CHAPTER 6

CONCLUSION

Since antiquity, man has used plants traditionally as medicines to treat diseases including common infectious ones wherein some of them are still included as part of the habitual treatment of various maladies (Rios and Recio, 2005). The most notable discoveries include the *Ipecacuanha* root from Brazil which yields a chemical called emetine, that kills *Entamoeba histolytica*, the cause of amoebic dysentery, and the Cinchona bark from Peru, which yields quinine, an alkaloid that kills *Plasmodium*, the protozoa that causes malaria (Modi, 1995).

The phytochemical screening of species of *Phyllanthus* has yielded to identify 299 compounds wherein biological activity has been reported for about 28 compounds only. In the case of antimicrobial activity, 4 species each have been reported for antibacterial activity, antifungal activity and 7 species for both antibacterial and antifungal activities.

The plant, *P. wightianus*, chosen for the present study is one such species. Though the Malayali tribals in the Vellore District of Tamil Nadu State in Peninsular India traditionally use to set bones (bone fractures) and to treat diarrhoea it has been not been investigated by phytochemical, antimicrobial and pharmacological studies prior to the present study. The name bears its efficacy such as Elumbotti in Tamil language combining two words such as Elumbu means bone and otti means pastes or sets right fractured bones. Therefore, the present study is aimed to prove the efficacy scientifically by isolating the bioactive principles/compounds responsible for efficacy and test them *in vivo*. Further screening of extracts and its compounds isolated from them for other biological activities could also prove useful. Keeping these objectives
in mind, the extracts of the plant and an isolated compound such as bergenin have been scientifically investigated.

The preliminary phytochemical analysis of the plant revealed the presence of glycosides, steroids, triterpenes, flavones, phenols in all the extracts such as hexane, chloroform and methanol extracts and catechins, coumarins, sugars, saponins and tannins in methanol extract.

The results of the fluorescent analysis were quite helpful to fix parameters in assessing quality and standardization. Analysis of ash for inorganic elements revealed the presence of 2.960 mg sodium and 1.200 mg potassium and 6.300 mg calcium, 0.003 mg cobalt, 0.060 mg copper, 2.130 mg iron, 1.089 mg magnesium and 0.345 mg manganese.

The compounds were isolated applying various chromatographic techniques (TLC, CC, HPLC, HPTLC and GC - MS) and identified by the analysis of spectral data (^H and ^C NMR, IR, UV - VIS, MS) and X-ray crystallographic analysis in specific cases. Confirmation was done by direct comparison with authentic sample (m.p., m.m.p. and superimposable IR and HPTLC).

The paper chromatographic analysis led to the identification of amino acids such as DL - Alanine (0.225), L - Arginine mono HCl (0.162), DL - Aspartic acid (0.347), L – Cystine (0.120), L - Glutamic acid (0.160), G - Lysine (0.138), DL - Methionine (0.154), DL - Tryptophan (0.624) and L - Tyrosine (0.375).

When the spectra were compared by superimposable methods the HPTLC chromatograms performed with marker compounds revealed the presence of friedelin in hexane extract, lupeol in chloroform extract, and gallic acid and bergenin in methanol extract.
The HPLC analysis done in comparison with the values of standards given in parentheses helped to identify and estimate tannins such as gallic acid (GA) - 5.158 (5.092), corillagin (C) - 18.900 (18.875), geraniin (G) - 20.292 (19.817) and ellagic acid (EA) - 27.617 (27.592). Estimation of area of peak facilitated to quantify corillagin - 3.89%, (the most abundant one), geraniin - 3.19%, followed by meager quantities of ellagic acid - 0.68% and gallic acid - 0.38%. The HPLC analysis of the methanol extract carried out along with marker compounds of lignans such as hypophyllanthin and phyllanthin indicated their absence.

A fraction of hexane extract subjected to GC - MS analysis showed 23 peaks with various retention times and molecular weights wherein only seven compounds such as 6,10,14-trimethyl-penta-decan-2-one, hexa-decanoic acid methyl ester, 3,7,11,15-tetramethyl-1-hexadecen-3-ol (isophytol), phytol, octa-decanoic acid methyl ester, 9-octa-decanoic acid methyl ester and di-n-octyl phthalate could be identified unequivocally.

GC - MS analysis of the hexane and chloroform extracts resulted to isolate and identify friedelin and lupeol and sterols such as stigmasterol, campesterol and β-sitosterol in a mixture form. Bergenin, corillagin, ellagic acid, gallic acid and geraniin were isolated and identified from the methanol extract.

Agar-well diffusion method followed to assess antimicrobial activity. Gram-positive, gram-negative and pathogenic fungi including dermatophytes were tested. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were estimated. When similar concentrations were compared it is revealed that the extracts were more potent against almost all the gram-negative bacterial strains than that of gram-positive bacterial strains. Out of three extracts, the methanol extract produced better inhibition with
stronger and broader spectrum. It was followed by the hexane extract. Moderate activity recorded for the chloroform extract. Dose-dependent inhibition and increased inhibitions at higher concentrations were observed. Inhibiting activity of the extracts might be due to the presence of different classes of compounds or their synergistic action. The observation of strong and potential activity against both bacterial and fungal agents including those of skin and wound infections causing microorganisms supports not only the traditional efficacy of bone setting but also provides the possibility of developing a good therapeutic agent for skin and wound infections.

The present study has revealed the possibilities of the test extracts to develop drugs for different diseases such as the potential inhibition against *Ps. aeruginosa, E. coli, Pr. mirabilis* and *Pr. vulgaris* gives scientific evidence to the test extracts for their ability to act against the agents causing urinary tract infections; the activity against the enteric agents such as *E. coli, V. cholerae* and *V. parahaemolyticus* provides scientific evidence to the test extracts as an effective antidiarrheal agent; antipseudomonal and antistaphylococcal activities of the test extracts are considered important against nosocomial infections; and anti-dermatophytic and anti-candidal role of the test extracts strongly supports the efficacy of the test extracts against the agents of bacteria and fungi causing skin infections.

Pharmacological screening such as oral acute toxicity studies, analgesic, anti-inflammatory, *in vitro* antioxidant, wound healing, antidiabetic, antiarthritic, immunomodulatory and hepatoprotective activities (*in vitro* and *in vivo*) for various test extracts was done based on the ethnobotanical claim, qualitative analysis of various test extracts and the presence of active compounds. Scrutiny of published literature facilitated to test bergenin, an isolated compound from methanol extract, for antiarthritic and hepatoprotective activities (*in vitro* and *in vivo*).
In the case of analgesic activity, hexane, chloroform and methanol extracts did not show significant increase in latency to hot plate method. Significant % of protection was recorded in the order of methanol, hexane and chloroform extracts against acetic acid-induced writhing in mice. The findings suggest that the analgesic activity of the test extracts might be due to peripheral analgesic mechanism rather than central analgesic mechanism and it appears to occur by mechanisms independent of activation of opioid receptors as there was a complete lack of analgesic effect to radiant heat in the hot plate test, a very sensitive opioid assay, where morphine caused a graded increase in paw latency. The presence of β-sitosterol, campesterol and stigmasterol in the hexane and chloroform extracts and gallic acid, ellagic acid and geraniin in the methanol extract might have contributed for analgesic activity.

Significant protection of oedema in the anti-inflammatory activity for all the test extracts might be due to significant *in vitro* free radical scavenging activity of the different classes of compounds present in them.

In the case of *in vitro* antioxidant activity, all the test extracts expressed DPPH and NO radical scavenging activity (except chloroform extract). Of which, the methanol extract exhibited strong free radical scavenging activity due to the presence of phenolics in it. It could be correlated to the significant hepatoprotective, antidiabetic and anti-inflammatory activities (both acute and chronic).

All the test extracts exhibited significant wound healing activity. Among the ointments prepared with test extracts, methanol extract ointment produced a highly significant activity to both the models of wound healing, i.e., more or less similar to that of standard drug, nitrofurazone (2% w/w). It may due to the presence of active constituents such as phenols, polyphenols, flavonoids, tannins, saponin and coumarins and their individualistic or synergistic action. The polyvalent activities of the test
extracts such as anti-inflammatory, antioxidant and antimicrobial activities would have hastened the wound healing activity. Thus, the present study confirms the promising wound healing activity and provides scientific validation to the ethnotherapeutic efficacy of the plant as a potential wound healing agent.

All the test extracts exhibited significant hypoglycemic activity. Among all, the methanol extract expressed better activity which might be due to the presence of flavonoids, ß-sitosterol, lupeol, gallic acid, ellagic acid, saponins and tannins and it could be correlated to the synergistic relationship in healing with antioxidant and free radical scavenging activities.

The plant chosen for the present study has not been scientifically investigated for arthritic and rheumatoid diseases. The findings against adjuvant - induced arthritis in rats revealed the significant protection of the test extracts. Among all, the methanol extract and its isolate bergenin exhibited better protection. Better protection exhibited by the methanol extract could mostly be due to the presence of bergenin in it as there was only a slight difference between the activity of methanol extract at 200 mg/kg/p.o. and bergenin at 50 mg/ kg/p.o.. Further, the activity of bergenin was comparable to that of standard, indomethacin. The significant protection might be due to the presence of ß-sitosterol in the hexane extract and lupeol in the chloroform extract. However, it is impossible to rule out the role of other active constituents for their antiarthritic activity. Invariably, all the extracts exhibited better protection at higher doses than lower doses. The results of the present study provide strong evidence for the possibility to develop antiarthritic and antirheumatic drugs and also suggest that the test extract and compound might be useful for the treatment of clinical rheumatoid arthritis while considering the similarities of this model to human rheumatoid arthritis.
In the case of immunomodulating activity, the methanolic extract is superior over hexane and chloroform extracts. The test extracts express not only potential non-specific immune response but also effective in improving humoral and cell-mediated immunity. The test extracts selectively suppress the cell-mediated immune response without inhibiting the humoral immune response that showed its worth as an anti-inflammatory agent against immunologically induced chronic inflammatory diseases such as arthritis.

The increase in survival rate is a general marker exhibiting potency of the test extracts to overcome infectious conditions. The active constituents such as bergenin in methanol extract, friedelin in the hexane extract and lupeol in the chloroform extract might be responsible for their potential immunomodulating activity. However, the role of other constituents present in them can not be ruled out for the immunomodulating potential.

As far as hepatoprotective activity is concerned the test extracts were active to both in vitro and in vivo models. Both methanol extract and bergenin exhibited dose-dependent inhibition of HBsAg at the tested temperature and incubation periods proving their antiviral capacity. As that of other Phyllanthus species, popularly known for antiviral properties, the plant chosen for the present study also has similar property. The findings of in vivo model revealed that all the test extracts and bergenin exhibited significant hepatoprotection against INH + RMP - induced hepatic damage. The hepatoprotective activity of bergenin is so comparable to that of standard silymarin. The methanol extract had been effective in offering protection which is more or less related to the influence of bergenin. Among all, the methanol extract exhibited superior activity than hexane and chloroform extracts. It may be due to the presence of bergenin or other polyphenols such as ellagic acid, gallic acid, corillagin
and geraniin and or their synergistic activity. The high potency of the methanol extract in hepatoprotection could be associated with its high medicinal value. The presence of different active constituents such as β-sitosterol in the hexane extract and lupeol in the chloroform extract or other active constituents may be responsible for hepatoprotection. The overall hepatoprotective effect of the test extracts and bergenin is probably due to a counteraction of free radicals by its antioxidant nature/or to its ability to inhibit lipid accumulation by its antilipidemic property.

**Salient findings of the present study include**

- Reporting of friedelin, lupeol, β-sitosterol, campesterol, stigmasterol, corillagin, ellagic acid, gallic acid, geraniin, 6,10,14,trimethyl penta decan-2 one, hexa decanoic acid methyl ester, isophytol, phytol, octa decanoic acid methyl ester, 9- octa decanoic acid methyl ester, and di-n-octyl phthalate from *P. wightianus* for the first time in science.
- Among all the *Phyllanthus* species, bergenin was reported only in *Phyllanthus flexuosus* (Tanaka and Matsunaga, 1988). **Next, it is reported here.**
- Significant activity against skin infection causing agents such as dermatophytes, *Staph. aureus*, *Staph. epidermis* and *Ps.* species is reported to *P. wightianus* here for the first time.
- Screening of plant extracts and bergenin to treat arthritis provides scientific evidence for the ethnobotanical claim for treating bone disorders and diseases.
- The potent activity recorded to wound healing models substantiates the efficacy of the ethnobotanical claim in the treatment of skin infections.
- Scientific evidence generated through screening against almost all the enteric bacteria by recording significant activity and ability of the plant to treat diarrhea.
The absence of lignans such as phyllanthin and hypophyllanthin reported as hepatoprotective agents (Thyagarajan et al., 1988) does not make any difference in hepatoprotection. The potent hepatoprotective activity may be attributed for the presence of other bioactive compounds. Hepatoprotective activity has been reported to bergenin and ellagic acid by Shin et al. (2005) and to bergenin isolated from Mallotus japonicus by Kim et al. (2000) and Lim et al. (2000 b) in vivo and in vitro. Ellagic acid may also be responsible for hepatoprotective activity.

- Hepatoprotective activity of the bergenin is reported here for the first time from P. wightianus by in vivo and in vitro models and those models that have not been tested so far were tested for the first time here to bergenin.

**Suggested perspectives**

As perspective of this work could suggest that in the antimicrobial studies the enteric bacteria can be analyzed individually for recognition of the largest prevalence antimicrobial in relation to the individualized bacterial species. For the anti-inflammatory activity, in the future it can be used culture of PBMCs and human macrophages activated with LPS of bacteria and molecules of protozoa-recognized inflammatory agents of several human pathologies – and like this to test the anti-inflammatory action that it can be analyzed by nitric oxide dosage and different pro-inflammatory proteins (interleukins). In the increase in humoral antibody (immunomodulatory activities) it should be defined the profile of the subclass of the antibody and in the delayed type hypersensitivity reaction (DTH) to determine the dosage of lymphocytes T helper and suppressor, since the immunological recognition of the immunomodulation demands the determination of this profile. Important, also, in the antiarthritic activity using Freund’s adjuvant
of this profile. Important, also, in the antiarthritic activity using Freund’s adjuvant it is the dosage of markers as protein C reactive (PCR), rheumatoid factor and latex, among others. In the hepatoprotective studies using INH + rifampicin, to test also with anesthetics that are pathological agents recognized of the hepatic cells and that are used with great frequency in practice medicine, where it would generate in the future a study control pre-anesthetic and post-anesthetic. In the antidiabetic activity using STZ could be used as safe marker the glycosylate hemoglobin and tests in vitro with cortisol – a recognized hyperglycemia agent. Finally, in the activity against skin infection it could be used *S. aureus* beta-lactamase positive, since that exists a high incidence in the world of these infections for this etiological agent without easy control for antibiotic.