PHARMACOGNOSTICAL EVALUATIONS

In past few years the use of medicinal plants has been considerably increased as there is an increase in demands of raw material for pharmaceutical preparation as well as for self medication in large population in the world (Abou-Arab and Abou Donia, 2001). It is very essential to obtain a proper quality control profile for various medicinal plant used in traditional system of medicine. This may be helpful in minimizing the adulteration of these plants which occurs mainly due to improper knowledge regarding the varied geographical conditions, associated problems of different vernacular names, its morphology and microscopy. It is also emphasized that, correct identification and proper quality assurance of the starting material is an essential prerequisite to ensure reproducible quality of herbal medicine which contributes to its safety and efficacy (Laloo et al., 2013). According to the World Health Organization (WHO), pharmacognostical standards are considered to be the primary step for diagnosis of the herbal drug, which includes macroscopic and microscopic evaluation of the particular plant/plant parts. Further, macroscopical examination of a plant/plant parts represents detailed information regarding the qualitative assessment of plant based on its morphological and sensory characters such as size, shape color, taste, odor etc while the evaluation of crude drugs on the basis of histological studies helps to broaden the views about the type of characters and their occurrence in plant tissues, which in turn is necessary for proper identification of plant/plant parts (Kumar et al., 2014; Prasad et al., 2013).

Different physicochemical parameters determined included water content, chemical assay of active ingredients, inorganic impurities (toxic metals), pesticides and others. Different physicochemical parameters evaluated in the present study includes determination of foreign matter which indicates about the presence of visible matter other than the crude drug itself. Foreign matter was found to be 2% w/w. The foreign matter should be as minimum as possible otherwise it has a huge impact which can produce error during latter stage of the standardization process. Loss on drying represents the moisture content present in the drug. The root tubers were estimated for their moisture content and were found to contain considerably low amount of moisture. The moisture level in the crude drug is important as high level of moisture may cause deterioration during storage condition and could alter the potency of the active constituents.

The ash values are quantitative standards that represent inorganic salts naturally occurring, adhering, or deliberately added to crude drug as a form of adulterant. Total ash includes both physiological which is derived from the plant tissue itself as well as non physiological ash which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface. Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth, whereas water-soluble ash is the water-soluble portion of the total ash (Kokate et al., 2006). The results indicated that acid insoluble ash was present in more amount as compared to water soluble ash.

The alcohol soluble extractive was found to be more compared to water soluble extractive. Extractive value is the amount of active chemical constituents present in plant material extracted through different solvents (Anonymous, 2002b). Presence of saponins in medicinal plants can be detected by their ability to form persistent foam when the plant material is shaken in water which is measured in terms of foaming index. Gums, mucilage, pectin or hemicelluloses imparts swelling properties to plants contributing to specific therapeutic or pharmaceutical utility which can be measured as swelling index (Anonymous, 2002b). The root powder of *Leea macrophylla* showed high swelling index. Haemolysis is a processes causing haemoglobin to diffuse in the surrounding medium which is mainly due to the presence of saponins in case of plants. This property is used for the determination of hemolytic activity of plant material that was found to be low due to the low saponin content of the plant. Fluorescence drug analysis have an immense value in qualitative determination of crude drug, since some chemical constituents of plant exhibit fluorescence in the visible range in day light while many others produce fluorescence in ultra violet range (e.g. alkaloids like berberine) (Prasad et al., 2012a).

Another serious issue associated with use of herbal medicines is intentional or accidental presence of heavy metals more than the permissible range set by the regulatory authorities (Sahoo et al., 2010). Lead, mercury, cadmium and arsenic are the predominant contaminants. Heavy metal poisoning from medicinal plant products have caused several health hazards like liver and kidney failure and even death. In order to prevent heavy metal toxicity requisite regulatory measures and quality control of medicinal plants is necessary (Street, 2012). Heavy metals like As, Pb, Cd and Hg in the roots of *Leea macrophylla* were found to be within the permissible range as proposed by WHO. Plants are prone to deteriorate either by insects or by disease therefore pesticides are used as a protective measure (Abou-Arab and Abou Donia, 2001).The presence of pesticide residue may be as a result of either agricultural practice or environmental contamination. Pesticide residue includes their metabolite and/or their degradation products which remain in plant or in the soil. Chlorinated and organophosphorus pesticides are the most harmful pesticides, which persist in enviournment and exhibit adverse health effects (Zhang et al., 2012). The presence of pesticide residue in herbal medicine seriously affects development and the process of internationalization of traditional herbal medicine (Sahoo et al., 2010). Hence WHO and other organizations have established requirements to limit pesticide residue in herbal material. The roots of *Leea macrophylla* were estimated for the presence of chlorinated and phosphate pesticides which were found to be within the prescribed limits as per WHO.

The DNA fingerprinting analysis of *Leea macrophylla* has been performed for the first time. Conventionally macroscopic, microscopic, physicochemical parameters, analytical chromatographic techniques such as thin-layer chromatography, High-Performance Liquid Chromatography have been used for authentication of plant material. However, from past twenty years, molecular markers have rapidly complemented the classical strategies. Molecular markers are generally referred to as biochemical constituents, including primary and secondary metabolites in plants and macromolecules, viz. proteins and deoxyribonucleic acids (DNA). Secondary metabolites as markers have been extensively used in quality control and standardization of herbal drugs. Focus is now, on development of markers based on genetic composition and hence it is unique, stable, and ubiquitous to the plants. These DNA-based markers are not influenced by age, physiological condition as well as environmental factors. Different types of DNA-based markers viz., RAPD, RFLP (Restriction Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeat), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeat) etc., are employed for plant species discrimination coupled with methods of plant identification involving taxonomy, physiology and embryology. The authentication of herbal drugs and discrimination of adulterants from genuine medicinal herbs are essential for both pharmaceutical company as well as public health (Kiran et al.,2010).

Soxhlet extraction is among the most commonly used extractive technique which facilitates through release of metabolites ingrained in complex matrices of plant with the help of various solvents. During processing the powdered root was screened for its extractive value in different solvent including petroleum ether, hexane, chloroform, ethyl acetate and ethanol. It was observed that maximum yield was obtained in ethanol, and consequently it was selected as extracting solvent for the experiment. Design of experiments (DoE) was used to determine the relevant influential factors and their admissible limit range, which was further utilized to develop a response surface model that in turn aided estimation of a descriptive mathematical model (Singh et al., 2015). The 3D response surface plots (Figure 9) of DoE for extraction efficiency and phenolic yield suggested the following outcomes. The powdered root was screened through sieve 20 (median sized openings) resulted in maximum extraction efficiency and phenolic yield while a decrease was observed when particles were screened with either sieve 10 (coarse particles) and 40 (very fine). Grinding of plant material into small particles enhances the surface area for efficient mixing with solvent, which in turn should hasten the mass transfer of active principle from plant material to the solvent, however this principle holds true up to a certain extent only. Particles sized small (those produced by sieve no. 40) create difficulty during extraction due to attainment of slimy character, clogging the sample plug and slowing down the extraction process.

During extraction, attainment of equilibration time is essential. The equilibration time is the time after which the amount of analyte extracted remains constant. Intuitively prolongation of exraction periods should give maximum yields, however this study showed that extraction efficiency and phenolic yield was maximum at 18 hrs while increase in time upto 24 hrs reduces the yield. Unnecessary continuous extraction for prolonged time may deteriorate the phytoconstituents due to excessive effect of heat which may reduce the yield (Handa et al., 2008).

The study indicated that when absolute ethanol (100%) or highly polar solvent blend (ethanol: water 85:15) was used the extraction efficiency and phenolic yield was lower as compared to the solvent blend of ethanol: water (92.5:7.5). The choice of solvent selected for extraction is vital as it is intended to isolate phytoconstituents. Solvents vary in their ability to extract phytoconstiutents from plant tissue due to variable degree of affinity to them (Cowan, 1999). Efficiency of extraction can also be enhanced by addition of co-solvent to liquid phase as it may alter the polarity of final solvent blend (Wang and Weller, 2006). Selection of solvent is based upon the polarity of targeted phytoconstituent and in turn depends on the molecular affinity of solvent towards solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility (Azmir et al., 2013).

Preliminary phytochemical analysis performed in the study gives an idea about the chemical nature of the active constituents present in that extract. Ethanolic extract of root of *Leea macrophylla* as well as its successive subfractions (hexane, chloroform, ethyl acetate, butanol and aqueous) were subjected to phytochemical screening.

Ethanolic extract and its aqueous fraction showed presence of alkaloids, glycosides, flavonoids, steroids, phenols, tannins, saponin, mucilage, protein, amino acid and sugar. Hexane fraction gave positive test for steroid only, whereas chloroform fraction showed the presence of flavonoids and steroids. Alkaloids, glycosides, steroids, phenols and tannins were present in ethyl acetate fraction. Along with alkaloids, glycosides, steroids, phenols and tannins, butanol fraction gave positive test for the presence of flavonoids, protein and sugars. The quantitative evaluation for phytochemical estimation showed presence of phenolic, tannins, flavonoid, steroids and alkaloid in ethanolic extract of *Leea macrophylla* and its butanol, aqueous and ethyl acetate fractions in decreasing order, while chloroform fraction exhibited presence of only flavonoids and steroids whereas hexane fraction demonstrated presence of steroids only.

Flavonoids have been found to increase capillary permeability and associated in treatment of various cardiovascular disease due to their antioxidant potential and anti-inflammatory properties (Crespy et al., 2002; Gabor, 1979). Phenols can neutralize oxygen derived free radicals by donating hydrogen atom or an electron to the free radicals and have ability to quench. Therefore, they are considered as strong antioxidant and free radical scavengers with anticarcinogenic, antibacterial, anti-inflammatory activities and are also used in coronary heart disease, some types of tumors and coronary artery disease (Yildiz et al., 2011; Shukla et al., 2012). Tannins have a strong astringent action and are reported to have anti-bacterial, anti-inflammatory, anti-viral and anti-oxidant activities (Prasad et al. 2012b; Kapu et al., 2001; Schulz et al., 2002). Alkaloids have been reported to possess wide range of therapeutic importance in the fields of cancer, malaria, pain, inflammation, parkinsonism, hypertension and number of central nervous system disorder (Rathbone and Bruce, 2002).

The HPLC analysis confirmed the presence of chlorogenic acid in quite considerable amount. Dietary polyphenols are thought to be beneficial for human health by exerting various biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, and alteration of signal transduction pathways. Polyphenols are mainly classified into phenolic acids and flavonoids. A major class of the former is hydroxycinnamic acids, and chlorogenic acid is the major representative of hydroxyl cinnamic acids. Chemically, chlorogenic acid is an ester formed between caffeic acid and quinic acid and is a natural antioxidant abundantly distributed among plant species which have been reported to possess antimicrobial, antimutagenic and anti-inflammatory activity (Sato et al., 2011; Xiang et al., 2008). Thus the presence of chlorogenic acid may contribute to the potent antioxidant and antibacterial potential of *Leea macrophylla.*

Different *in vitro* antioxidant model performed in the present study demonstrated a potent antioxidant potential of *Leea macrophylla* and its sub-fractions. The ethanolic extract exhibited the potent *in vitro* antioxidant activity followed by its fractions LMBU, LMAQ, LMEA, LMC and LMH in descending order. Antioxidants are considered as important nutraceuticals on account of many health benefits. Normal physiological processes results in generation of reactive oxygen species (ROS). Oxidative stress condition is a result of excessive ROS production which overcome cellular antioxidant defences this in turn leads to progression of several degenerative diseases such as aging related disease, cancer, cardiovascular diseases, diabetes mellitus and various neurodegenerative disease, via DNA mutation, protein oxidation and/or lipid peroxidation. Thus antioxidants play pivotal role either by preventing or delaying the oxidative damage caused by ROS in various ways and hence medicinal plants having antioxidant potential have attained extensive relevance in treating such chronic diseases (Amessis-Ouchemoukha et al., 2014; Chiang et al., 2015). Recently, interest has been developed in medicinal plants containing antioxidants as active phytochemical, such as phenol compounds, terpenoids, and vitamins, for their potential use as nutraceuticals and/or food additives in the prevention of many diseases (Craig, 1999). Dietary polyphenols are thought to be beneficial for human health by exerting various biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, and alteration of signal transduction pathways (Sato et al., 2011). From the overall observation, the potent *in vitro* antioxidant activity of the roots may be attributed due to phenolics, tannins and flavonoids which were found to be present in considerable high amount in the root tuber of *Leea macrophylla* (Souza et al., 2008).

Plants have an ability to survive from microbial attacks through an arsenal of chemicals which may act as either physical barriers or chemical ones (Prasad et al., 2013). At present, numerous antibiotics are being used for treatment of infections, however they have been associated with adverse effects and have also been found ineffective against these pathogens (Joshi et al., 2013). Interest in ethnopharmacy as a source of these compounds has increased worldwide, particularly in the search for drugs to counter multi-resistant microorganisms. The extract of *Leea macrophylla* was found to have wide range of activity against both Gram positive and some of Gram negative bacteria like *S. flexneri*, *P. aeruginosa* and *S. boydii*. This antimicrobial activity may be attributed possibly to a cumulative action of various phytochemicals detected during phytochemical screening and which are known to cause damage to cell membranes, causing leakage of cellular material and ultimately leading to the death of the microorganism (Marzouk et al., 2010).

Free radical scavenging activity of phenolics and flavonoids imparts them antioxidant potential and major phytoconstituents from plant source responsible for antimicrobial activity includes phenolics, phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids (Gyawali and Ibrahim, 2014). The phytochemical profiling thus clearly explains potent antioxidant and antimicrobial activity of ethanolic extract of *Leea macrophylla* followed by its butanol, aqueous, ethyl acetate, chloroform and hexane fractions in decreasing order owing to the phytoconstituents and their quantity present.

PHARMACOLOGICAL EVALUATIONS

Biogels fabricated for topical delivery should have low hardness and compressibility yet high adhesiveness. Low gel hardness will reduce the mechanical input required to take out gel from a storage container, whereas low compressibility will improve the flow property of gel (relatable to spreadability) and application onto the topical biologic membrane. High gel adhesiveness will ensure prolonged contact of the biogel onto the wounded area. BG2 aptly combined these three properties with low magnitude of compressibility, hardness and adequate adhesiveness prompted us to select it for further preclinical evaluation. The presence of distorted irregular entities in case of lyophillized BG2 (Fig. 1D) are probably the evidence of precipitated microparticles of *Leea macrophylla* extract. This is the most critical distinction, between blank gel and BG2, suggesting proper incorporation of extract in bioadhesive gel network.

The results obtained from excision and incision model as well as from biochemical estimations demonstrated potential wound healing effect of *Leea mcrophylla* via both oral and topical route. But significant improvement was considerably more when the ethanolic extract was applied topically with means of a bioadhesive gel. In incision model, the wound breaking strength was assessed after oral and topical application. During initial stages of wound processing, only the formed blood clot holds edges of wound together, and consequently little breaking strength is required to disrupt or reopen the wound (Kumara Swamy et al., 2007). With progression of time, breaking strength increases via rearrangement of collagen and production of stable intra and intermolecular crosslinked fibre (Mallefet and Dweck, 2008). From the study it was evident that topical treatment was decisively more effective than oral administration.

Excision wound model showed progressive lessening of wounded area amongst the treated groups. The fastest and most complete wound healing (100% wound contraction) was observed with ELMT (5% w/v) within 20 days whereas orally treated rats showed complete healing within 22 days as compared to the control group where complete healing took more than 22 days.

Hence, topical treatment explicates faster rate of wound contraction, epithelisation period and reduced scar area. The success of any medicament professing wound healing hinges on its ability to cause immediate wound closure without scar formation (Clark, 2013). Contraction is characterized by reduction in wound, thus accelerating healing without new tissue formation; and centripetal movement of the contracting wound edges to expedite closure of wound opening determines the period of epithelialization (Upadhyay et al., 2013).

Regular immunological representatives (monocytes) and connective cells like fibroblasts are crucial for normal wound closure (Kant et al., 2013). To validate their role, the data from excision model was substantiated by histological finding which showed re-appearance of skin structure with distinct layers of dermis and epidermis in treated groups (Figure 10). On comparison: the control group had lesser intact tissue in dermal layer than the treated group animals. Formation, alignment and contraction of extra cellular matrix molecules determine the intactness of tissue. Re-establishment is critically dependent on efficiency of reorganization of extra cellular matrix molecules (Yariswamy et al., 2013). The rise in granulation tissue mass and protein amount signifies increased cellular proliferation resulting in increased synthesis of collagen which is the principal extracellular protein in granulating tissue (Fikru et al., 2012). Increase in collagen in orally and topically treated drug is also evident through histological studies.

Our studies on SOD, CAT, GSH, LPO, NO, and MPO status in granulation tissue revealed significant anti-oxidant activity which would reduce free radicals stress and nominal MPO level which would prevent oxidative damage and thereby accelerate healing process. Inflammatory actions instigated by phagocytic cells during healing often lead to generation of reactive oxygen species (ROS). Balance between reactive oxygen species (ROS) and free radical scavenging enzymes (FRSE) is essential for completion of wound healing (Joshi et al., 2013). Although originally unintended, these responses thrust an oxidative stress on the damaged tissue and delay wound healing as a result of imbalance between free radical generation and antioxidants. Such is the dilemma with the ubiquitousness of inflammatory responses that both their over expression and under expression lead to deleterious consequences like susceptibility to innocuous environmental factors, severe damage and even tumorogenesis. Therefore, curtailing the influence of ROS on wound openings could be an important alternative strategy (White and White, 1990; Mikhal'chik et al., 2006). ROS can be eliminated by enhancing expression of antioxidant enzymes like SOD, GSH and CAT or by scavenging pre-exisiting free radicals such as LPO products and NO. SOD carries out disproportionation of superoxide radicals and quenches generation of any new free radicals. MPO, on the other hand generates free radicals. Its elevated status in any milieu (for instance granulation tissue or infiltrating neutrophils) is indicative of bourgeoning inflammation. MPO activity and neutrophil derived oxidants subject tissue to significant oxidative stress and contribute significantly towards wound recovery induced chronic inflammation (Zhang et al., 2011). GSH assists in detoxification of radicals by acting as a helper participant in the glutathione peroxidase (GPx)-instigated reduction of H_2O_2 and lipidperoxides (Roy et al., 2012).

Biochemical estimation elucidated increased levels of hydroxyproline, hyaluronic acid and hexosamine in topically treated animals as compared to oral treatment. The implications of these finding can be discerned in the following explanation. Collagen is made up of hydroxyproline and its relative concentration is an indicator of collagen production (Wang and Weller, 2006). The enhanced production of collagen is stabilized by hexosamine content which provides collagen sites for electrostatic bonding. Since, collagen critically controls the healing process by making up a major portion of connective tissue and also manages its construction, deposition and ensuing evolution; higher concentration of hydroxyproline and hexosamine, therefore promote faster wound healing. Collagen is also vital for re-epithelialisation of cellular-matrix and inter-cellular interactions thereby strengthening and integrating the wound matrix (Pather and Kramer, 2012). Collagen not only provides the tissue matrix with strength and integrity, it also caters to the homeostic demands in the latter portion of wound healing timeline (Roy et al., 2012). The above stated functions of collagen are complimented by hyaluronic acid, which does not participate in its synthesis or maintenance but instead it is involved in water retention, nutrient exchange, cell differentiation and cell mobilization. Hyaluronic acid plays an active role in variety of physiologic and pathophysiologic developments including embryo implantation, cutaneous wound healing and osteoarthritis. Historically, dermatologists and cosmetic practitioners has been investigated hyaluronic acid for its contributory roles which help in regaining elasticity, turgor and moisture of skin (Chen et al., 2014).

The results supported the finding as treatment with ethanolic extract of *Leea macrophylla* reduces the level of proinflammatory cytokines including IL-1β, IL-6 and TNF-α which are involved in the process of inflammation, trauma and wound healing regulated by activity released by macrophages, endothelials and keratinocytes (Wolfgang, 2002). VEGF levels were enhanced by treatment. VEGF is another imperative growth factor involved in wound healing produced from keratinocytes smooth muscle cells, fibroblasts, thrombocytes, macrophages and neutrophils and endothelial cells during wound healing which plays key role in angiogenesis (Muthukumar et al., 2014).

Proliferation during wound healing is indicative of multifaceted events occurring in time of course. Proliferation occurs during different phases including epithelialization, angiogenesis, granulation tissue formation, and collagen deposition in order to restore barrier function, protection against fluid loss and bacterial intrusion. Impaired proliferation, migration and contraction during wound healing advances towards inefficient repair of wound, reluctant epithelization, reduced tensile strength and increased susceptibility towards bacterial infection (Broughton et al., 2006; Jeffrey and Bridget, 2014). Ki67 is a commonly used proliferation marker. The expression of Ki67 protein is thought to be an indicator of growing cells within the overall cell population (Choi et al., 2012).

The study demonstrated that treatment with ethanolic extract of *Leea macrophylla* causes an increase in cellular proliferation as indicated by level of Ki67 which was found to be highest on day 7 post wounding while a decline was observed on 14th day, however the effect was more pronounced with topical treatment as compared to oral administration. Increased cellular proliferation is essential aspect in healing of wound (Lee et al., 2012) Increased cellular proliferation in the study demonstrated early wound healing in treated animals as compared to control while decline in Ki67 expression may due to the fact that cellular proliferation might have been subsided as a result of transit from proliferation phase to remodeling phase (Xian et al., 2015) which was evident from the study as topical treatment exhibited highly significant decrease in cellular proliferation on 14th day produced earlier wound healing on 20th day as compared to oral treatment where complete wound healing was achieved on 22nd day.

Significant amount of tannins, phenols, flavonoids, carbohydrate and saponins were present in the extract. Phenolics (flavonoids and phenolic acids) have been reported for their redox potential, which allows them to chip in significantly as oxygen quenchers and reducing agents and in some cases even metal chelators (Pattanayaka et al., 2008) imparting them potential in tackling inflammation (Sengar et al., 2015) and thereby wound healing (Akkol et al., 2012) Moreover phenolic compounds have ability to facilitate wound healing by different stage of wound healing either by stimulating collagen synthesis, cell proliferation and angiogenic effect (Agar et al., 2015). Chlorogenic acid quantified in present study is a phenolic acid representing the class of hydroxycinnamic acid polyphenolic compounds and is a dimer of quinic acid and caffeic acid connected with an ester bond (Sato et al., 2011). It is a natural antioxidant found abundantly amongst plant species reportedly possessing antimicrobial, antimutagenic, anti-inflammatory activity (Xiang and Ning, 2008) and wound healing properties (Chen et al., 2012). Wound healing potential of chlorogenic acid is accredited to its ability to increase cellular proliferation and its antioxidant potential accelerating the process (Chen et al., 2012). Earlier studies have also reported that honey which exhibit presence of chlorogenic acid also illustrated wound healing potential through free radical scavenging activity promoting cell proliferation in acquisition of concurrence approaching rapid wound healing (Chaudhary et al., 2015). Furthermore tannins act by chelating free radicals, facilitating wound contraction, accelerating capillary vessel and fibroblast formation and by instigating keratinocyte proliferation thus hastening the process of wound healing (Prasad et al., 2010). Hence all these class of phytochemicals have a significant role in wound healing processes. Therefore the observed potent wound healing activity of the extract may be attributed to the presence of chlorogenic acid along with the cumulative effect of other phytochemicals present in the extract. Probably such effect is the result of a synergy with all the molecules contained in the extract.

The study demonstrated that topical application of ethanolic extract of *Leea macrophylla* showed faster and more significant healing as compared to oral treatment. This can be explained due to the fact that topical delivery is mainly used for achieving local effect. It is expected that systemic administration would require high doses in order to be secreted substantially at a peripheral location. Skin being a large organ, switching to alternative routes of administration, such as the topical route, can, in principle provide, predicable, effective and reliable drug delivery. Topical application allows local drug targeting with minimal systemic effects and is mainly used for delivery of anti-inflammatory, anti-histaminic, antibiotics, wound healing and analgesic drugs (Liu et al., 2014).