SUMMARY AND CONCLUSION

“Research is creating new knowledge”

Neil Armstrong (1930-2012)
First person to walk on the Moon
SUMMARY

The use of herbal preparations for treating disorders and diseases has been growing in the developing and developed countries. Among the complementary or alternative medicines, traditional Indian Ayurvedic medicine is becoming one of the most widely used therapies throughout the world, next to Traditional Chinese medicine. The increasing interest in herbal medicines is due the belief that, being natural and traditionally used, they are safe and harmless, however the natural origin of a medicine is not a guarantee of safety. Recent reports point out the risks associated with the use of herbal medicines. The adverse effects of herbal drugs may be broadly grouped into two main groups, intrinsic and extrinsic. Extrinsic effects includes the quality of the herbal medicinal products due to microbial contamination. Microbial contaminants are known to alter the chemical property of the herbs thereby affect the medicinal potential of the drug. Thus one of the major reasons for the poor quality of herbal drugs is the contamination plant materials with bacteria, fungi, and microbial toxic metabolites such as aflatoxins, fumonisins etc. Of late, many of the developed countries are imposing quality criteria for import of herbal drugs. Hence a thorough purity check of herbal drug plant materials and herbal products for microbial contamination in general and microbial toxins in particular is of considerable interest. Southern parts of Karnataka is one of the important region of the state known for collection processing of herbal drug plant materials, and for manufacture and sale of herbal formulation. Considering these, it was envisaged to screen the herbal drug plant materials and herbal products of this region for microbial contamination and toxic microbial metabolites to understand the gravity of the problem and suggest management practices with the following objectives.

OBJECTIVES

- To know the diversity and concentration of aerobic bacteria associated with the herbal drug plant materials and herbal medicines
- To understand the diversity and concentration of culturable fungi associated with the herbal drug plant materials and herbal medicines
- To assess the aflatoxin contamination of the herbal drug plant materials and herbal medicines.
- To understand the effect of gamma irradiation on the microflora, phytochemistry and bioactive potential of herbal drug plant materials and herbal medicines.
The results of the thesis is presented in five chapters. Each chapter is with an introduction, review of literature, materials and methodology followed and results illustrated with tables and figures followed by discussion. References cited are provided at the end of the thesis as bibliography.

Chapter-1: General introduction

This chapter provides an insight into the aspects of traditional medicines and its prevalence in various parts of the world including India. Apart from highlighting the major role of traditional medicines in health care, it also discuss about the quality issues associated herbal drug plant materials and herbal preparations. This section provides information on microbial and mycotoxin contamination of herbal drug plant materials and herbal products including maximum permissible limits prescribed by various organizations such as WHO and FSSAI. Further it illustrates the various management strategies currently employed for decontamination of herbal medicines and their advantages and dis advantages. This chapter highlights an overview of the work done by various workers with respect to bacterial, fungal and mycotoxin contamination of medicinal plants, herbal materials and herbal medicines. The chapter also consists information about the test samples. A total of 12 herbal drug plant materials viz., *Acorus calamus* rhizome, *Cassia angustifolia* leaves, *Centella asicatica* leaves, *Hemidesms indicus* roots, *Myristica fragrans* mace, *Phyllanthus emblica* fruits, *Piper longum* inflorescence, *Terminalia bellerica* fruits, *Terminalia chebula* fruits, *Tinospora cordifolia* stem, *Viteveria zizanioides* roots and *Withania somnifera* stem, 6 paste form of herbal medicines Amalaki choorn, Kashayada pudi, Kemmu choorna, Mruduvirechana choorna, Trikatu choorna and Thriphala choorna, 6 paste form of herbal medicines such are Agatsya harithaki rasyana, Amalaka rasayana, Ashwagandha rasayana, Brihatvasava lehya, Chyavanaprsha and Drahshadi rasayana, and 6 liquid form of herbal medicines viz., Amritharista, Ashvagandharista, Dashamoolarista, Panchali herbal syrup, Sogadeberina juice and Triphalarista were selected for the study. The test samples were selected based on their wide usage and availability.

Chapter 2: Diversity and concentration of aerobic bacteria associated with the herbal drug plant materials and herbal medicines

This chapter comprises the screening of 12 herbal drug plant materials, 6 powder, 6 paste and 6 liquid form of herbal medicines for bacterial contamination employing serial dilution method
(Koch, 1883). Bacteria associated with each test sample was enumerated in terms of Colony Forming Unit (CFU). The bacterial contaminants were isolated by streaking method, pure cultures were maintained on NA media and they were grouped into Gram positive and Gram negative bacteria through Gram staining method (Gram, 1884) and KOH string test (Fluharty and Packard, 1967). Diversity of bacterial contaminants was studied by considering various morphological characteristic features viz., shape, margin, elevation, color, surface texture, optical feature and growth pattern (Seeley and Vandemark, 1962). All the test herbal drug plant materials and herbal products were also screened for the incidence of E. coli and Salmonella using selective media such as MacConkey agar and EMB for E. coli and brilliant green agar and bismuth sulphite agar for Salmonella (Anon., 2007). Further all the bacterial isolates were tested for antibiotic susceptibility to 4 antibiotics having different mode of action viz., Gentamicin, Ciprofloxacin, Cefotaxime and Trimethoprim through disc diffusion method (Bauer et al., 1966). Most frequently occurring Gram positive bacteria and Gram negative bacteria, as well as the multidrug resistance bacteria were subjected to molecular identification and structure was recorded through SEM imaging technique (Dougall et al., 1994).

All the test samples were found to be contaminated by bacteria except liquid form of herbal medicines. Test herbal drug plant materials and powder form of herbal medicines were associated with large number of bacteria exceeding the maximum permissible limit prescribed by WHO (Annon, 2007), whereas paste form of medicines were within the maximum permissible limit. A total of 366 bacteria were isolated, among which 303 were Gram positive comprising 171 cocci and 132 bacilli, and 63 bacteria were found to be Gram negative consisting 19 cocci and 44 bacilli. Among 366 bacteria, most of the bacteria (147) exhibited circular shape followed by irregular (143) and punctiform (51). Maximum number (156) of bacteria showed entire margin, followed by curled margin (126), lobate (29) rhizoidal shape (28). Raised type of elevation was found to be predominant, recording in 222 bacteria followed by flat (50) and umbonate (43). Six different colored bacteria were recorded of which 160 were whitish, 135 bacteria were white, 36 were Yellow, 26 were orange and 8 were pink. Three type of surface textures were observed of which 186 were smooth, 186 were rough and 32 were wrinkled were recorded. Optical feature of bacteria were found to be Opaque (236) and Transluscent (95) and transparent (35). Four different type of bacterial growth pattern viz., uniform, sediment, flocculent and pellicle on nutrient broth was observed.
Among all the test samples, rhizomes of *A. calamus*, leaves of *C. asiatica*, mace of *M. fragrans*, fruits of *P. emblica* and *T. bellerica*, Stem of *T. cordifolia*, Kashayada pudi, Kemmu choorna and Triphala choorna were found to be contaminated by *E. coli*. All the test samples were free from *Salmonella* contamination. All the isolates were susceptible to one or more test antibiotics. Among 366 bacterial isolates two isolates viz. MGB-CISHDT-RR-Tco-M-003 (Gram positive bacteria) isolated from *T. cordifolia* stem was resistant to all the test antibiotics, and MGB-CISHDT-RR-Ca-M-0032 (Gram negative bacilli) isolated from *C. asiatica* was resistant to Gentamycin, Cefataxime and Trimethoprim. Through 16s rRNA sequencing and molecular study the two most frequently occurring bacteria were identified as *Micrococcus luteus* and *Aeromonas hydrophila*. Similarly the two multidrug resistant bacteria were identified as *Bacillus nealsonii* and *Aeromonas schubertii*. The sequences were submitted to NCBI-Gen bank and authentic accession numbers were obtained as follows.

<table>
<thead>
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<th>Sl. No.</th>
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<th>GenBank accession number</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td><em>Aeromonas hydrophila</em></td>
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<tr>
<td>3</td>
<td><em>Bacillus nealsonii</em></td>
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<td>4</td>
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</table>

Present study is the first report on bacterial contamination of selected herbal drug plant materials and products of this region, and the first report on isolation and molecular identification of *Micrococcus luteus* from *A. calamus*, *C. angustifolia*, *C. asiatica*, *T. bellerica*, *V. zizanioides* and *W. somnifera*, and *Aeromonas hydrophila* from *A. calamus*, *C. angustifolia*, *C. asiatica*, *H. indicus*, *P. emblica*, *T. bellerica*, *T. chebula*, *T. cordifolia*, *V. zizanioides*, Kashayad pudi, Triphala choorna and Amalaki choorna. The study has also reported the incidence of multidrug resistant bacteria viz., *Bacillus nealsonii* and *Aeromonas schubertii* in *Tinospora cordifolia* and *Centella asiatica* respectively for the first time.

Chapter-3: Assessment of diversity and concentration of culturable fungi associated with the herbal drug plant materials and herbal medicines

The chapter comprises of information about the fungal contaminants associated with test herbal drug plant materials and different forms of herbal medicines. Density and diversity of fungi was recorded by serial dilution method (Koch, 1883). Most of the fungi were identified based on their cultural and morphological characteristic features by referring to standard manuals (Thomb
and Raper, 1951; Singh et al., 1991; Burnett and Hunter, 1998; Gilman, 1998; Mathur and Korgsdal, 2003) and research articles (Navi et al., 1999; Schubert et al., 2007; Webster et al., 2007; Ellis et al., 2007; Pornsuriya et al., 2008; Pitt et al., 2009; Samson et al., 2011; Asgari et al., 2011). Fungal contamination of the test samples was further confirmed through detection and quantification of ergosterol following the procedure of Seitz et al. (1977) and Jambunathan et al. (1991).

All the herbal drug plant materials, powder and paste medicines were found to be contaminated by a broad spectrum of fungi. In case of liquid medicines out of 6 medicines, only panchali herbal syrup and sogadeberina juice were found in association with fungi. All the contaminated samples harboured fungal concentration that exceeded the maximum permissible limit prescribed by WHO (Annon, 2007). A total of 1817 isolates of 211 fungal species belonging to 82 genera were isolated. Among all the isolates, 1802 were identified based on their cultural and morphological characteristic features whereas 15 fungi were nonsporulating sterile fungi and hence labeled as unidentified fungi with respective serial numbers. Aspergillus niger and A. flavus were found to be the predominant fungi.

Among different forms of herbal drugs screened, herbal drug plant materials recorded high amount of ergosterol compared with paste and powder form of drugs. Highest ergosterol was observed in T. cordifolia (965.23 µg/kg) followed by C. asiatica (563.09 µg/kg) and M. fragrans (509.31 µg/kg). In case of powder form of herbal drugs Amalaki choorna recorded high ergostrol (196.03 µg/kg) followed by Trikatu choorna (154.33 µg/kg) and Kemmu choorna (148.52 µg/kg). Among 6 paste form of herbal drugs tested Drahshadi rasayana recorded high ergosterol (170.15 µg/kg) followed by Brihatvasava lehya (150.42 µg/kg) and Ashwagandha rasayana (122.51 µg/kg).

Positive correlation was observed between the concentration of fungal contaminants and quantity of ergosterol quantity. H. indicus recorded the highest CFU of fungi (22.22 CFU/g) and it also recorded the highest ergosterol (304 µg/kg) content. Similarly Amalaki rasayana recorded least fungal CFU (0.66 CFU/g) with least ergostrol (36 µg/kg) content.

The study reveals the association of a wide variety of fungi (211) with the test samples. The results reveals that the fungal contamination status of test samples is an important factor to determine the quality of herbal drug plant materials and herbal products, understanding of which is essential for the development of management strategies and good manufacturing practices.
Chapter-4: Assessment of the microbial metabolites contamination of the herbal drug plant materials and herbal medicines with special reference to mycotoxins

This chapter deals with the methodology and results of the screening of all the *Aspergillus flavus* isolates for Aflatoxin B₁ production. Detection of toxigenic strain was done by TLC method (Singh *et al.*, 2008) and quantification was done by spectroscopic method with reference to standard Aflatoxin B₁. Further all the herbal drug plant materials and herbal products were subjected to screening for the incidence of Aflatoxin B₁ following TLC and LCMS method (Thomas *et al.*, 1975). Among the total 120 isolates from test herbal drug plant materials and herbal medicines, 79 (65.83%) isolates tested positive for aflatoxin B₁ production.

High quantity of aflatoxin B₁ was recorded from isolates of *T. cordifolia* (5008.20 µg), *Myristica fragrans* (1343.19 µg), *Vetiveria zizanioides* (1329 µg) and *H. indicus* (1000.41 µg). *V. zizanioides* (132 µg/kg) was highly contaminated by aflatoxin B₁ followed by *T. cordifolia* (104.19 µg/kg), *H. indicus* (81.86 µg/kg) and *A. calamus* (13.73 µg/kg). Incidence of Aflatoxin B₁ in herbal drug plant materials was confirmed by LCMS analysis.

Based on the results it is concluded that *A. calamus*, *H. indicus*, *M. fragrans*, *T. cordifolia* and *V. zizanioides* are not fit for human consumption as they possess high aflatoxin B₁ exceeding the maximum permissible limit prescribed by European Pharmacopoeia (4 µg/kg) and FSSAI (30 µg/kg).

Chapter-5: Effect of gamma irradiation on the microflora of herbal drug plant materials and herbal medicines

This chapter provides a detail account of the effect of ⁶⁰Co gamma irradiation and fungal infestation on nutritional, phytochemical and bioactive potential of *A. calamus* rhizome and *M. fragrans* mace. Microbial contamination of herbal drug plant materials takes place during pre and post-harvest practices. Considering this the test materials were aseptically collected from the field at harvesting stage. Collected samples were hermetically sealed and used for the treatment studies. Based on the availability of samples, rhizome of *A. calamus* and *H. indicus* were selected for further studies. Pilot experiment conducted with one herbal drug plant material *Hemidesmus indicus* roots revealed that gamma radiation at 1, 2 and 3 kGy was ineffective for complete decontamination of bacteria and fungi, hence higher doses of 5, 10, 15 and 20 kGy were selected for the study.
Irradiation treatment with $^{60}$Co gamma radiation

*Acorus calamus* rhizomes and *Myristica fragrans* mace were collected from the field and treated with 5, 10, 15 and 20 kGy of gamma irradiation at dose rate of 7.83 kGy/hr. The untreated samples served as control. All samples were analyzed for microflora, moisture content, extract yield, nutritional value, phytochemical constituents and bioactive potential. Total elimination of bacteria and fungi was observed at 15 kGy in *M. fragrans*. In case of *A. calamus* total elimination of fungi was observed at 15 kGy while bacterial contamination ($2 \times 10^5$ CFU/g) persisted, however at 20 kGy treatment total elimination of both bacteria and fungi was observed.

For further study the samples were treated with only 20 kGy. Both irradiated and control samples were stored for a period of 6 months. Samples were drawn at regular intervals of one week, 12 weeks and 24 weeks and subjected to nutritional, phytochemical and bioactive potential analysis following standard procedures. Moisture content was determined as per the procedure described by ISTA (2015). Nutritional value of the test samples was determined through estimation of total carbohydrate (Hedge and Hofreiter, 1962), protein (Lowry *et al.*, 1951) and lipid (Fabbri *et al.*, 1980) content which is expressed in terms of energy value (FAO, 2003). Qualitative (Sofowara, 1993; Tease and Evans, 1989) and quantitative phytochemical analysis was done with special reference to alkaloids (Harborne, 1973), flavonoids (Muthulakshmi *et al.*, 2015), phenols (Hagerman *et al.*, 2000), tannins (Prabhavathi *et al.*, 2016), terpenoids (Ferhuson, 1956) and saponins (Obadoni and Ochuko, 2001). Bioactive potential of test samples was studied by antibacterial activity assay following disc diffusion method (Bauer *et al.*, 1966) and antioxidant activity by DPPH scavenging activity (Blois, 1958).

*A. calamus* rhizomes recorded the bacterial count of $27 \times 10^7$ CFU/gm and fungal count of $14 \times 10^5$ CFU/gm in the analysis done after a week of storage, while $18 \times 10^7$ CFU/gm bacteria and $14 \times 10^5$ CFU/gm fungi was observed after 12 weeks and, $15 \times 10^7$ CFU/gm bacteria and $13 \times 10^5$ CFU/gm fungi was observed after 24 weeks of storage. Bacterial concentration of *A. calamus* rhizomes control samples was found to decrease with increasing storage period and insignificant variation was observed in case of fungal concentration.

In case of *M. fragrans* mace, control samples both bacterial and fungal concentration was found to increase with increasing storage period recording $21 \times 10^7$ CFU/gm bacteria and $16 \times 10^5$ CFU/gm fungi in the analysis done after a week of storage, $26 \times 10^7$ CFU/gm bacteria and $25 \times 10^5$
Summary and Conclusion

CFU/gm fungi in the samples after 12 week and $20 \times 10^7$ CFU/gm bacteria and $33 \times 10^5$ CFU/gm fungi after 24 weeks storage. Irradiated samples of both *A. calamus* rhizomes and *M. fragrans* mace was found to be free of microbial contamination in all the analysis.

Results of moisture content determination revealed that the irradiated samples of both *A. calamus* rhizomes (7.6%) and *M. fragrans* mace (2.98%) was lesser than that of control samples of *A. calamus* (8.02%) and *M. fragrans* (5.13%). Insignificant variation in moisture content was recorded during storage period of 24 weeks.

Extract yield of control samples of *A. calamus* rhizomes (3.98 mg/gm) and *M. fragrans* mace (10.69mg) was lesser than the irradiated samples of *A. calamus* (5.02gm/100gm) and *M. fragrans* (10.97mg/100gm). Extract yield of all the samples was found to be increased with increasing storage period.

Nutritional value of irradiated samples of both *A. calamus* (153.7 kcal/100gm) and *M. fragrans* (129.7 kcal/100gm) was found to be more than that of control samples of *A. calamus* (148 kcal/100gm) and *M. fragrans* (115.6 kcal/100gm). Marginal variation in the energy value was observed with all the samples during the storage period of 12 weeks and 24 weeks.

Qualitative phytochemical analysis revealed the presence of alkaloids, flavonoids, phenols, tannins, terpenoids and saponins in both control and irradiated samples of *A. calamus* and *M. fragrans* in samples stored for a week, 12 weeks and 24 weeks. Results of qualitative analysis revealed that the irradiated samples possessed comparatively more quantity of all the tested phytochemicals than that of the control samples in both *A. calamus* and *M. fragrans*. Insignificant variation in phyto-constituents was observed during storage period of 24 weeks.

Antibacterial potential of irradiated *A. calamus* was found to be high when compared with control samples, recording inhibition zone of 7-9mm, 6mm, 6-8mm, 20-24mm, 7-18.5 mm, 19-20mm and 7mm against *Xanthomonas compestris oryzae*, *Xanthomosa compestris vasicatoria*, *Pseudomonas syringae*, *Bacillus cereus*, *Staphylococcus aureus* and *pseudomosa aeruginosa* respectively, over a storage period of 24 weeks.

In case of *M. fragrans* antibacterial activity of irradiated samples was found to be same as that of control samples exhibiting zone of inhibition against *Xanthomonas compestris vasicatoria* (6 mm), *Pseudomonas syringae* (26 mm), *Bacillus cereus* (6 mm), *Staphylococcus aureus* (8 mm). However variation was recorded in *Pseudomonas syringae* against which control samples did not
Summary and Conclusion

recorded any activity but irradiates samples showed 17 mm zone of inhibition in samples stored for 24 weeks.

DPPH scavenging activity of irradiated samples of *A. calamus* showed insignificant difference when compared with that of control samples. In case of *M. fragrans* mace irradiated samples recorded marginally high DPPH scavenging activity recording less IC$_{50}$ value of 19.5 mg/ml than that of control samples (24 mg/ml) in a week of stored samples.

**Artificial fungal infestation treatment**

Both the test samples were artificially infested with selected fungi viz., *Aspergillus niger*, *A. flavus* and *Penicillium citrinum*. Method followed by Onfrog *et al.* (1999) was adopted for preparation of spore suspension and 3 ml of each fungal spore suspension having $10^5$/ml spore concentration is used for inoculation of each sample (150 gm).

Both control and artificially infested samples were hermetically sealed and stored for a period of 6 months. Samples were drawn at regular intervals of a week, 12 weeks and 24 weeks and subjected to various analyses as described earlier.

Bacterial and fungal count of infested samples was higher in both *A. calamus* and *M. fragrans* than that of the control. Control samples of *A. calamus* recorded bacterial CFU of $27 \times 10^7$ (1 week), $18 \times 10^7$ (12 weeks) and $15 \times 10^7$ CFU/g (24 weeks) and fungi was $14 \times 10^5$ (1 week) $15 \times 10^5$ (12 weeks) and $13 \times 10^5$ CFU/g (24 weeks). Bacterial CFU of infested *A. calamus* rhizomes was found to be decreased with increasing the storage period recording $34 \times 10^7$ CFU/g (1 week), $25 \times 10^7$ CFU/g (12 weeks) and $22 \times 10^7$ CFU/g (24 weeks). However insignificant difference was observed in fungal concentration. In case of infested *M. fragrans*, bacterial concentration was found to be decreased recording $35 \times 10^7$ CFU/g (1 week), $30 \times 10^7$ CFU/gm (12 weeks) and $28 \times 10^7$ CFU/g (24 weeks). However considerable increase in the fungal concentration was observed with increasing storage period recording CFU of $18 \times 10^5$ (1 week), $22 \times 10^5$ (12 weeks) and $35 \times 10^5$ (24 weeks).

Infested samples of *A. calamus* rhizomes recorded higher moisture content than that of the control (7-8%) with increasing storage period of a week (13.4%), 12 weeks (16.2%) and 24 weeks (37.6%). The moisture content also increased in case of *M. fragrans*. Extract yield of infested samples were found to be higher than that of the control in both *A. calamus* and *M. fragrans* and the same also increased with increasing storage period.

Energy value of infested samples of *A. calamus* found to be less than that of control. The energy value decreased with increasing storage period.
Phytochemical analysis of control and infested samples recorded slight increase in quantity of alkaloids, flavonoids and phenols in the infected samples than that of control. However fungal infestation was found to reduce the saponins and terpenoids content in both *A. calamus* and *M. fragrans*.

Antibacterial activity of infested samples of *A. calamus* was found to be higher than that of control samples recording 3mm more zone of inhibition against *Xanthomonas oryzae*, 4.5mm more against *Xanthomonas campestris* pv. *vesicatoria*, 1 mm more against *Pseudomonas syringae* and 7mm more against *Pseudomonas aeruginosa*. The *M. fragrans* infested samples recorded antibacterial activity against *Xanthomonas oryzae* (9mm) while control sample did not record any activity. Similarly only the infested samples exhibited activity against *Pseudomonas syringae* (26mm), *Bacillus cereus* (6 mm) and *Staphylococcus aureus* (8 mm) in a week stored samples.

Antioxidant activity assay revealed that the infested samples have less DPPH scavenging activity than that of control samples of both *A. calamus* and *M. fragrans*.

**Irradiation treatment of artificially fungal infested samples**

In order to understand the effect of gamma irradiation on the selected storage fungi, *A. calamus* rhizomes and *M. fragrans* mace were subjected to artificial fungal infestation as described earlier and incubated for a week at room temperature. After the period of incubation of 7 days samples were subsequently treated with 20 kGy of $^{60}$Co gamma radiation. Irradiated samples were stored for a period of 6 months and analyzed at regular intervals of 1 week, 12 weeks and 24 weeks and subjected to various analyses as described earlier.

Microflora analysis revealed that the control samples were contaminated with bacteria and fungi, whereas the irradiated samples were found to be free of contaminants. Moisture content of irradiated *A. calamus* rhizomes was lesser than that of control samples and it also decreased with increasing storage period of a week (7.19%), 12 weeks (7.7%) and 24 weeks (7.6%). In case of *M. fragrans* moisture content of irradiated samples was slightly higher than the control (5.13%) in a week stored samples (5.15%) and lesser than control in 12 weeks (5.13 %) and 24 weeks (5.11 %) stored samples. Energy value of irradiated *A. calamus* rhizomes and *M. fragrans* mace was found to be lesser than control samples.

Phytochemical analysis showed insignificant difference in alkaloid content between irradiated and control samples of both *A. calamus* rhizomes and *M. fragrans* mace. Flavonoid contents of irradiated *A. calamus* (208 µg/g) was higher than that of control samples (185.21-
186.66 µg/g). In case of *M. fragrans* mace flavonoid content of irradiated samples (225 µg/g) was lesser than that of control sample (231.66-233.33 µg/g). Phenol content of irradiated *A. calamus* and *M. fragrans* was higher than control in a week stored samples which decreased with increasing storage period, while the phenol content of control samples was found to be increased with increasing storage period. Tannin content of irradiated sample was higher than that of control samples in both *A. calamus* and *M. fragrans*.

Terpenoid content of irradiated samples was found to be lesser than control and it increased with increasing the storage period. In case of *M. fragrans*, terpenoid content of control samples decreased with increasing storage period, but irradiated samples showed insignificant variation over storage period. Saponin content of irradiated *A. calamus* was lesser than the control samples. In case of *M. fragrans* saponin content of irradiated samples (0.6 µg/g) was found to be consistent over the storage period, while control samples recorded increase in saponin content with increasing storage period of a week (0.58 µg/g), 12 weeks (0.6 µg/g) and 24 weeks (0.61 µg/g).

Antibacterial activity of irradiated *A. calamus* samples was found to be same as that of control in case of *Xanthomonas oryzae* (9 mm), *Bacillus cereus* (7mm) and lesser than control in case of *Pseudomonas syringae* (1 mm), *Staphylococcus aureus* (2 mm). It also recorded more zone of inhibition of 0.5 mm and 9mm against *Xanthomonas compestris vascicatoria* and *Pseudomonas aeruginosa* respectively. Insignificant variation in zone of inhibition was observed over the storage period of 24 weeks. In case of *M. fragrans* antibacterial potential of both control and irradiated samples were found to be same against all the test bacteria except *Pseudomonas syringae* against which irradiated samples recorded 0.5 mm more zone of inhibition than control.

Antioxidant activity of irradiated samples was lesser than that of control samples stored for a week and 24 weeks, whereas insignificantly high in 12 weeks stored samples. In case of *M. fragrans* DPPH scavenging activity of the irradiated samples was found to be higher than that of control samples.

Findings of the investigation clearly showed that the gamma irradiation treatment with 20 kGy dose have significant effect on the phytochemical constituents of the *A. calamus* rhizomes and *M. fragrans* mace and nutritional value of the irradiated sample was also found to be unchanged. Present work also revealed that the fungal infection can cause loss of energy value in herbal drug plant materials and it also causes reduction in saponin and terpenoid quantity. Results also points out that the gamma radiation treatment have positive effect on DPPH scavenging
activity recording low IC\textsubscript{50} value in irradiated sample than the control. The increased DPPH scavenging activity can be attributed to the increase alkaloids, flavonoids, phenols, tannins, terpenoids and saponin content in irradiated samples, which are active ingredients possessing antioxidant activity.

Based on the results obtained it can be stated that the gamma irradiation is an efficient method for the management of microbial contamination of herbal drug plant materials.

**CONCLUSION**

Presence of high density of fungi and bacteria in the tested herbal drug plant material and herbal medicines indicate the risk associated with the use of such contaminated plant materials and products. Occurrence of *Micrococcus luteus* and *Aeromonas hydrophila* like opportunistic pathogens, and incidence of multidrug resistant bacteria viz., *Bacillus nealsonii* and *Aeromonas schubertii* in the herbal materials clearly indicated the poor quality of the samples. Concentration of AB\textsubscript{1} in *A. calamus*, *H. indicus*, *M. fragrans*, *T. cordifolia* and *V. zizanioides* exceeds the maximum permissible limit prescribed by WHO, should be taken seriously because of its proven carcinogenic and immunosuppressive activities. The study reveals the need to educate the herbal drug practitioners regarding good manufacturing practices. Findings of the gamma irradiation treatment studies, showed the potential of 20 kGy irradiation treatment for complete elimination of microbes associated with *A. calamus* rhizomes and *M. fragrans* mace. Based on the results obtained, 20 kGy gamma irradiation can be recommended as an efficient method for the decontamination of *A. calamus* rhizome and *M. fragrans* mace, as the nutritional value, phyto-constituents and bioactive properties of the irradiated samples were found to be unharmed by the treatment.
SIGNIFICANCE OF THE WORK

- Concentration and morphological diversity of bacterial contaminants of herbal drug plant materials and herbal products is illustrated.
- Most frequently occurring Gram negative (*Aeromonas hydrophila*) and Gram positive (*Micrococcus luteus*) bacteria were identified through 16 s RNS sequencing.
- All the bacterial contaminants were tested for antibiotic susceptibility, and incidence of 2 multi drug resistant bacteria viz., *Bacillus nealsonii* and *Aeromonas schubertii* is reported.
- The study demonstrated the density and diversity of fungi associated with selected herbal drug plant materials, powder, paste and liquid forms of herbal products.
- All the *Aspergillus flavus* isolates (120) were screened for their ability to produce aflatoxin B₁ and 79 isolates showed the ability to produce aflatoxin B₁.
- *Aspergillus flavus* contaminated samples were assessed for the incidence of Aflatoxin B₁ by which biosafety of the test samples was determined.
- Present work states the optimum dose of ⁶⁰Co gamma radiation required for the complete decontamination of *H. indicus* roots (20 K Gy), *A. calamus* rhizomes (20 K Gy) and *M. fragrans* mace (15 K Gy).
- It reveals the effect of fungal infection as well as gamma irradiation on the nutritional phytochemical and bioactive potential of rhizomes of *A. calamus* and mace of *M. fragrans*.