1. ABSTRACT

Plants have formed the basis for traditional medicinal systems for thousands of years, with the first records dating from about 2600 B.C. in Mesopotamia. People used oils from cedar and cypress, licorice, myrrh, and poppy juice, among other substances that are still in use today for the treatment of a variety of illnesses and infections. Ancient Egyptian, Chinese and Indian documents show that medicine in these societies included numerous plant-based remedies and preventives. Ayurveda, which literally means the science of life, is one of the oldest systems of medicines in India. The origin of Ayurveda has been lost in prehistoric antiquity, but its concepts and approaches have been perfected between 2500 and 500 B.C. in India. Besides Ayurveda there are several other complementary and alternative systems of medicine like Homeopathy, Siddha and Unani systems of medicine, which are also practiced and developed with the course of time in India, where plants and plant-based formulations are employed for health care and disease treatments.

Today approximately 80 % of the world’s population relies on traditional plant based medicines for primary health care. About 25 % of prescription drugs dispensed in the United States contain plant extracts or active ingredients derived from plants. Out of a total of 520 new drugs approved for commercial use between 1983 and 1994, 30 were new natural products and 127 were chemically modified natural products.

In almost all the traditional systems of medicine, the medicinal plants play a major role and constitute their backbone. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices. It is difficult to get reliable figures for the total number of medicinal plants on earth; according to some estimation, around 35,000–70,000 plant species are being used worldwide in health care systems.

Despite the great successes already achieved in natural products chemistry and drug development, we have barely begun to tap the potential of our molecular diversity. Only an estimated 5 % to 15 % of the 250,000 species of higher terrestrial plants in existence have been chemically and pharmacologically investigated in systematic fashion.
Approaches like high-throughput screening, phytochemical profiling, quality controls and standardization of raw materials and finished products, clinical trials, herbal therapeutics, pharmacokinetics and herbal pharmacovigilance will not only help to prove the rationale of using these systems but also to get maximum benefits of the natural resources.

Interest in medicinal plants has burgeoned due to increased efficiency of new plant-derived drugs and the growing interest in natural products. Because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades. The use of plants as medicines dates from the earliest years of man’s evolution. Medicinal plants serve as therapeutic alternatives, safer choices, or in some cases, as the only effective treatment. A larger number of these plants and their isolated constituents have shown beneficial therapeutic effects, including anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial and immunomodulatory effects. Several plant products are known to exhibit immense medicinal value against human diseases. It is tempting to speculate that the restorative and rejuvenating power of these herbs may be due to their action on the immune system. Oxidative damage to biological structures has also been implicated in the toxicity-induced pathophysiology of several diseases. It has been reported that the health promotive, disease preventive and rejuvenation approach based on using medicinal plants in ‘Ayurveda’, is due to the anti-oxidant effects of these plants.

In spite of the tremendous advances made in the modern sciences, there are still a large number of ailments for which suitable drugs are not found. With the potential of uncovering new compounds with idealistic pharmacological profiles (i.e., no side effects, no addictive potential), natural products still hold great promise for the future of drug discovery especially in the treatment of pain disorders and potentially drug addictions. The alternative and complementary systems of medicine have not adequately been explored for the safe and effective anti-inflammatory drugs. Plants and other natural products described in historical ethnobotanical and ethnopharmacological literature are being utilized to aid in the identification of natural products that have been historically employed in the alleviation of pain. Over the last several decades, more analgesic substances have been purified from natural
products such as polysaccharides, terpenes, curcuminoids, alkaloids, etc. resulting in novel structural classes and mechanisms of actions. Many of these act by their radical scavenging effect or their immunomodulatory activity. Immunomodulators act by stimulating or suppressing both the humoral and cellular immune system thereby promoting the phagocytosis and further neutralizing the toxins whereas antioxidants protect, by preventing the harmful effects of free radical mediated chain reactions in the cell membranes and by reducing the susceptibility of the tissues to oxygen stress.

*Inula cappa* (Buch.-Ham. ex D. Don) DC., (F:Compositae), a perennial shrub commonly known as ‘Chirchitta’ and ‘Poshkanmul’, is found mainly in the temperate region from Kashmir to Sikkim. In the traditional system of medicine the plant is mainly used as an anti-inflammatory agent, anodyne and in the treatment of gastric disorders. It is reported to contain flavonoids and germacrene class of the sesquiterpenes, certain lipids like capric acid, myristic acid, inositol angelates etc. Since the plant is not investigated phytochemically and pharmacologically in detail, the present study was undertaken to investigate the plant within the context of the modern scientific framework. Elfin scientific reports regarding phytochemical and pharmacological aspects led us to investigate the plant for anti-inflammatory, immunomodulatory and antioxidant activity with an aim to substantiate claims made of this plant in traditional system of medicine.

In herbal research, it is essential to authenticate the plant and to establish its pharmacognostical and phytochemical standards. The roots of *Inula cappa* were identified by the morphological and microscopical studies which included the powder study and were also authenticated by a taxonomist.

The qualitative parameters like the extractive value, ash value, microbial load and foreign matter were studied according to the methods described by the Indian pharmacopoeia and the Ayurvedic Pharmacopoeia. The alcohol soluble extractive value was almost the half of the water soluble extractive value whereas the n-hexane soluble extractive value was very less (less than ten times compared to the alcohol soluble extractive value). Various chemical tests were performed to analyze the plant qualitatively for the presence of different chemical groups and it was observed that the major components of the *Inula cappa* roots were phenolics, including tannins and
flavonoids. It also showed the presence of terpenoids, saponins and coumarins. However, alkaloids were found to be absent.

The presence of good amount of the phenolics and flavonoids prompted us to perform the antioxidant activity of the roots. For the activity one kilogram powder of the roots of *Inula cappa* was extracted with methanol (Extract A) and the marc was further extracted with water to give the water soluble portion (Extract A1). The methanolic extract was further portioned between ethyl acetate and water to give ethyl acetate soluble (Extract A2) and insoluble fraction (Extract A3). Extracts were tested for their antioxidant activity in both *in-vitro* and *ex-vivo* experiments.

Proton-radical scavenging action is an important attribute of antioxidants, which is measured by α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay. It was found that Extract A2 exhibited equipotent activity to the Extract A, whereas extracts A1 and A3 did not show any activity. The suppressive effect of *Inula cappa* on the superoxide radical was also confirmed in this study. The capacity of both the extracts (A and A2) to scavenge superoxide radical revealed that the extracts possessed superoxide dismutase like activity. Although, the activity was found to be lower than scavenging activity of ascorbic acid in entire concentration ranges.

Both the extracts A and A2 possessed the ability to reduce iron III (Fe^{+3}) and also in a linear concentration dependent fashion when compared with standard reducing agents like gallic acid and tannic acid. The reducing power property may be contributing to its antioxidant activity. Both the extracts (A and A2) also inhibited lipid peroxidation which was evident from their ability to decrease the malondialdehyde (MDA) formation, shown by a decrease in the absorbance.

*Inula cappa* has been traditionally used in some regions of Nepal as medicinal remedy. The roots of *Inula cappa* have been utilized by these populations against pains, rheumatism and gastrointestinal ailments. Extract A, A1, A2 and A3 were thus evaluated for their effects on acute inflammation using carrageenan and histamine induced paw oedema. It was observed that Extract A and Extract A2, when administered orally at a dose of 300 mg kg^{-1} body weight, inhibited carrageenan-induced rat paw oedema. Extract A showed significant anti-inflammatory activity
which was comparable with indomethacin, but the inhibition was significant after 3 hour, whereas Extract A2 exhibited the activity in the initial phase. Extracts A and A2 also inhibited the histamine induced hind paw oedema at the dose of 300 mg kg\(^{-1}\) per oral. However, the activity of Extract A2 was stronger compared to Extract A.

The cotton pellet test is considered a model for studies on chronic inflammation and inflammatory granuloma is considered as a typical feature of established chronic inflammatory reaction. Both, Extract A and Extract A2 were effective in suppressing granuloma formation in this model. However, Extract A exhibited a stronger activity compared to Extract A2. Extract A was further studied to find out the mechanism of its action as it showed a better activity compared to Extract A2 and also because the extractive value of Extract A2 was very less it was not studied further. Extract A was evaluated for its ability to inhibit prostaglandin synthesis and found to inhibit the amplitude of the contraction of the uterus isolated from the diestrus rats demonstrating the activity. Extract A also possessed potent membrane stabilizing properties apparent from its inhibitory activity on heat-induced haemolysis in a concentration dependant manner. The activity of Extract A was comparable to the standard drug aspirin at all the dose levels tested.

Carrageenan-induced pleurisy is considered to be an excellent acute inflammatory model in which activity of drugs against increased vascular permeability, fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate. It was observed that Extract A not only produced significant inhibition of fluid exudation but was also effective in reducing leukocyte infiltration and protein extravasation when compared to the control. This indicated that the Extract A reduces the capillary permeability thus leading to a reduced dilation of arterioles and venules and to a decreased vascular permeability.

*Inula cappa* roots were also evaluated for immunomodulatory activity using various experimental models to identify a possible role in the mechanism of its anti-inflammatory activity. In the preliminary tests, all the extracts A, A1, A2 and A3 were tested for their effect, as many rasayana drugs are known to possess immunomodulatory properties. In the carbon clearance model it was observed that
Extract A and Extract A2 enhanced the activity compared to control groups whereas, Extract A1 and A3 were found to be inactive.

Extract A stimulated the humoral response against sheep red blood cells (SRBC) and produced a dose dependant increase in the antibody synthesis. The maximum effect was observed at 300 mg kg\(^{-1}\) of oral dose. This result was also supported by the \textit{in-vitro} plaque forming assay, where nearly three-fold increase in the IgM antibody plaque formation was observed in the spleen cells of Extract A (300 mg kg\(^{-1}\), orally) treated mice as compared to control animals. A model of cell-mediated inflammation, SRBC induced delayed type hypersensitivity (DTH), was used to investigate the immunomodulatory effect of Extract A, and a significant difference in paw thickness was observed between Extract A fed mice and normal saline fed control mice. Extract A inhibited the paw thickness by 85\% compared to the control group. Hence its effect was evaluated at different dose levels and the study revealed that oral administration of the Extract A (75–300 mg kg\(^{-1}\)) produced a dose dependant decrease in the DTH reaction with a maximum at a dose of 300 mg kg\(^{-1}\). In immunoprophylactic assay, when mice of treated and untreated groups were subjected to fatal dose of \textit{E.coli} (2.5 \ \times \ 10^8 \ \text{cells suspended in normal saline}), Extract A treated groups showed significantly less percentage of mortality compared to the control group.

Lymphocyte proliferation is a complex event that involves interaction of interleukins (IL-1 and IL-2) and expression of their receptors. Colorimetric MTT assay was used for evaluating the ability of Extract A to enhance lymphocyte proliferation, using the splenic and thymic lymphocytes, since the cleavage of MTT has several desirable properties for assaying cell survival and proliferation.

In this study, it was observed that the Extract A exhibited immunomodulatory potential by inhibiting the proliferation of the splenocytes in the \textit{ex-vivo} experiment, whereas in the \textit{in-vitro} test the proliferation increased dose dependently. A similar effect was also observed in the thymocytes.

Extract A also inhibited the production of the proinflammatory cytokines, IL-2 in Con A-activated splenocytes. Immunostimulatory effects of a drug or nutritional supplement are difficult to evaluate in healthy people and animals. Therefore, the
effects of Extract A were evaluated in the mice immunocompromised with hydrocortisone acetate. It was observed that Extract A could bring about a significant recovery of the IL-2 production and lymphocyte proliferation. The fact that Extract A could bring about a significant recovery of the IL-2 production and lymphocyte proliferation in HCA immunocompromised mice was also supported by its tendency to bring back the leukocyte count to the normal in the cyclophosphamide induced myelosuppression experimental model. From this study it can be assumed that the Extract A may be having some immunostimulant properties.

Besides this, Extract A did not reveal any untoward effect on behavioral response, body weight, normal reflexes and visceral appearance in the mice. It did not produce any histopathological changes in the major organ tissues and the hematological parameters were found to be normal too.

Extract A was found to be more potent than the fractions derived from it (Extract A2 and A3) and Extract A1. Extract A, being a whole extract, contains all the constituents and so probably synergism and potentiation is a possible rationale for its stronger activity.

Chemoprofiling and marker compound analysis using modern analytical techniques like HPTLC and HPLC have emerged as one of the tools for the quality assessment of the ayurvedic drugs. HPTLC can be used for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. In the present study, TLC finger printing was developed for the methanolic extract using various solvent system and multiple markers setting the standards for the identification of the roots of *Inula cappa.*

For the fingerprinting, 6 solvent systems ranging from highly non-polar to an increased polarity were selected. After developing the plate they were visualized in UV 254, UV 366 and after derivatisation with anisaldehyde in sulphuric acid. The documentation was done and the Rf values and the color of bands were noted. A co-chromatography was also done with the marker compounds wherever resolved.
The solvent systems evolved for different marker compounds resolved well, the compound/s of interest in each case, and the bands were pure as confirmed by overlapping the absorption spectra recorded at start, middle and end portion of each band corresponding to the standard of the respective markers.

In the solvent system of hexane: ethyl acetate (9: 1, v/v) when observed under UV 254 nm, Extract A showed 7 bands, with 2 major bands, of which one was of thymol isobutyrate at R_f 0.51. β-sitosterol, lupeol and α-amyрин resolved well in the solvent system II, Toluene: Methanol (9:1, v/v), which is relatively polar, after derivatization with anisaldehyde in sulphuric acid at R_f 0.44, 0.58 and 0.92, respectively.

By increasing the polarity a little more with toluene: ethyl acetate: formic acid (7: 3: 0.5, v/v/v), 6 bands were observed under UV 254, 7 bands under UV 366 and 8 bands after derivatization with anisaldehyde sulphuric acid reagent. In a relatively more polar system of chloroform: methanol: water (7: 3: 0.5, v/v/v), seven bands were seen in the UV 254 nm. Under UV 366, the maximum (10) bands were resolved in this system, however, after derivatization only 4 bands were observed.

In solvent system of toluene : ethyl formate : formic acid : water (3: 3: 0.8: 0.2, v/v/v/v), a total of 6 bands were resolved under UV 254, 9 bands under UV 366 nm and five bands after derivatization with anisaldehyde in sulphuric acid reagent. In the solvent system containing ethyl acetate: formic acid: glacial acetic acid: water (10: 1.1: 1.1: 2.6, v/v/v/v) four bands were observed after derivatization. Under UV 254 and UV 366 only 3 and 4 bands respectively were observed. A band at R_f 0.55, was confirmed to be chlorogenic acid.

Volatile oil was isolated from the roots of *Inula cappa* using hydrodistillation. The oil was investigated by GC-MS. *Inula cappa* oil was mainly composed of thymol, and its derivative thymol isobutyrate. A TLC fingerprint was also developed and showed two major bands in the UV 254 nm and after derivatisation with anisaldehyde in sulphuric acid reagent.

Two compounds were isolated from the Extract A by column chromatography using a solvent system of petroleum ether and increasing concentrations of ethyl acetate. The
isolated compounds were characterized using UV, IR, EIMS and NMR spectroscopy. The structure elucidation of the compound revealed that it belonged to the sesquiterpene lactone series and were identified as isoalantolactone and germacrolide.

TLC densitometric quantification methods were developed for the estimation of 7 marker compounds viz., β-sitosterol, lupeol, α-amyrin, chlorogenic acid, thymol isobutyrate, germacrolide and isoalantolactone using HPTLC. Various solvent systems were tried to resolve the marker compounds from the other compounds. It was possible to resolve β- sitosterol and lupeol in the same solvent system (toluene: methanol, 9.4: 0.6, v/v) and hence quantified in one system. α- amyrin was quantified in toluene: ethyl acetate (9.8: 0.2, v/v). Chlorogenic acid was resolved from methanolic extract of \textit{Inula cappa} in the solvent system of ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6, v/v), while thymol isobutyrate was quantified in toluene: ethyl acetate (9.3: 0.7, v/v).

Chlorogenic acid and α- amyrin are reported for the first time in this particular plant. The amount of chlorogenic acid found was 0.045 % w/v. Thymol isobutyrate was also identified as a major constituent of the volatile oil of the roots. For the quantification of isolated compounds, isoalantolactone and germacrenolide, a solvent system of toluene: methanol (9.4: 0.6, v/v) was used.

Efforts were made to simplify the sample preparation step and resolve more than one marker in the same solvent system. The sample clean-up requirement was minimal. The methods were validated in terms of accuracy, precision, limit of detection, limit of quantification, specificity, linearity and repeatability.

From the recovery studies which were carried out at three different levels i.e. 50 %, 100 % and 125 % addition of marker compounds, the average percentage recovery obtained was found to be between 97-100 %. The methods developed were found to be precise, reproducible and selective, as revealed from the limit of detection (LOD) and limit of quantification (LOQ), the linearity range which reflects the sensitivity, and the average percent recovery. The purity of the standard compounds and the isolated compounds was tested by the recording the spectra at start, middle and end
positions and also an overlapping spectrum recorded from the resolved band in the test sample and the standard compound.

In summary, the present study sets the standards for *Inula cappa* roots in terms of its microscopical studies and phytochemical analysis. Two sesquiterpenes, isosalantolactone and germacrolide were isolated and quantified in the roots. Our study gives the first report on the presence of chlorogenic acid and *α*-amyrin in the roots of *Inula cappa*. Thymol isobutyrate was quantified using a new HPTLC method. Lupeol and β-sitosterol were also quantified in the roots for the first time. TLC fingerprinting developed can be a useful tool for the identification of the raw material. The identification and quantification of the marker compounds provides the data for the standardization for the drug. Pharmacological investigations revealed that Extract A had profound immunomodulatory activity on humoral and cell mediated immune responses. Further, the anti-inflammatory activity could be due its ability to inhibit prostaglandin synthesis, scavenge the free radicals and to an extent because of its immunomodulatory potential-to inhibit the lymphocyte proliferation. Moreover, Extract A was found to be non-toxic up to a dose level of 1.5 g kg\(^{-1}\) body weight.

In conclusion, our studies provide supportive evidence towards the traditional claims made for the roots of *Inula cappa* as a potent anti-inflammatory and immunomodulatory agent, adding value to its actual utility in the treatment of various ailments.