SUMMARY

Medicinal plants are integral part of human civilization since ancient times. Herbal drugs are in great demand in developing as well as developed countries for basic health care. These are widely used because of their safety and less costs. Herbal extracts are used for curing various human ailments because of their medicinally important active compounds. Although these compounds have enormous structural diversity and activity, only a small portion of that diversity has been explored for their pharmacological potential. Among important medicinal plants *C. borivilianum* Sant. et Fernand., belongs to the family Liliaceae, commonly known as *Safed Musli* in India, is one of the most important medicinal herbs which offer a cure for many ailments. The tubers of this plant have many therapeutic applications in Ayurvedic, Unani, Homeopathic and Allopathic systems of medicine and are an important constituent of more than 100 herbal drug formulations. Tubers of the *C. borivilianum* have been used for their aphrodisiac properties since centuries and referred as 'Natural Viagra'. Because of its health restorative and promotive properties, it is referred to as ‘revitalizer’.

This herb is also used to cure other diseases like diabetes, fever, bronchitis, ophthalmic conditions, vomiting, diarrhea, dysentery, dyspepsia, gonorrhea, leucorrhea etc. It has antinflammatory properties so used to cure arthritis and rhumatism. Because of these properties in Ayurveda, it is referred as *Roganut* (disease curative). It helps in normalizing various functions of the body such as blood pressure, blood sugar, and improves the overall immunity and delays aging. Therefore, it acts as an antioxidant and immunomodulator. The demand of this drug is increasing at global level, and is sold in the world market as "Indian Viagra", "Herbal Viagra", "White gold", or "Wonder crop".

The demand for *C. borivilianum* in the world market is resulting in it’s over exploitation in the wild state, pushing the plant to the verge of extinction. Therefore, it is enlisted among the list of 59 selected priority medicinal plants, the cultivation and export of which is being actively encouraged by the Government of India through the National Medicinal Plant Board, and subsidies are provided by the Government for its cultivation. The global demand for *C. borivilianum* is over 35,000 tonnes per annum, while its supply is just to the extent of 5,000 tonnes/year.
The main problems with the cultivation of *C. borivilianum* are linked to its poor seed germination percentage, low seed viability and long dormancy period. Secondly, the cultivation cost of this plant is very high, *i.e.*, Rs. 3,12,500/ha.

1. **AIMS AND OBJECTIVES**

Keeping in mind the immense medicinal importance of this plant and problems related to its cultivation, the present work was planned to increase its biomass and yield by giving treatments of different plant growth regulators, steroids and metals etc. to make the cultivation of this crop cost effective. Since Zn(II) and Cr(III) are important in human health for reproductive potential and cure of diabetes, these metals were applied to the tubers to enhance their curative properties. Besides, scanty information is available in literature on the antioxidative and antimutagenic potential of *C. borivilianum*. Therefore, an attempt has also been made to study its antimutagenic and antioxidative properties of different parts of the plant. Keeping in view the dearth of literature on the subject, the study was framed to meet the following objectives:

1. Effect of plant growth regulators (kinetin and IBA, 24-Epibrassinolide), mammalian steroids (testosterone and cholesterol), and metals (Zn(II) and Cr(III)), in single and binary combinations on the growth of plant biomass especially the tubers by giving various treatments viz., pre-soaking of seeds/tubers and foliar spray.

2. Effect of treatments on biochemical parameters (chlorophyll, carotenoid, saponin, carbohydrate, protein, Zn and Cr content) on plants grown in sand/soil culture.

3. Effect of treatments on antioxidant defense system of seedlings and leaves of plants on lipid peroxidation, protein content, and different antioxidant enzyme activities viz., superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR).

4. Effect of treatments of metals, PGRs and steroids on shoot apices of *C. borivilianum* through tissue culture technique and to establish a protocol for easy propagation of plants.

5. Antimutagenic potential of methanol and water extracts of tubers, peels, leaves and seeds of *C. borivilianum* with Ames assay using TA98 and TA100 strains of *Salmonella typhimurium* (Maron and Ames, 1983).
6. Antioxidative potential of different extracts of *C. borivilianum* by employing following *in vitro* assays:

(i) 2, 2-Diphenyl-1-picrylhydrazyl assay (Blois, 1958).

(ii) Lipid peroxidation assay (Halliwell and Guttridge, 1989).

(iii) Deoxyribose degradation assay (Halliwell *et al*., 1987 and Arouma *et al*., 1987).

(iv) Reducing power assay (Oyaizu, 1986).

(v) Chelating power assay (Dinis *et al*., 1994).

8. Quantification and identification of saponins in tubers of *C. borivilianum*.

2. METHODOLOGY

In order to study the effects of plant growth regulators (kinetin and IBA, 24-EpiBr), mammalian steroids (testosterone and cholesterol), and metals like Zn(II) and Cr(III) on plant growth and metal uptake seeds or tubers were grown in Petri plates, sand and soil cultures.

2.1 Seed germination studies

In seed germination studies, seed viability was checked using tetrazolium test (Cotrell, 1947). Single and binary treatments were given at various concentrations (0, 10^{-10}, 10^{-8}, 10^{-6} M) through pre-soaking of seeds. The seedlings were grown in solutions of different treatments in Petri plates and kept in seed germinator at at 30±1°C for 21 days for a photoperiod of 8 h dark and 16 h light. The seedlings were observed for percent germination, number of roots, root length, shoot length, and fresh seedling weight and were also analyzed for total protein content and various antioxidant enzymes.

2.2 Sand culture

In sand culture, treatments were given through pre-soaking of tubers in solutions of different concentrations (0, 10^{-10}, 10^{-8}, 10^{-6} M) of PGRs, steroids and metals for six hours before sowing. Tubers were grown in sand and pots were kept in the culture room under a regime of 8 h dark and 16 h light at 25±1°C temperature, and relative humidity of 75-80%. Plants were studied for various growth parameters like number of leaves, leaf length, leaf width and number of tubers. The leaves were analysed for various biochemical parameters like total chlorophyll, total carotenoid, lipid peroxidation, total protein content and various antioxidants.
2.3 Soil culture

In soil culture single treatments were given through pre-soaking of tubers as well as foliar spray. Morphological parameters like number of leaves, leaf length, leaf width, number of inflorescences, length of the inflorescence and number of fruits per inflorescence were studied for 60 days old plants whereas tubers were harvested after 9 months and analyzed for fresh weight of tubers, number of tubers per plant and % increase in tuber length, as well as for various biochemical parameters like total carbohydrate, protein and saponin contents.

2.4 Tissue Culture

The effect of different PGRs (BAP, IBA and 24-EpiBr) and steroids (testosterone and cholesterol) metals Cr(III) and Zn(II) were studied on shoot apices of C. borivilianum using tissue culture technique. The main steps involved in this technique are:

(i) In vitro shoot induction

Surface sterilized shoot apices were inoculated on MS medium supplemented with BAP (5 μM, 10 μM and 15 μM, 20 μM and 25 μM), 3% (w/v) sucrose and 0.8% (w/v) agar. The cultures were maintained in the culture room under a regime of 16 h photoperiod at 25±1°C. Observations were made for fresh weight of shoot biomass/explant, percent shoot induction, number of shoots/explant and shoot length.

(ii) In vitro root induction:

The isolated surface sterilized shoot apices were transferred to MS medium containing BAP 20 μM with IBA (0 μM, 5 μM, 10 μM and 15 μM, 20 μM and 25 μM). In this process, the best concentration of IBA (15 μM) was selected on the basis of maximum root growth. This concentration was used for further experiments. Observations were made for percent root formation, number of roots, root length and root biomass.

(iii) Effect of steroidal hormones and metals on shoot and root induction

Sterilized shoot apices were transferred to MS medium supplemented with BAP 20 μM and IBA 15 μM having different concentrations (0, 10^{-10}, 10^{-8}, 10^{-6} M) of 24-EpiBr, testosterone, cholesterol, Cr(III) and Zn(II) with 3% (w/v) sucrose and 0.8% (w/v) agar. Observations were made for fresh weight/plant, number of shoots, shoot length, percent shoot induction, number of roots, root length, and percent root induction.
(iv) **Hardening and field transfer of in vitro raised plant**

All the 4-week rooted plants were transferred to small earthen pots containing a mixture of sterilized sand and soil (1:1) and kept in the culture room at 25±1°C for seven days. The plants were then transferred to large earthen pots containing garden soil and vermicompost (9:1) and kept in the net house under high humidity for further acclimatization. Finally, the plants were transferred to the natural field conditions and after one month the survival rate was calculated.

**2.5 Antimutagenic studies of *C. borivilianum***

1. Plant materials (tubers, peels, leaf and seeds) were ground and extracted with solvents (methanol and water) through maceration method at room temperature. Total phenolic content of the extracts was determined using Folin-Ciocalteu method (Singleton and Rossi, 1965). Total saponin content of extracts was determined in n-butanol fraction using gravimetric method.

2. The antimutagenic potential of methanol and water extracts of tubers, peels, seeds and leaves of *C. borivilianum* were evaluated by employing Ames assay, also known as plate incorporation assay as proposed by Maron and Ames (1983). TA98 and TA100 strains of *Salmonella typhimurium* were used for the present study.

3. The antimutagenic ability of the extract at different concentration (100 µg/0.1 ml, 200 µg/0.1 ml, 400 µg/0.1 ml, 800 µg/0.1 ml, 1000 µg/0.1 ml, 1500 µg/0.1 ml, 2500 µg/0.1 ml) was determined against three known mutagens. The experiments with metabolic activation (S9 mix) were carried out with 2-AF (2.5 µg/0.1 ml) in both the tester strains, while in experiments without metabolic activation NPD (20 µg/0.1 ml) was used for TA98 and sodium azide (20 µg/0.1 ml) was used for TA100.

4. In this experiment, 0.1 ml mutagen was added to the test tubes containing 0.1 ml bacterial culture and 2 ml soft agar supplemented with histidine. After mixing, the contents of the test tubes were poured onto minimal agar plates, and the plates were incubated in an inverted position at 37°C for 48 h. In S9 experiments, 0.5 ml S9 mix was also added in top agar. Number of colonies/plate was counted with the help of colony counter.
5. Two types of experiments were designed for antimutagenic screening of the extracts i.e., co-incubation (bioantimutagenicity) and pre-incubation (desmutagenicity). Gallic acid and rutin were used as standards.

2.6 Antioxidant activities of *C. borivilianum*

The *in vitro* antioxidant activities of different extracts of tubers, leaves, peels and seeds at different concentrations (50-1000 µg/ml) were evaluated by employing DPPH, lipid peroxidation, deoxyribose degradation (site specific and non-site specific), reducing power and chelating power assays. The radical scavenging activity of the extracts was determined as inhibition percentage.

2.7 Statistical Analysis

The data were analyzed for mean, standard deviation, linear, logistic and multiple regressions, one-way and two-way analysis of variance (ANOVA) and path analysis. The differences (p ≤ 0.05) among means were compared by honestly significant difference (HSD) using Tukey’s test (Meyers and Grossen, 1974), and the values were expressed as Mean±SD.

3. RESULTS

The important findings of the present study are:

3.1 Effect of PGRs, steroids and metals on seedling growth

1. In single treatment, germination percentage was observed to increase with increase in the concentration of PGRs, steroids and metals, except for chromium where a slight decrease occurred with increase in its concentration. 24-EpiBr, testosterone, cholesterol and Zn enhanced the germination percentage at all the concentrations with all other treatments.

2. In most of the binary combinations, the interactive effects of all the treatments caused increase in germination percentage, except chromium. The most effective order of treatments for germination percent enhancement at 10⁻⁶ M concentration was found to be testosterone > cholesterol > 24-EpiBr > Zn > Kn > IBA.

3. In single treatments, maximum number of roots was observed in 24-EpiBr, testosterone and cholesterol. In binary combinations, at 10⁻⁵ M concentration, combinations of (testosterone+24-EpiBr), (testosterone+cholesterol) and (24-EpiBr+cholesterol), produced maximum number of roots.
4. Maximum root length was observed in $10^{-6}$ M concentration of 24-EpiBr followed by cholesterol. In binary combination maximum root length was observed in combinations of (testosterone+cholesterol) at $10^{-6}$ M concentration followed by (testosterone+24-EpiBr) and (24-EpiBr+cholesterol) at the same concentration tested.

5. The maximum increase in shoot length was observed in (testosterone+cholesterol) combination.

6. The maximum enhancing effect on seedling weight was found in combination of (testosterone+cholesterol) followed by (testosterone+24-EpiBr) at $10^{-6}$ M concentration.

7. In statistical analysis of results, multiple regression models with and without interactions were used to determine which treatment have a unique contribution in enhancing the growth parameters of *C. borivilianum*.

8. In statistical analysis of results, path analysis was also used to examine the comparative strength of direct and indirect relationship among treatments and growth parameters.

3.2 **Effect of PGRs, steroids and metals on plants grown in Sand culture**

1. At the highest tested concentration, the increase in relative weight of different treatments was observed to be in the order of cholesterol>testosterone>24-EpiBr>Zn>Cr.

2. The order of enhancement in number of leaves was found to be in the order of 24-EpiBr>testosterone>cholesterol>Zn>Cr.

3. At $10^{-6}$ M concentration cholesterol showed maximum increase in leaf length followed by testosterone.

4. At $10^{-6}$ M concentration the increase in number of roots was observed to be in the order of enhancement as testosterone>cholesterol>24-EpiBr>Zn>Cr.

3.3 **Effect of PGRs, steroids and metals on plants grown in soil culture Tuber treatments**

1. In tuber treatments, testosterone at $10^{-6}$ M concentration, enhanced the leaf length maximum followed by cholesterol. No significant effect on leaf width and number of inflorescences occurred with any of the treatments given to the tubers.
Maximum stimulating effect for inflorescence length was observed with testosterone and cholesterol at $10^{-6}$ M concentration.

2. Maximum enhancement in tuber weight was observed with $10^{-6}$ M concentration of Zn and testosterone and $10^{-10}$ M concentration of kinetin.

3. Maximum enhancement in number of tubers was observed in plants treated with $10^{-6}$ M 24-EpiBr.

4. Maximum percent increase in tuber length was observed in $10^{-6}$ M testosterone followed by $10^{-8}$ M kinetin.

**Foliar treatments**

1. In foliar treatments, maximum increase in leaf length occurred with cholesterol. No significant increase in leaf width and number of inflorescences was observed in foliar treatments of metals, PGRs and steroids.

2. Maximum enhancement in length of inflorescence was observed for treatments with testosterone and cholesterol.

3. The maximum number of fruits/plant was produced at $10^{-6}$ M concentration of testosterone followed by cholesterol at the same concentration.

4. All the treatments showed stimulating effect on tuber weight. The maximum increase in weight was observed in $10^{-6}$ M 24-EpiBr followed by cholesterol at the same concentration.

5. All the treatments showed stimulating effect on number of tubers. The maximum enhancement was observed in $10^{-6}$ M and $10^{-8}$ M concentrations of 24-EpiBr.

6. Zn(II), 24-EpiBr and testosterone showed maximum enhancement in percent increase in tuber length. The most effective concentration for Zn and testosterone was found to be $10^{-6}$ M while 24-EpiBr showed maximum enhancement at $10^{-8}$ M.

### 3.4 Effect of PGRs, steroids and metals on shoot apices of *C. borivilianum* in tissue culture

1. Maximum increase in fresh weight, number of shoots/plant and shoot length was observed in MS basal medium supplemented with 20 µM BAP.

2. Maximum number of roots/plant was observed in MS basal medium supplemented with 25 µM IBA. Maximum root length was found in MS+15 µM IBA, while maximum percent root induction was observed in MS+10 µM IBA.
3. Maximum enhancement in fresh weight was observed in $10^{-6}$ M concentration of testosterone followed by cholesterol.

4. The maximum number of shoots were observed for $10^{-6}$ M concentration of 24-EpiBr followed by testosterone at the same concentration.

5. The maximum stimulating effect on shoot length was observed in $10^{-6}$ M concentrations of testosterone followed by cholesterol at the same concentrations.

6. Percent shoot induction was found maximum for testosterone followed by cholesterol at $10^{-6}$ M concentration.

7. Maximum increase in number of roots was observed in treatments of 24-EpiBr followed by testosterone at $10^{-6}$ M concentration.

8. Maximum increase in root length was observed in $10^{-6}$ M testosterone followed by cholesterol.

3.5 Antimutagenic potential of *C. borivilianum*

The search for antimutagenic agents from plant origin is very important, since mutagenic and carcinogenic factors are omnipresent in human environment and their elimination is impossible. Moreover, the enzymes responsible for the activation of promutagens are present in mammalian cells and their activation happens frequently and can induce genotoxic effects. Therefore, exploration of plants for their antimutagenic potential is very important. The important findings of this work are as follows:

1. The tuber methanol and water extracts were found more effective against 2-AF induced mutations in pre-incubation mode in TA98 strain of *S. typhimurium*.

2. Peel methanol extract showed strong inhibitory activity in S9 dependent mutagen in pre-incubation mode in TA100 strain, while peel water extract exhibited more % inhibition against 2-AF in co-incubation mode in TA98 strain.

3. Leaf methanol extract exhibited maximum inhibition in S9 dependent pre-incubation mode in TA100 strain, while leaf water extract showed maximum inhibition against 2-AF in pre-incubation mode in TA98 strain.

4. Seed methanol extract showed maximum percent inhibition against 2-AF in co-incubation mode in TA100 strain, while seed water extract exhibited higher effect against 2-AF in TA98 than TA100 in pre-incubation mode.
5. In statistical analysis, along with one-way and two-way ANOVA results were also analyzed through logistic regression models that showed better correlation among percent inhibition in mutagenicity and concentration of extract of *C. borivilianum*.

3.6 **Antioxidant Potential of *C. borivilianum***

Plants are the potent sources of many bioactive compounds such as phenolics, flavonoids, carotenoids etc., which have antioxidative properties. After critical analysis of results, it has been corroborated that the extracts of *C. borivilianum* possess significant antioxidative potential in various *in vitro* assays. The important findings of this work are as follows:

1. In DPPH assay, tuber water extract showed highest hydrogen donating capacity, while tuber methanol extract exhibited maximum hydroxyl radical scavenging potential than tuber water extract. The extracts showed poor percent inhibition in lipid peroxidation, reducing power and metal chelation assay.

2. In DPPH assay, peel methanol extract showed maximum percent inhibition. Peel methanol extracts of *C. borivilianum* also exhibited high lipid peroxidation activity. In deoxyribose degradation assay, the hydroxyl radical scavenging efficacy of peel methanol extract was found comparable with peel water extract. Peel methanol and water extracts exhibited moderate reducing power. Methanol peel extract showed significant chelating effect.

3. Leaf methanol extract was found to be more potent antioxidant than leaf water extract in DPPH assay. Both the leaf extracts of *C. borivilianum* have similar dose dependant trend in inhibition of lipid peroxidation. The leaf methanol and water extracts have high potent ability to scavenge hydroxyl radicals as indicated in deoxyribose non-site specific assay. The reducing ability of both the leaf extracts is comparable or similar at all the concentrations. Leaf extracts exhibited maximum inhibitory effect in chelating power assay than all other extracts of *C. borivilianum*.

4. The seed methanol extract showed highest DPPH radical scavenging activity, while seed water extract showed highest activity in lipid peroxidation. The results revealed good hydroxyl radical scavenging ability of seed extracts in site and non-
site specific deoxyribose degradation assays, while low to moderate effect in reducing and chelating power assays.

4. CONCLUSIONS
The different findings of the present study have lead to the following conclusions:

1. In growth studies, in single treatments, 24-EpiBr, testosterone, cholesterol and Zn exhibited maximum enhancing effect, while in binary combinations (testosterone+24-EpiBr), (testosterone+cholesterol) and (24-EpiBr+cholesterol) enhanced the maximum growth of plant. Cr (III) mostly showed phytotoxic effect in seed germination and growth of plant in sand and tissue culture studies but showed slight enhancing effect in soil culture.

2. Among biochemical parameters, total protein content was significantly enhanced in seedlings and plants grown in sand and soil culture in all the treatments. The treatments also affected the antioxidant enzymes in a dose response manner.

3. Maximum uptake of Zn(II) in tubers grown in soil culture was found in testosterone, Zn and Cr treatments, whereas Cr(III) uptake in roots was found maximum in Cr, 24-EpiBr and testosterone treatments.

4. Maximum antimutagenic activities were observed in tuber methanol, tuber water, peel methanol and leaf methanol extracts of *C. borivilianum*.

5. Maximum antioxidant effect was observed in tuber water, peel methanol, peel water and leaf methanol extracts.

6. The antimutagenic and antioxidant effect of *C. borivilianum* may be attributed due to the presence of phenols and spirostanol and furostanol saponins.

The present study, therefore, established that growth and tuber output of *C. borivilianum* may be enhanced by the use of selective single or binary treatments of PGRs, steroids, Zn(II) or Cr(III). The study will provide baseline data for cultivation, growth enhancement, quality improvement and utilization of *C. borivilianum* as a curative and therapeutic plant. Since the plant is a threatened species, the study will help in its conservation by promoting its cultivation in the field with the use of growth promoters, thereby protecting this threatened plant in the wild state.