CHAPTER - 8

SUMMARY
AND
CONCLUSION
During past two decades there has been a significant interest in the use of herbal medicines. World Health Organization has issued guidelines for assessment of quality, safety and efficacy of these medicines and emphasized on the need of standardized preparations for health benefits from consumer and health care point of view. The botanicals employed in herbal medicines should be well authenticated and documented. Desirability of good manufacturing practices, knowledge of active and characteristic constituents, standardization of manufacturing process, identification of active or characteristic substances by chromatographic printing to ensure consistence quality of the preparation are some of the approaches suggested for quality and safety of medicaments. Physical and chemical stability of the product, establishment of shelf life and studies from standpoint of pharmacological and toxicological activities may provide further strength in safety of herbal drugs.

A country like ours, which have a very strong tradition in Ayurvedic, Unani and Siddha medicine can be beneficial of renewed global interest in herbal medicine by taking extensive research work in standardization and quality control of herbal products. Drugs affecting CNS are high demand areas where herbal medicine may contribute substantially. The research work on drugs affecting CNS was taken up in the present investigations.

A survey of Ayurvedic literature and marketed Ayurvedic formulation revealed that *Evolvulus alsinoides, Convolvulus pluricaulis, Centella asiatica* and *Bacopa monniera* are extensive employed for CNS disorders. Looking to their importance these plants were selected with a view to provide parameters for the standardization and formulation.
STUDIES ON CRUDE DRUGS

Collection of *Evolvulus alsinoids* and *Convolvulus pluricaulis* was made during the month of January-February from area adjoining Dr. Harisingh Gour Vishwavidyalaya Sagar campus. *Centella asiatica* and *Bacopa monniera* were procured from crude drug suppliers of New Delhi. The identity of collected/procured drug was confirmed at National Botanical Research Institute (NBRI), Lucknow, India.

Macrosopic and microscopical examination of the drugs in whole and powdered form was undertaken and the characters were recorded. The detailed diagrams of tissues in sections as well as microscopic details of the powder are giving in chapter 3. Studies of characters suggest that the drugs collected/procured are authentic as they matched well with descriptions reported in monographs for the drugs in pharmacopoeia/reports.

Ash values of the four drugs under investigation were determined by the method described in pharmacopoeia. Total ash, acid insoluble ash and water soluble ash values for these drugs were determined. *Evolvulus alsinoids* yielded 10.422±0.101 total ash, 3.212±0.051 acid-insoluble ash and 2.112±0.031 water soluble ash. Values for *Convolvulus pluricaulis* were total ash 8.159±0.046, acid insoluble ash 3.062±0.124, water soluble ash 2.258±0.052. In case of *Centella asiatica* the values were total ash 7.904±0.024, acid insoluble ash 3.083±0.130, water soluble ash 1.036±0.011 whereas the values of *Bacopa monniera* were total ash 9.623±0.013, acid insoluble ash 5.132±0.074, water soluble ash 0.952±0.024.

As compared to values prescribed in pharmacopoeia the values obtained in our study are far less and calls for a review of values giving in Indian Herbal Pharmacopoeia as ours was carefully collected material.

Extractive profile of the drugs was prepared by extracting them with chloroform, petroleum ether, benzene, ethanol and water. High extractive
values were obtained in ethanolic and aqueous extracts, which clearly indicates high proportion of saponin glycosides, tannins, carbohydrates etc. The extractive values can serve as a helpful parameter in making in-house quality control standards for the drug/s and use of consistent extractive profile of material may achieve in biopharmaceutically similar formulations.

The moisture contents of a crude drug should be minimized in order to prevent decomposition of crude drug either due to chemical changes or microbial contamination. Excess moisture also indicates that the purchaser is paying a high price for unwanted water. Loss on drying or heating to constant weight can be determined for material, which do not contain compounds, which are volatile at the temperature of drying. The percent loss on drying was found to be 3.162±0.121 for *Evolvulus alsinoides*, 3.841±0.0143 for *Convolvulus pluricaulis*, 4.241±0.0476 for *Centella asiatica* and 5.032±0.461 for *Bacopa monniera*.

Many crude drugs contain mucilage in their cells and can be evaluated by measuring the volume of mucilage produced within 24 hrs from 1 gm of the drug. This is termed as swelling factor and it reflects on the mucilage content of the drug. Approximately, 1 gm of accurately weighed drug sample was transferred into a 25 ml glass stoppered measuring cylinder and 20 ml of water was added into the cylinder. The mixture was thoroughly shaken at intervals of 10 minutes for 1 hr and allowed to stand for twenty three hrs at room temperature. The volume occupied by the swollen sample was measured. The swelling factor was found to be 4.820±0.621 for *Evolvulus alsinoides*, 5.641±0.243 for *Convolvulus pluricaulis*, 5.201±0.426 for *Centella asiatica* and 5.132±0.232 for *Bacopa monniera*.

Foaming index is one of the parameter included in WHO guidelines for crude drug standardization as it is directly related to saponin content of the drug. Powdered drug were thus studied for foaming index and the values
obtained for *Evolvulus alsinoids* was 142.85±1.341, *Convolvulus pluricaulis* 153.72±3.630, *Centella asiatica* 111.41±2.624 and for *Bacopa monniera* 133.42±3.032.

**PHARMACOLOGICAL SCREENING OF EXTRACTS**

*Evolvulus alsinoids, Convolvulus pluricaulis, Centella asiatica* and *Bacopa monniera* have been recognized in Ayurvedic literature as Medhya- meaning drugs that potentiate memory. Although considerable pharmacological and phytochemical studies have been undertaken on *Centella asiatica* and *Bacopa monniera*, very little attention has been paid to *Evolvulus alsinoids* and *Convolvulus pluricaulis*. Many Ayurvedic formulations containing these drugs are available in the market for improving memory in which these drugs are used exclusively or in combination. It was thus considered desirable to screen *Evolvulus alsinoids* and *Convolvulus pluricaulis* for their effect on memory behaviour and learning and to compare them with *Centella asiatica* and *Bacopa monniera*.

Pharmacological investigations of different extracts of the drug viz. petroleum ether, chloroform, methanol and aqueous extracts were undertaken with a view to know the most active extract of the drug/drugs. LD<sub>50</sub> values determined as per guidelines of Organization for Economic Co-operation and Development (OECD) was found to be more than 2000 mg/kg body wt. for extracts of all drugs. 200 mg/kg body wt. were thus selected for screening pharmacological efficacy of the drugs.

Elevated plus maze, two way active avoidance with negative (punishment) reinforcement: shuttle box, measurement of pentobarbitone induced sleep and spontaneous motor activity experiments were undertaken to screen activity on CNS. The experiments were carried out using Wistar strain rats of either sex. For elevated plus maze experiment six groups of six animals were used for every extract. Group I was administered with vehicle,
Group II administered with scopolamine (0.3mg/kg body wt.), Group III, IV, V and VI were given scopolamine and 200 mg/kg body wt. of petroleum ether, chloroform, methanol and aqueous extracts. After 24 hrs and 7 days transfer latency and retention latency of animals was noted. Only methanolic and aqueous extract of the drugs exhibited significant activity in this experiment.

As compared to scopolamine treated group which has ITL, 1st RTL and 2nd RTL value as 70.42±2.60, 100.02±2.56 and 77.25±1.64, methanolic extract of Evolvulus alsinoides has values of 69.68±3.47, 55.86±1.02 and 36.11±1.60, for aqueous extract the values recorded were 63.77±2.16, 54.45±2.82 and 42.60±3.51. Similarly methanolic extract of Convolvulus pluricaulis exhibited ITL, 1st RTL and 2nd RTL values of 68.45±3.60, 57.86±2.82 and 39.16±2.60 whereas aqueous extract of the drug demonstrated 61.74±2.03, 53.15±2.33 and 40.60±3.54 values respectively. Centella asiatica aqueous extract and methanolic extract showed ITL, 1st RTL and 2nd RTL values of 63.74±2.14, 53.15±1.53, 46.42±2.27 and 75.46±4.22, 58.86±1.82, 38.16±2.61 respectively. The values recorded for these parameters in case Bacopa monniera aqueous and methanolic extracts were 65.74±1.05, 56.15±2.54, 46.60±2.61 and 79.46±4.60, 59.86±1.52, 45.46±2.24 respectively. The studies suggest methanolic extracts to be most active in this experiment. The experiments also suggest that activities of drugs in following order.

Evolvulus alsinoides > Bacopa monniera > Centella asiatica> Convolvulus pluricaulis.

Learning and memory behaviour of test drug extracts was also tested in shuttle box wherein learned behaviour of animals is evaluated for avoidance response. The experiment was performed in five groups of six animals. Group I was given vehicle whereas group II, III and IV and V were administered with 200 mg/kg body wt. of petroleum ether, chloroform, methanol and aqueous extracts. Amongst the extracts tested, as in case of plus
maze experiment, methanolic extracts of the drugs exhibited highly significant activity as compared to that of other extracts.

As compared to control group in shuttle box experiment which has IT, 1<sup>st</sup> RT, 2<sup>nd</sup> RT and 3<sup>rd</sup> RT values as 9.24±1.26, 10.52±1.58, 11.24±1.58 and 11.82±1.82, *Evolvulus alsinoids* methanolic extract showed values of 22.82±3.24, 30.68±3.64, 32.62±2.41 and 35.58±2.41 whereas *Convolvulus pluricaulis* methanolic extract exhibited the values 24.45±3.12, 30.24±3.14, 34.32±2.35 and 38.24±2.21 respectively. Similarly methanolic extract of *Centella asiatica* showed the IT, 1<sup>st</sup> RT, 2<sup>nd</sup> RT and 3<sup>rd</sup> RT values of 25.77±2.12, 30.24±4.14, 36.33±2.37 and 39.36±2.74 whereas *Bacopa monniera* extract demonstrated 26.77±1.68, 31.24±2.34, 34.33±2.21 and 38.36±1.08 values respectively. The studies suggest that methanolic extract of each drug to be most active in this experiment.

The methanolic extract of *Evolvulus alsinoids* and *Convolvulus pluricaulis* showed significant response in learning and memory behavior in both elevated plus maze and shuttle box experiments. In case of *Centella asiatica* and *Bacopa monniera*, methanol as well as aqueous extract showed the significant improvement in learning and memory in both elevated plus maze and shuttle box paradigms but the effect of methanolic extracts was more prominent as compared to that of aqueous extract.

To optimize the dose for formulation the further experiments were conducted using three doses i.e. 100, 200 and 300 mg/kg body wt. for methanolic extract of each drug. In plus maze experiment, the 300 mg/kg dose of methanolic extract of *Evolvulus alsinoids* exhibited 26.52 and 50.66% reduction in 1<sup>st</sup> RTL and 2<sup>nd</sup> RTL respectively whereas in case of *Convolvulus pluricaulis* methanolic extract (300 mg/kg body wt.) the reduction was found to be 20.38 and 49.43% respectively. Similarly the 300 mg/kg body wt. dose of methanolic extract of *Centella asiatica* demonstrated 30.30 and 48.65%
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reduction in 1<sup>st</sup> RTL and 2<sup>nd</sup> RTL respectively whereas 30.76 and 48.86% reduction was found to be in case of 300 mg/kg body wt. dose of Bacopa monniera methanolic extract. Among the doses of methanolic extract tested, as in case of plus maze experiment, the dose 300 mg/kg body wt. was most prominent in shuttle box experiment. The increase in number of avoidance response was found to be more in the dose of 300 mg/kg body wt. for each drug. The percentage increase in number of avoidance in terms of 1<sup>st</sup> RT, 2<sup>nd</sup> RT and 3<sup>rd</sup> RT was found to be 19.83, 35.32 and 63.22 for 300 mg/kg dose of methanolic extract of Evolvulus alsinoids, 25.32, 44.50 and 67.82 for Convolvulus pluricaulis methanolic extract (300 mg/kg body wt.), 29.91, 46.47 and 60.36 for 300 mg/kg body wt. dose of methanolic extract of Centella asiatica and for Bacopa monniera methanolic extract at dose 300 mg/kg body wt. the percentage increase was found to be 15.16, 34.98 and 57.62 respectively.

Pentobarbitone induced sleeping time was studied for difference doses of the methanolic extract. 100 mg, 200 mg and 300 mg/kg of methanolic extract of each drug was administered to group of rats alongwith pentobarbitone sodium and increase in sleep time was compared with control group of rats receiving pentobarbitone sodium only. As compared to pentobarbitone treated group which has sleeping time 28.35±2.37 min., the sleeping time found for methanolic extract of Evolvulus alsinoids methanolic extract at 100, 200 and 300 mg/kg body wt. was 28.27±3.21, 36.63±3.68 and 49.62±3.54 respectively. For methanolic extract of Convolvulus pluricaulis the values were found to be 30.43±2.62, 32.43±3.72 and 48.26±2.68. Similarly in case of methanolic extract of Centella asiatica the sleeping time was found to be 29.71±2.64, 39.23±2.75 and 46.32±2.44 whereas Bacopa monniera methanolic extract demonstrated the sleeping time 34.73±3.42, 41.65±2.71 and 49.62±2.34 respectively. All the drugs tested prolonged the sleeping time suggesting mild tranquilizing effect. Increase in dose, however, did not increase sleeping time proportionately.
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The spontaneous motor activity was measured by using Photoactometer. The animals were divided in six groups for each drug. Group I served as control and administered with vehicle only. Group II was administered with amphetamine (50 mg/kg body wt. i.p.) whereas Group III was administered with phenobarbitone at 30 mg/kg body wt. i.p. Group IV was administered with methanolic extract at 100 mg/kg body weight. Group V was administered with methanolic extract at 200 mg/kg body wt. whereas Group VI was administered with methanolic extract at 300 mg/kg body wt. The number of motility count was recorded with reference to time intervals. The data obtained were compared with that of amphetamine and phenobarbitone.

In spontaneous motor activity experiment, methanolic extract of *Evolvulus alsinoides* at dose 300 mg/kg body wt exhibited phenobarbitone like activity as it reduced motility counts from 85.78±4.21 to 50.46±2.06 after 120 minutes of drug administration. The motility count 85.63±2.14 was reduced upto 55.26±4.32 in the group treated with methanolic extract of *Convolvulus pluricaulis* at 300 mg/kg body wt. whereas in case of *Centella asiatica* the reduction was found to be from 84.56±3.65 to 50.46±3.61 after 120 minutes of drug administration. Similarly the motility count was reduced from 84.56±2.43 to 51.66±2.82 after 120 minutes of drug administration in group treated with methanol extract of *Bacopa monniera* at 300 mg/kg body weight.

ANTIOXIDANT ACTIVITY OF EXTRACTS

The mechanism by which medicinal plant enhances learning and memory performance in behavioural task is yet unknown. Reports suggest that the varying degrees of behavioural impairments are associated with aging and age-associated neurodegenerative diseases and oxidative stress due to free radicals is responsible for producing the neuronal changes mediating
these behavioural deficits. This refers to the cytotoxic consequences of oxygen radicals like superoxide anion, hydroxyl radical and hydrogen peroxide, which act on polyunsaturated fatty acids in brain, thereby propagating the lipid peroxidation.

Earlier reports have shown that a number of natural drugs, which improve cognition, also have antioxidant properties. Therefore considering the antioxidant efficacy involved in the CNS activity, the most active methanolic extracts of each drug were subjected for in-vitro antioxidant activity. The role of free radical reactions in disease pathology is well established, suggesting that these reactions are necessary for normal mental health but can be detrimental to aerobic life as well. Diseases caused by free radical reaction are ageing, immunosupression, neurogenerative diseases and many others.

Three in-vitro tests, the DPPH radical scavenging action, hydroxyl scavenging and the lipid peroxidation assay for antioxidant activity were used to assess the antioxidant properties of methanolic extracts of each plant. All the three tests together provide a better assessment of antioxidant properties.

In DPPH test ascorbic acid was used as control and different concentration of methanolic extracts of the drugs were evaluated for antioxidant potential. IC\(_{50}\) value of ascorbic acid recorded was 3.46±1.21 whereas IC\(_{50}\) value recorded for *Convolvulus pluricaulis* was 8.62±0.11, *Evolvulus alsinoides* 10.28±0.24, *Centella asiatica* 17.32±0.31, *Bacopa monniera* 30.42±0.43.

In hydroxyl radical scavenging test the methanolic extract of drugs were evaluated by generating the hydroxyl radical using ascorbic acid-iron EDTA. IC\(_{50}\) value recorded for *Evolvulus alsinoides* was 13.68±1.70, *Convolvulus pluricaulis* 24.61±2.31, *Centella asiatica* 29.24±2.33, *Bacopa monniera* 48.14±2.68 whereas for standard it was found to be 8.42±0.78.
In lipid peroxidation assay, vitamin E was used as standard, which had IC₅₀ value recorded for *Centella asiatica* was 98.42±2.82, *Bacopa monniera* 76.48±3.68, *Evolvulus alsinoides* 103.24±4.22, *Convolvulus pluricaulis* 71.66±3.82.

It is known that free radical cause auto-oxidation of unsaturated lipids in food. On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable endproduct, which does not initiate or propogate further oxidation of lipid. The data obtained reveal that the activity of selected plants in DPPH test was in order of *Convolvulus pluricaulis* > *Evolvulus alsinoides* > *Centella asiatica* > *Bacopa monniera*.

Hydroxyl radical is an extremely reactive species formed in biological systems and has been implicated as highly damaging in free radical pathology, capable of damaging almost every molecule found in living cells. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. The hydroxyl radical scavenging activity of the MeOH extract of tested plants was in order of *Evolvulus alsinoides* > *Convolvulus pluricaulis* > *Centella asiatica* > *Bacopa monniera*.

The ability of the extracts to quench hydroxyl radicals seems to directly relate to the prevention of propagation of the process of lipid peroxidation, and the extract of Bacopa monierra seems to be a good scavenger of active oxygens species, thus reducing the rate of chain reaction.

The extracts of *Centella asiatica* and *Evolvulus alsinoides*, however, were not as effective in the lipid peroxidation assay as in the DPPH test. The possible explanation for this could be the lipid insolubility of tannins, where peroxidation takes place. The percentage inhibition in lipid peroxidation by
various MeOH extract was found to be in order of *Convolvulus pluricaulis* > *Bacopa monniera* > *Centella asiatica* > *Evolvulus alsinoides*.

Antioxidant activity has been ascribed earlier to only two classes of compounds, i.e. flavonoids and polyphenols isolated from various extracts. Since a wide variety of constituents are known from the extract studied here, it is difficult to ascribe the antioxidant properties selectively to any one group of constituents without elaborate studies that are beyond the scope of this investigation. However, the present results suggest that all the tested plant materials have moderate to potent antioxidant activity.

The present study, therefore demonstrate that methanolic extracts of each drug has two pronounced effects, i.e., improving learning and memory and antioxidant properties by decreasing the lipid peroxidation and augmenting antioxidant enzymes. The mechanism by which methanolic extracts enhances cognition can be attributed at least in part to antioxidant properties. The serotonergic receptor antagonist property or ability to decrease the levels of norepinephrine, dopamine and serotonin, and its metabolites in the brain, can be other mechanisms, which need exploration.

Based on the comparison of results obtained, the studies revealed that the overall pharmacological efficacy of tested drugs was found to be in order of *Evolvulus alsinoides* > *Bacopa monniera* > *Centella asiatica* > *Convolvulus pluricaulis*.

**QUALITY CONTROL AND STANDARDIZATION OF HERBAL EXTRACTS**

Dried extract of each drug was evaluated for different plant constituents and physical parameters such as nature of extract, color, odour, taste etc. Total yield of extract was also determined for individual extract.

Qualitative chemical tests showed the presence of steroids in methanolic extract of each drug whereas in aqueous, petroleum ether,
chloroform and benzene extract the steroid test was negative. The foam test was positive in aqueous extract of each drug. Alkaloids are found to be present in methanolic and aqueous extract of both *Evolvulus alsinoids* and *Convolvulus pluricaulis*. The tests for glycosides were positive in methanolic extracts of *Centella asiatica* and *Bacopa monniera* whereas it was negative in each extract of *Evolvulus alsinoids* and *Convolvulus pluricaulis*. Triterpenoids are found to be present in methanolic and chloroform extracts of each drug.

The methanolic, aqueous, petroleum ether extracts of each drug were found to be semisolid in nature whereas chloroform and benzene extracts were solid in nature. The methanolic extracts of *Evolvulus alsinoids*, *Convolvulus pluricaulis* and *Centella asiatica* were greenish black in color whereas it was reddish brown in case of *Bacopa monniera*. The aqueous extracts of each drug were found to be blackish brown whereas chloroform and benzene extracts were brownish black in color.

The methanolic and aqueous extracts of each drug were found to have characteristic odour except in *Centella asiatica*. Each extract of *Centella asiatica* was found to have aromatic odour. Chloroform extract of *Evolvulus alsinoids* and *Convolvulus pluricaulis* were found to have pungent odour. Benzene extracts of each drug were found to have characteristic odour except in *Centella asiatica*. The extracts of each drug were found to have bitter taste except petroleum ether and chloroform extract of *Centella asiatica* and *Bacopa monniera*, which were found to have astringent taste.

The total yield of different extracts of *Evolvulus alsinoids* were 2.9280 to 8.6747% (w/w) indicating maximum in methanolic and minimum with petroleum extract whereas the yield of *Convolvulus pluricaulis* was between 2.9406 and 9.200% (w/w). The total yield of different extracts of *Centella asiatica* were 3.2813 to 9.5623% (w/w) whereas the yield of *Bacopa monniera* was between 3.2853 and 9.0815% (w/w) indicating maximum in methanolic and minimum with petroleum extract.
The extracts were investigated for chemical profiling of their constituents by thin layer chromatography. Best resoluting mobile phases and detection conditions were selected experimentally. On the basis of TLC and solvent systems used in chromatographic analysis, HPTLC studies were performed. The extracts of *Evolvulus alsinoides* and *Convolvulus pluricaulis* were chromatographed in mobile phase, Butanol : Glacial acetic acid : Water (4:1:3) whereas the extracts of *Centella asiatica* and *Bacopa monniera* were chromatographed in Acetonitrile : water : Ammonia (12:18:0.6) and detected under UV light (Camag UV lamp dual wavelength). The chromatograms were scanned and densitometrically evaluated. The methanolic extract of different drugs showed the presence of 10, 11, 12 and 11 components in *Evolvulus alsinoides*, *Convolvulus pluricaulis*, *Centella asiatica* and *Bacopa monniera* respectively.

HPTLC analysis thus, was successful in characterizing the extracts of the four drugs investigated. The fingerprints obtained may be utilized for the purpose of quality control as well as standardization of extracts for formulations and other uses.

In contrast to chemically defined drug products, the biopharmaceutical quality and behaviour of extracts and their herbal medicinal products often are not well documented. Thus according to FIP recommendations the extracts must be examined for their biopharmaceutical profile. This can be achieved by the investigations on chemical composition and classification of active pharmaceutical ingredients in extracts, bioavailability & bioequivalence and solubility tests in buffers with different pH at 37°C. Therefore we have considered to investigate the methanolic and water extract of each drug which were found most active in experiments of chapter 4, for solubility in buffers of different pH by gravimetry and partitioning studies of methanolic extract in octanol/water system. Two gm accurately weighed amount of extract placed in beakers containing 250 ml of buffer I (pH 1.2), II (pH 4.6) and III (pH 6.8).
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After stirring for 60 minutes the residues were filtered through G-4 crucible and then the crucible was subjected for drying at 100°C for two hours.

The percentage solubility of drugs in buffer I (pH 1.2) was 90.5312±0.7411 to 93.5035±0.2432 indicating maximum for *Evolvulus alsinoides* and minimum for *Bacopa monniera* whereas in buffer II (4.6) it was 93.8642±0.7262 to 95.6281±0.8246 indicating maximum for *Evolvulus alsinoides* and minimum for *Convolvulus pluricaulis*.

The percentage solubility of drugs in buffer III (pH 6.8) was 95.6822±1.0281 to 96.2412±0.2671 indicating maximum for *Evolvulus alsinoides* and minimum for *Centella asiatica*. The partition coefficient of drugs in octanol/water system was found to be 0.610 to 0.838 indicating maximum for *Bacopa monniera* and minimum for *Convolvulus pluricaulis*.

ISOLATION AND CHARACTERIZATION OF MARKERS

During last two decades or so, emphasis has been laid on chemical methods of standardization based on physical, chemical assays, chromatographic analysis and various spectroscopic methods. Most of these are qualitative methods, though many plant materials have been standardized quantitatively using HPLC & HPTLC and these methods are used as an effective quality control parameter. In such cases, chemical markers have been isolated and characterized. In several plant materials spectroscopic methods of standardization have also been found to be useful. However, marker compound based standardization in many cases has not been as effective since these markers are not biologically active constituents in most of cases.

All these methods account for a single chemical entity or group of chemical compounds, but in many plants the activity may be attributed to different types of compounds that act synergistically to show the desired biological activity. Therefore, standardization by chemical methods, although
used widely may not prove to be a complete way of standardization. Sometimes the use of bioassay has been found to be an effective method in standardization and quality control of herbal materials. Though this method of standardization is not yet popular as compared to chemical methods, the trend all over the world is now on use of biological assay methods in addition to chemical methods, which ensures consistent clinical efficacy of the herbal product from batch to batch.

As methanolic and aqueous extracts of *Evolvulus alsinoides* and *Convolvulus pluricaulis* showed maximum CNS activity when compared to other extracts of the same drug, the methanolic extracts of both the drugs were explored for isolation of marker compounds. As Vardan and co-workers reported the presence and method for isolation of alkaloids, the same, however, not successful during our investigation even after repeated trials of method as well as with modifications of the method. During thin layer chromatographic studies, it was also noticed that the orange coloured spot detected with dragendorff's reagent, was very faint even in case of concentrated sample application. Search for alternative marker compound was thus desirable. Fluorescence producing single components were isolated from methanolic extracts of *Evolvulus alsinoides* and *Convolvulus pluricaulis* using preparative thin layer chromatography.

Standard asiatic acid and bacoside-b were procured from Chromadex phytochemical standards, USA and used as marker compounds for *Centella asiatica* and *Bacopa monniera* respectively. EA1 and CP1 were used as a chemical marker compound for standardization of *Evolvulus alsinoides* and *Convolvulus pluricaulis* respectively.

A simple and precise method has been developed for quantification of EA1 and CP1. Best results were obtained when chromatographic analysis was performed on silica gel 60 F254 plates and mobile phase, Butanol : Glacial
acetic acid : water, 4+1+3 (v/v). In UV light EA1 appeared as yellow band with $R_f$ 0.8 whereas CP1 appeared as yellow band at $R_f$ 0.7. After the treatment with ethanolic sulphuric acid (5% v/v) followed by heating at 110°C for 5 minutes, asiatic acid appeared as a violet band with $R_f$ 0.32 whereas bacoside-b appeared as violet band with $R_f$ 0.45. The developed method was reproducible and densitometric detection measured the concentration of different markers to percentage recovery more than 95% in all cases.

The peaks of markers on densitograms were well developed and retained its characteristics shape and peak area irrespective of the presence of other constituents in the tested extracts. Linearity was maintained throughout a broad range of tested constituents, from 5 to 50 μg/ml. Satisfactory results were obtained from study on the precision of the method, as it was confirmed by statistical data such as standard deviation, confidence interval, and relative standard deviation. Determination performed by two independent analyts confirmed the reliability of results obtained by use of the method. Small changes in condition had no effect on the final results.

The isolated marker compounds viz. EA1 and CP1 were subjected for preliminary characterization by IR, MASS, $^1$H NMR and $^{13}$C NMR. EA1 was found to be an amorphous powder, m.p. 187°C. It gave a positive Liebermann Burchard test, indicating it to be a steroidal/triterpenoidal compound. The UV spectrum of EA1 showed $\lambda_{\text{max}}$ at 132 nm. IR spectrum of EA1 showed peaks at cm$^{-1}$ 1454.2 and 1415.7 (C-C bending of methyl group), 2947.0 (C-H of cyclo alkanes), 1696.8 (carboxyl group) together with strong hydroxyl absorption bands at 3355.9 cm$^{-1}$.

FAB-MASS of EA1 has shown a peak at m/z 488 due to loss of CO$_2$ from the molecule (M$^+$- CO$_2$), indicating its molecular formula as C$_{30}$H$_{48}$O$_3$. It had peaks characteristic of a triterpenoids (bunches of peaks). Its $^1$H and $^{13}$C
NMR spectra suggest that it to be an oleanane type triterpene with two hydroxymethyl groups. This was consistent with the information provided by its FAB-MASS. Two characteristic peaks at m/z 240 and 248 denoted the Retro-Diels-Alder cleavage fragments commonly observed for olean-12-ene or urs-12-ene derivatives possessing three hydroxyl groups in rings A/B and a carboxyl group in ring D/E. Two ions at m/z 222 and 203 indicated further loss of water and COOH from m/z 240 and 248 respectively.

The $^1$H NMR spectrum of EA1 was recorded in D$_2$O at 300 MHz. Signals are δ 3.527 (2H, t, H-1), δ 6.480 (2H, t, H-2), δ 4.404 (1H, dd, H-3), δ 2.383 (1H, s, H-5), δ 2.123 (2H, q, H-6), δ 2.327 (2H, t, H-7), δ 1.638 (1H, t, H-9), δ 1.444 (2H, q, H-11), δ 5.346 (1H, t, H-12), δ 2.080 (2H, t, H-15), δ 2.894 (2H, t, H-16), δ 3.272 (1H, s, H-18), δ 1.153 (2H, d, H-19), δ 7.838 (2H, t, H-21), δ 6.480 (2H, t, H-22), δ 4.852 (2H, d, H-23), δ 3.720 (2H, s, H-24), δ 0.843 (3H, s, H-25), δ 1.255 (3H, s, H-26), δ 1.027 (3H, s, H-27), δ 0.843 (3H, s, H-29), δ 0.926 (3H, s, H-30). Signals between δ 0.8 to δ 1.9 are characteristic for pentacyclic triterpenoid moiety. The peaks at δ 0.826 (s), 0.843 (s), 1.081 (s), 1.153 (s), 1.758 (d) and 1.827 (s) can be assigned to the six methyl groups. These peaks are characteristic of the proton at C-24, C-25, C-26, C-27, C-29 and C-30 of a pentacyclic triterpenoid moiety. A signal between δ 5.2 and δ 5.4 is characteristic of olefinic proton signal of Δ$_{12}$-oleanane type of triterpenes. The four signals between δ 4.0 and δ 4.4 are characteristic of proton of C-2, C-3, C-23 and C-6. A singlet between δ 2.4 and δ 2.6 may be assigned to the proton of C-5. The peak at δ 5.346 (d) indicating the presence of double bond. Due to substitution at H-3, H-5 and H-17, they resonate at down field δ4.852, 3.494 and 5.632 respectively.

$^{13}$C NMR spectrum of EA was recorded at 75 MHz in D$_2$O. The signals are δ 217.383 (C-1), δ 48.789 (C-2), δ 76.623 (C-3), δ 38.577 (C-4), δ 41.227 (C-5), δ 32.873 (C-6), δ 168.889 (C-7), δ 127.485 (C-8), δ 56.766 (C-9), δ 49.824 (C-10), δ
20.191 (C-11), δ 34.445 (C-12), δ 36.945 (C-13), δ 45.135 (C-14), δ 29.662 (C-15), δ 167.144 (C-16), δ 77.469 (C-17), δ 22.639 (C-18), δ 15.830 (C-19), δ 120.738 (C-20), δ 141.900 (C-21), δ 109.718 (C-22), δ 143.005 (C-23), δ 174.075 (C-24), δ 139.623 (C-25), δ 138.499 (C-26), δ 11.608 (C-27), δ 21.678 (C-28), δ 20.641 (C-29), δ 123.041 (C-30). A total of 30 carbon signals were seen. A signal at δ 217.383 indicates the presence of carboxyl group. Carbon signal at δ 127.485 indicating the presence of double bond. Down field signal at δ 76.623 indicate the attachment of substitution in the molecule. Signal at δ 174.075 indicate that additional hydroxyl group must be located at C-24.

On the basis of elemental analysis, IR spectra, 1H NMR and 13C NMR the compound EA1 was identified as 3β, 23, 24-trihydroxyolean-12-en-28-oic acid.

CP1 was a amorphous powder m.p. 92-94°C. It gave a positive Lieberman-Burchard test, indicating it to be a steroidal/triterpenoidal compound. The UV spectrum of CP1 showed at 226 nm (λ_{max}).

IR spectrum of CP1 showed peaks at cm^{-1} 1458.1 and 1465.5 (methyl groups), 1697.4 (carboxyl group), 2842.9 (C-H of cyclo alkanes) together with strong hydroxyl absorption bands at 3417.6 cm^{-1}.

FAB-MASS of CP1 has shown a peak at m/z 488 due to loss of CO₂ from the molecule (M^{+} - CO₂), indicating its molecular formula as C₃₀H₄₈O₅. The FAB-MASS of CP1 indicated that it to be an oleanane type triterpene with one hydroxyl group and two secondary alcoholic groups on ring A/B, but in its mass spectrum, the peak of rings A/B possessing three alcoholic groups corresponding to the Retro-Diels-Alder cleavage at m/z 240 was not observed. However there was a peak at m/z 222, indicating the loss of one molecule of water.

The proton NMR spectrum of CP1 was recorded in D₂O at 300 MHz. The signals are δ 3.29206 (2H, t, H-1), δ 4.86699 (2H, t, H-2), δ 3.36065 (1H, dd,
H-3), δ 2.42013 (1H, s, H-5), δ 2.39685 (1H, t, H-6), δ 2.38738 (2H, t, H-7), δ 1.75859 (1H, t, H-9), δ 1.73095 (2H, q, H-11), δ 1.22020 (1H, t, H-12), δ 3.49109 (2H, t, H-15), δ 5.55498 (2H, t, H-16), δ 1.08929 (1H, s, H-18), δ 1.16766 (2H, d, H-19), δ 7.57437 (2H, t, H-21), δ 6.48689 (2H, t, H-22), δ 7.45699 (3H, s, H-23), δ 6.93120 (2H, s, H-24), δ 1.84416 (3H, s, H-25), δ 0.82380 (3H, s, H-26), δ 0.77728 (3H, s, H-27), δ 3.39105 (3H, s, H-29), δ 3.73132 (3H, s, H-30). Signals between δ 0.8 and δ 1.9 are characteristic for pentacyclic triterpenoid moiety. The peaks at δ 0.777 (s), 0.823 (s), 1.089 (s), 1.167 (s), 1.843 (d) and 1.893 (s) can be assigned to the six methyl groups. These peaks are characteristic of the proton at C-24, C-25, C-26, C-27, C-29 and C-30 of a pentacyclic triterpenoid moiety. A signal between δ 5.2 and δ 5.4 is characteristic of olefinic proton signal of Δ12-oleanane type of triterpenes. The four signals between δ 4.0 and δ 4.4 are characteristic of proton of C-2, C-3, C-23 and C-6. A singlet between δ 2.4 and δ 2.6 may be assigned to the proton of C-5. The peak at δ 5.346 (d) indicating the presence of double bond. CP1 has shown ring units at H-3, H-5 and H-17, as their δ values are similar to those of EA1.

\[^{13}\text{C} \text{NMR}\] spectrum of CP1 was recorded at 75 MHz in D₂O. The signals obtained are δ 218.145 (C-1), δ 29.163 (C-2), δ 77.201 (C-3), δ 38.703 (C-4), δ 41.066 (C-5), δ 32.918 (C-6), δ 170.511 (C-7), δ 128.496 (C-8), δ 52.968 (C-9), δ 29.030 (C-10), δ 48.822 (C-11), δ 48.981 (C-12), δ 53.689 (C-13), δ 129.589 (C-14), δ 53.871 (C-15), δ 168.106 (C-16), δ 78.127 (C-17), δ 27.833 (C-18), δ 15.293 (C-19), δ 120.767 (C-20), δ 142.399 (C-21), δ 109.930 (C-22), δ 143.510 (C-23), δ 174.949 (C-24), δ 142.361 (C-25), δ 140.073 (C-26), δ 12.099 (C-27), δ 21.476 (C-28), δ 20.956 (C-29), δ 52.835 (C-30). A total of 30 carbon signals were seen. A signal at δ 218.422 indicates the presence of carboxyl group. Carbon signal at δ 120.767 indicating the presence of double bond. Down field signal at δ 77.201 indicate the attachment of substitution in the molecule. Like EA1, CP1 shown signal at δ 174.949 indicate that additional hydroxyl group must be located at C-24.
SUMMARY AND CONCLUSION

From the $^1$H NMR and $^{13}$C NMR of CP1 and by comparing with literature values, it can be deduced that CP1 is a pentacyclic triterpene with an endocyclic double bond and six methyl groups. Thus by analysis of IR and Mass spectral data of CP1, it was identified as oleanane triterpenoids. CP1 was chemically a 3β, 6β, 24-trihydroxyolean-12-en-28-oic acid.

EA1 and CP1 were first time isolated from the methanolic extract of *Evolvulus alsinoides* and *Convolvulus pluricaulis* respectively. Mass spectrum (FAB-MASS) of the compounds was recorded and the molecular weight and molecular formula were obtained. Peaks in the mass spectrum could be readily assigned to the different fragments arising from the molecule. All proton and carbons in the molecule could be readily discerned and values assigned unequivocally by $^1$H NMR and $^{13}$C NMR. One of the striking features of these compounds was that its $^{13}$C NMR spectrum contained 30 peaks, indicating that all carbons in this molecule are nonequivalent.

The spectral study revealed that CP1 is a positional isomer of EA1 differing only in the position of the hydroxyl groups. The number of methyl carbons is the same in both the compounds. This difference in different kinds of carbons could be readily discerned by a comparison of the $^{13}$C NMR spectra of two compounds. The $^1$H NMR spectra of two compounds also substantiate findings regarding the difference EA1 and CP1.

A precise method was developed for determination of concentration of chemical marker present in the polyherbal formulations using HPTLC and HPLC. This method was slight modification in sample preparation and assay conditions developed for estimation of marker component present in individual extracts.

The linear dependence of peak area on concentration was observed in both the methods throughout the concentration range tested. Thus the method resulted in accurate, reproducible and expected concentrations of
marker components, EA1, CP1, asiatic acid and bacoside-b in polyherbal formulation. The accuracy of estimation procedures was confirmed by recovery experiments.

**PREPARATION AND EVALUATION OF HERBAL FORMULATION**

Polyherbal syrup and tablet formulation were prepared using four drug extracts. Formulation containing varying amount of extracts (25 mg to 75 mg) were prepared and subjected for investigations. The tablets were evaluated for weight variation, hardness, friability, disintegration, content uniformity and dissolution test. In the weight variation test the percentage weight variation in all the tablet formulations was within the pharmacopoeial limit. The tablets were also satisfactory in the pharmacopoeial limit of hardness and friability.

The content uniformity in the syrups and tablets was determined by the estimation of marker components present in different polyherbal formulations. With a view to standardise the syrups and tablets, estimation of markar component was undertaken using standard procedure of HPTLC and HPLC. The concentrations of marker components present in prepared formulations were in range of expected theoretical concentration.

The dissolution test tablets was performed using a USP 25 dissolution apparatus 1 (rotating basket method) model Veego DA-6D, 6-vessel assembly. As described in the FIP recommendations and WHO guidelines for biopharmaceutical characterizations of herbal medicinal products, three buffers with pH 1.2, 4.6 and 6.8 should be taken as dissolution medium. For pH 1.2 gastric fluid, simulated and intestinal fluid, simulated was used for pH 6.8. An acetate buffer was used to maintain pH 4.6. The utilization of gastric fluid (simulated) and intestinal fluid (simulated) as dissolution medium was aimed with a view to investigate the biopharmaceutical profile of formulations. The tablet of each formulation was placed into the basket, and
subjected to dissolution test. Four samples of each formulation were tested. 10 ml samples were withdrawn from the dissolution vessel at the end of 30 min., 1 hour, 2 hours, 4 hours and 6 hours. The quantity of EA1 and CP1 dissolved at each sampling time was estimated by HPLC method.

All the five formulations showed comparable release pattern from the 2nd hour onwards with differences only in the 30 min. and 1st hour, and the reason for the latter appear to be differences in the rate of burst effect.

It was observed that the components other than markers are also releases at the same pattern and rate as it was found in markers in all the formulations with only minute differences, which were insignificant statistically.

ESTIMATION OF MARKERS IN MARKETED PRODUCTS

The concentration of chemical markers present in the marketed products was also determined by HPTLC and HPLC. Three marketed syrup formulations selected for the estimation of marker component MSYRUP1 is manufactured by Baidyanath Ayrveda Bhawan, Jhansi (U.P.), MSYRUP2 manufactured by Unjha Herbal Products, Bangalore and MSYRUP3 is manufactured by Himalaya Drugs Co., Bangalore (Karnataka). HPTLC analysis exhibited the concentrations of EA1 in MSYRUP1, MSYRUP2 and MSYRUP3 was 0.4352, 0.2311 and 0.2433 μg/ml respectively while CP1 was present in the concentrations of 0.2321, 0.6343 and 0.2462 μg/ml respectively.

The concentration of marker components, EA1, CP1, asiatic acid and bacoside in marketed polyherbal tablet formulation was also determined by HPTLC. The marketed products selected for the estimation of marker component by HPTLC were coded as FORMULATION1, FORMULATION2, FORMULATION3, FORMULATION4, FORMULATION5. Each tablet was ground and pass through 40 mesh sieve and transferred into 100 ml conical
flask. 15 ml aqueous methanol (1:1) was added to the contents of flask and heated (60°C) on water bath for 10 minutes and then filtered into a separate conical flask using Whatman filter paper no. 41. The extraction was repeated for four times as above using 15 ml of solvent each time. The filtered extracts were pooled and transferred into 100 ml volumetric flask and the volume made upto the mark with methanol. This solution was used for HPTLC analysis.

In FORMULATION1 the concentration of asiatic acid, bacoside-b and EA1 was 0.4328, 0.3427 and 0.3007 μg/tablet while CP1 was absent. As manufacturer of FORMULATION1 reported that they have used extracts of Brahmi, Sheelajit and Shankhpushpi but the variety of Shankhpushpi does not mentioned in the literature leaflet of FORMULATION1. The results confirms that manufacturer had used Evolulus alsinoides as Shankhpushpi and not the drug of Convolvulus pluricaulis as its marker constituent CP1 was absent.

FORMULATION2 is marketed as BRAHMI VATI. The manufacturer of FORMULATION2 reported in their literature leaflet of product that they have used extract of Brahmi Patra, Ras Sindhur, Shuddh Shiljatu, Abhrak Bhasma and other ingredients as per RTS. In the marker estimation it was found that asiatic acid and bacoside-b present in concentrations of 0.3807 and 0.2979 μg/tablet whereas EA1 and CP1 were absent. The result suggests that manufacturer have used both Centella asiatica and Bacopa monniera as Brahmi in the manufacturing of FORMULATION2.

FORMULATION3 is also marketed as BRAHMI VATI. The manufacturer of FORMULATION3 reported in their literature leaflet of product that they have used pure extracts of Bacopa monniera. The results of present investigation also favors the details of presence of constituent in product as it resulted the presence of only bacoside-b at a concentration of 0.3730 μg/tablet.
FORMULATION 4 is marketed as SHANKHPUSHPI TABLETS. The manufacturer of FORMULATION 4 reported in their literature leaflet of product that they have used extracts of Shankhpushpi and Neer brahmi. In the marker estimation it was found that bacoside-b and EA1 present at concentrations of 0.4289 and 0.2104 μg/tablet whereas asiatic acid and CP1 were absent. The result suggests that manufacturer have used both *Bacopa monniera* as Neer Brahmi and *Evolvulus alsinoides* as Shankhpushpi in the manufacturing of FORMULATION 4.

FORMULATION 5 is marketed as SHANKHPUSHPI VATI. The manufacturer of FORMULATION 5 claimed in their literature leaflet of product that they have used extracts of Brahmi, Shankhpushpi and Swarn Bhashm. In the marker estimation it was found that bacoside-b, CP1 and EA1 present at concentrations of 0.3853, 0.2467 and 0.3688 μg/tablet whereas asiatic acid was absent. The result suggests that manufacturer have used both *Bacopa monniera* as Brahmi and both *Evolvulus alsinoides* and *Convolvulus pluricaulis* as Shankhpushpi in the manufacturing of FORMULATION 5.

The concentration of chemical marker present in the marketed syrups and tablets was also determined by HPLC. The results obtained were accurate and yielded expected concentration of markers.

The results obtained were highly accurate and reproducible, therefore this method is recommended and suggested to use in future for the evaluation of commercial herbal products.

**STABILITY STUDIES OF HERBAL FORMULATIONS**

The stability was studied for the period of three months. The different parameters such as nature, colour, odour, texture were studied for tablet formulations whereas pH, colour, odour and nature were studied for syrup
formulations. There was no marked change found to be in syrup and tablet formulations after the storage for three months.

Temperature dependent degradation behaviour of syrups and tablets was investigated to study the shelf life of marker components. The time required for degradation upto 10% was determined with reference to residual marker constituents. The data obtained were subjected for curve fitting and it was observed that the degradation behaviour was following first order kinetics. A graph, residual concentration versus time was plotted and k values were obtained from the regressed line slope.

The shelf life of syrups and tablets were determined with reference to residual concentration of marker components, EA1 and CP1 and was expressed in terms of days. At RT (room temperature maintained at 25±2°C) the shelf life of EA1 in syrups was found to be 1100 to 1120 days whereas in tablet it was found to be 1280 to 1343 days. The shelf life of EA1 in syrups at 30°C was found to be 1100 to 1110 days whereas in tablet it was 1276 to 1335 days. At 40°C shelf life of EA1 in syrups was found to be 1082 to 1117 days whereas in tablets it was found to be 1263 to 1340 days. The shelf life of EA1 in syrups at 50°C was found to be 1026 to 1086 days whereas in tablets it was 1236 to 1322 days.

At RT (room temperature maintained at 25±2°C) the shelf life of CP1 in syrups was found to be 1101 to 1123 days whereas in tablets it was found to be 1283 to 1338 days. The shelf life of CP1 in syrups at 30°C was found to be 1112 to 1116 days whereas in tablets it was 1278 to 1334 days. At 40°C shelf life of CP1 in syrups was found to be 1079 to 1118 days whereas in tablets it was found to be 1259 to 1336 days. The shelf life of CP1 in syrups at 50°C was found to be 1023 to 1082 days whereas in tablets it was 1241 to 1331 days.
To conclude, the investigations undertaken for four drugs viz. *Evolvulus alsinoides, Convolvulus pluricaulis, Centella asiatica* and *Bacopa monniera* set parameters for standardization of raw material. Chemical markers for *Evolvulus alsinoides* and *Convolvulus pluricaulis* were isolated and their structures arrived by chemical and spectral data. HPTLC and HPLC methods for determination of chemical markers in individual herb and in combined extracts were developed. Efficacy of drugs under investigations in learning and behaviour was studied.

Syrup and tablet formulations of drugs were prepared and evaluated for their pharmaceutical and biopharmaceutical properties. Methods for standardization of herbs in dosage forms were developed.

The studies, thus, were successful in development and standardization of polyherbal formulations incorporating the four herbs.