of irradiation, which clearly indicates that the shelf life of the products can be increased upto 12 months.

The effect of gamma irradiation on the elimination or control of microorganisms on Kalmegh powder is in agreement with that of Migdal et al according to whom an ionizing radiation of 10kGy could give satisfactory results pertaining to microbiological decontamination of medicinal herbs.

Thus, gamma irradiation at doses of 5kGy and 10kGy helps in extending shelf-life of Kalmegh plant samples, ensures its microbial safety and helps in overcoming quarantine barriers to international trade. Further, gamma irradiation has got the advantages of its Dosimetric release, where the products can be sterilized in its final pack and released to the market even without post sterility testing, its single exposure makes it economical and these products can compete in global market.

Table 1: Compiled results for pathogen detection in Kalmegh samples during 0, 6 and 12 months of storage.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of the sample</th>
<th>Pathogens tested</th>
<th>E. coli</th>
<th>Salmonella</th>
<th>Pseudomonas</th>
<th>Staphylococcus aureus</th>
</tr>
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<tr>
<td>1</td>
<td>K-1</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>K-2</td>
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<td>-</td>
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<td>K-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

"+" = Present  
"-" = Absent
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