5.1 PHYTOCHEMISTRY

5.1.1 Qualitative Analysis

The extractive values of *P. wightianus* which positively correlate the nature of the constituents and to evaluate the total content of the secondary metabolites present in the crude extracts are useful to fix pharmacopoeial standard. It was higher in methanol extract such as 14.70% whereas lower values were registered for chloroform and hexane extracts such as 3.60% and 2.30%.

The total ash value was found to be 8.29%, which showed the presence of inorganic constituents. The low value of 1.25% for acid insoluble ash indicated the presence of negligible amount of siliceous matter. The water soluble ash was found to be 1.40%. These studies form the basis for judging the identity and purity of the crude drugs.

5.1.2 Quantitative Analysis of Inorganic Elements (Salts and Minerals)

Determination of elements in the medicinal plant drugs and its extracts is of special importance. Therapeutic applications introduce these minerals into the human body. The favorable effect of the extract is presumable considering the combined effect of organic and inorganic compounds such as metal iron complexes of organic constituents.

There are fourteen trace minerals thought to be essential. They are chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc. Barthakur *et al.* (2005) and Gennaro (2004) have documented the importance of minerals in human nutrition and health. Even though
they are needed in minute quantities they play a bigger role for the normal and better health of a person. Such minerals include calcium, chlorine, magnesium, phosphorus, potassium, sodium and sulphur. According to Recommended Dietary Allowances (RDA, 1989), per day intake-need of an adult is 2000 mg potassium, 800 mg calcium, 350 mg magnesium, 15 mg zinc, 2.5 mg manganese and 1.5 – 3.0 mg copper. In the present study, a one g plant material contained high amounts of sodium (2.960 mg), potassium (1.200 mg), calcium (6.300 mg), iron (2.130 mg) and magnesium (1.089 mg) while other minerals such as manganese (0.345 mg), copper (0.060 mg) and cobalt (0.003 mg) were present in trace quantities. So, it is concluded that the plant extract is a good source of calcium, potassium and sodium and iron.

5.1.3 HPLC Analysis and Estimation of Tannins and Lignans

5.1.3.4 Tannins

The HPLC fingerprint of the methanol extract of *P. wightianus* (Fig. 9) showed 36 peaks with various retention times (quantified by the area of peak) along with standard tannins. The following tannins were identified in comparison with retention time of the standards such as gallic acid (GA) - 5.158 (standard 5.092), corillagin (C) - 18.900 (18.875), geraniin (G) - 20.292 (19.817) and ellagic acid (EA) - 27.617 (27.592). Corillagin was present most abundantly (3.89%), followed by geraniin (3.19%). Ellagic acid (0.68%) and gallic acid (0.38%) were present in minute quantities.

5.1.3.5 Lignans

Analysis of the HPLC fingerprint of the methanol extract of *P. wightianus* showed 8 peaks with various retention times wherein absence of peaks at *R*ₜ = 13.20 for hypophyllanthin and 16.50 for phyllanthin indicated their absence (Fig. 11).
5.1.4 GC-MS Analysis of Lipids

GC-MS analysis of hexane: benzene (4:1) fraction of the hexane extract showed 23 peaks (Fig. 12) wherein 7 compounds could be identified unequivocally (Table 8).

5.1.5 Isolation and Characterization of Compounds from Various Extracts (Figs. 52-70)

The compound PW1 eluted with hexane: benzene (1:1) gave a colourless material, which was crystallized from acetone (m.p. 263°C). IR spectrum showed no absorption for hydroxyl group. It showed absorption at 1716 cm\(^{-1}\) assignable to cyclohexanone and 1389 cm\(^{-1}\) assignable to gem-dimethyl group (Fig. 52). The \(^1\)H NMR spectrum showed seven tertiary methyl groups in this region \(\delta\) 0.65 -1.1 including the secondary methyl at 80.80 appearing as doublet \(J = 6.8\ \text{Hz}\) (Fig. 53). The \(^13\)C NMR spectrum also showed the relevant signals for friedelin (Ali et al., 1999). The three keto carbonyl carbon appeared at \(\delta\) 213.18 (Fig. 54). The IR, \(^1\)H and \(^13\)C NMR spectral data determined the structure of the compound as friedelin. The compound PW1 further confirmed by superimposibility of HPTLC chromatogram of the authentic compound (Fig. 3).

The compound PW2 eluted with benzene gave an amorphous powder, which was crystallized from acetone as colourless needles (m.p.213 - 215°C). The IR spectrum showed an intense band at 3360 and 1643 cm\(^{-1}\) corresponding to hydroxyl group and vinylidene group at 1637 and 790 cm\(^{-1}\) (Fig. 55). The \(^1\)H NMR spectrum showed the presence of 6 tertiary methyl groups at \(\delta\) 0.76 - 1.68 (Fig. 56). The vinylic methyl group attached to C – 20 position. The H – 3 axial protons appeared at \(\delta\) 3.20 as a multiplet. H-29 methylene protons appeared as broad singlet at \(\delta\) 4.56 and 4.68. H – 19 appeared as multiplet at \(\delta\) 2.40. The \(^13\)C NMR spectrum (Fig. 57) also
Fig. 52. IR spectrum of friedelin
Fig. 53. $^1$H NMR spectrum of friedelin
Fig. 54. $^{13}$C NMR spectrum of frieulin
Fig. 56. $^1$H NMR spectrum of lupcol
Fig. 57. $^{13}$C NMR spectrum of lupicol
confirmed the structure of lupeol (Wang et al., 1992). The compound PW2 further confirmed by superimposibility of HPTLC chromatogram of the authentic compound (Fig. 5).

The compounds PW3a – PW3c were eluted with benzene: chloroform (1:1) as sterol mixture. The IR spectrum of the sterol mixture showed the presence of hydroxyl (3431 and 1063 cm\(^{-1}\)) tri-substituted double bond (1638 and 838 cm\(^{-1}\)) and trans di-substituted double bond (970 cm\(^{-1}\); Fig. 58). The tri-substituted double bond peak arises due to \(-\Delta^5\) double bond. The trans di-substituted double bond peak arises due to \(\Delta^{\text{22 (23)}}\) double bond of stigmasterol. In the \(^1\)H NMR spectrum of the sterol mixture, the methyls of the sterols appeared in the region \(\delta 0.68 - 1.25\) methyl doublets appeared at \(\delta 0.84\) and \(0.91\) (\(J \approx 6.4\) Hz) due to secondary methyls in the side chain (Fig. 59). H - 22 and H - 23 of stigmasterol appeared as \(dd\) at \(\delta 5.15\) and \(5.01\), the coupling contacts being 5.4, 8.6, 1.5 and 8.7 Hz respectively (Hung and Yen, 2001). The broad multiplet at \(\delta 5.35\) corresponds to H – 6 of the sterols. The multiplet at \(\delta 3.52\) corresponds to H – 3 of the sterols. The \(^{13}\)C NMR also showed the presence of three sterols. C – 3 being the hydroxyl in all the sterols appeared at \(\delta 71.8\). C – 5 and C - 6 representing the tri-substituted double bond appeared at \(\delta 140.7\) and 121.7 (Fig. 60). The side chain trans di-substituted double bond of stigmasterol gave C -22 and C -23 at \(\delta 129.2\) and 138.3, C -18 and C -19 appeared at \(\delta 21.0\) and 23.0 and C-2 appeared at \(\delta 19.8\). The C – 29 methyl groups appeared at \(\delta 11.9\).

The compound PW4 eluted with ethyl acetate (100%) yielded a white amorphous powder (m.p. 250°C). The IR spectrum showed peaks for phenol (3364 and 3288 cm\(^{-1}\)), aromatic carboxylic acid (1713 cm\(^{-1}\)) and an aromatic peaks (1617, 1541, 1470, 1339, 1245, 1202, 1054, 1027, 866, 790, 763, 731 and 701 cm\(^{-1}\); Fig. 61).
Fig. 58. IR spectrum of sterol mixture
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**Remark:**

- The table shows the frequency (FREQ) and position settings (POSITION) along with the corresponding PPM values.
- The bar graph indicates the level of each setting, with higher bars representing higher settings.
- The INT (%) column indicates the percent of the total PPM value.
Fig. 59. $^1$H NMR spectrum of sterol mixture
Fig. 60. $^{13}$C NMR spectrum of sterol mixture
Fig. 61. IR spectrum of gallic acid
\(^1\)H NMR spectrum showed only a singlet at \(\delta\) 6.92 corresponding to the two equivalent aromatic protons of gallic acid (Fig. 62). The \(^{13}\)C NMR also confirmed the compound to be gallic acid. The carboxylic acid carbon appeared at \(\delta\) 167.63 (Fig. 63). The Para carbon to the carboxylic acid appeared at 138.13. The two adjacent carbons being equivalent to appeared at \(\delta\) 145.54. The two carbon atoms, unsubstituted and equivalent appeared at \(\delta\) 108.88.

The compound PW5 eluted with ethyl acetate: methanol (4:1) gave a colourless crystalline solid on crystallization from acetone (m.p. 360°C). It gave a positive ferric reaction on adding alcoholic ferric chloride by producing bluish green colour. The IR spectrum showed peaks for hydroxyl (3143 cm\(^{-1}\); Fig. 64), \(\alpha-\beta\)-unsaturated-\(\delta\)-lactone (1720 cm\(^{-1}\)) and aromatic systems (1610, 1509, 1457 and 806 cm\(^{-1}\)). The compound was identified as ellagic acid in comparison with authentic sample (m.p., m.m.p. and superimposable IR.

The compound PW6 eluted with ethyl acetate: methanol (9:1) gave acetate crystals (m.p. 140 – 141°C) on several recrystallizations from methanol extract yielded pale yellow prisms, and confirmed by TLC using an ethyl acetate: methanol: water (88: 12: 5) as the mobile phase (Rf = 0.7). Mass spectrum showed molecular ion peak of \(m/z\) 346.28 having molecular formula C\(_{14}\)H\(_{18}\)O\(_{10}\). The UV spectrum showed maxima at 275 and 310 nm (Fig. 65). The IR spectrum showed peaks for hydroxyl at 3391 cm\(^{-1}\), coumarin lactone carboxyl group at 2949, 2895 and 1701 cm\(^{-1}\) and an aromatic system at 1612, 1528, 1464 cm\(^{-1}\) (Fig. 66). All the above spectral data confirmed compound PW6 to be bergenin. The structure of the compound was further confirmed by X-ray crystallographic data (Tables 34 & 35) and spectrum (Fig. 67).
Fig. 62. 1H NMR spectrum of gallic acid
EXMOD SGCOM
DFREQ  100.40
OBNUC 13C
SCANS  1024
ACQTM  0.655
PD     1.689
PW1    4.0
RESOL  1.53
TEMP.  23.0 c
SPEED  15
SLVNT DMSO
YG     3.46
YG2    3.4594
RGAIN  27
XE     22000.0000
XS     1100.0000
Hz/cm  .
VALUE   1100.0000
DFILE DU1: [100.130
INSTRUMENT: JEOL
MODEL: GSX 400
Fig. 65. UV-VIS spectrum of bergenin
Table 34. Atomic Coordinates x10^4) and Equivalent Isotropic Displacement Parameters (Å^2 x 10^3) for newlxl2m. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor

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Table 35. Hydrogen Coordinates (x 10^4) and Isotropic Displacement Parameters (A^2 x 10^3) for newlxl2m

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Fig. 67. Perspective view of the molecular structure of bergenin monohydrate
Fig. 68. Compounds isolated from hexane and chloroform extracts of *Phyllanthus wightianus*
Fig. 69. HPLC identification of compounds from methanol extract of *Phyllanthus wightianus*
1. Gallic acid (PW4)  
2. Ellagic acid (PW5)  
3. Bergenin (PW6)  

Fig. 70. Compounds isolated from methanol extract of *Phyllanthus wightianus*
5.2 ANTIMICROBIAL STUDIES

The screening of plant extracts and plant products for antimicrobial activity has shown that the higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003). There are many published reports on the effectiveness of traditional herbs against gram-positive and gram-negative microorganisms, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases (Evans et al., 2002).

In the present study, the various test extracts of *P. wightianus* such as hexane, chloroform and methanol were evaluated for their antibacterial, antifungal and antidermatophytic properties.

The presence of antibacterial substance in the higher plants is well established (Srinivasan et al., 2001). Several workers have reported that gram-positive bacteria are more susceptible towards plant extracts compared to gram negative bacteria (McCutcheon et al., 1992; Lin et al., 1999; Rios and Recio, 2005; Parekh and Chanda, 2006, 2007). In the present study, the test extracts of *P. wightianus* were active against both gram-positive and gram-negative bacteria. The potency was higher in gram-negative bacteria such as *Escherichia coli* (33 mm, 30 mm, 13 mm, 10 mm), *Pseudomonas aureoginosa* (31 mm, 26 mm, 25 mm, 23 mm), *Proteus mirabilis* (29 mm, 25 mm, 15 mm, 13 mm), *Proteus vulgaris* (30 mm, 25 mm, 15 mm, 13 mm), *Salmonella paratyphi* (31 mm, 26 mm, 24 mm, 20 mm), *Salmonella typhi* (31 mm, 26 mm, 24 mm, 20 mm), *Vibrio cholerae* (30 mm, 27 mm, 15 mm, 13 mm) and *Vibrio parahaemolyticus* (27 mm, 24 mm, 23 mm, 9 mm) for methanol extract than that of gram-positive bacteria such as *Bacillus cereus* (28 mm, 16 mm, 0, 0), *Bacillus subtilis* (28 mm, 25 mm, 13 mm, 10 mm), *Staphylococcus aureus* (17 mm, 16 mm, 15 mm, 13 mm), *Staphylococcus epidermidis* (20 mm, 17 mm, 13 mm, 10 mm), *Aeromonas*
hydrophila (25 mm, 20 mm, 13 mm, 0), Enterobacter aerogenes (20 mm, 19 mm, 16 mm, 15 mm) at 100, 50, 25, 12.5 mg/ml respectively (Tables 9 & 10; Figs. 24 - 26).

According to McCutcheon et al. (1992), the activity against both gram-positive and gram-negative bacteria indicates the presence of broad spectrum of antibiotic compounds or simply general metabolic toxins. Action of the test extracts against almost all the gram-positive and gram-negative bacteria also corroborate the same. Further, the test extracts were highly potent against fungal strains that strongly support the broad spectrum of the test extracts (Tables 11 & 12; Figs. 27 - 29).

Verpoorte and Dihal (1987) reported the antimicrobial activity of P. amarus and P. urinaria. Ethanol extract of P. amarus showed activity of <15 mm at 50 mg/ml concentration against B. subtilis and Staph. aureus. P. urinaria was active only against Staph. aureus and produced <15 mm activity at 50 mg/ml. In the present study, methanol extract at 50 mg/ml exhibited 16 mm inhibition against Staph. aureus, and chloroform and hexane extracts produced 15 and 22 mm inhibition, respectively. In the case of B. subtilis, methanol extract produced 25 mm inhibition and hexane extract exhibited 10 mm and the chloroform extract was inactive at 50 mg/ml. Comparisons reveal that action against Staph. aureus is more or less (16 mm) similar as that of P. amarus (<15 mm) whereas against B. subtilis, the activity is superior (25 mm) that of P. amarus (<15 mm). Variation in activity may be due to the presence or absence of different types of active constituents in them. P. amarus and P. discoideus did not exhibit any kind of activity against E. coli, Ps. aeruginosa, A. niger and C. albicans. In turn, all the test extracts in the present study were active against them. The results proved its high potency than that of P. amarus, P. urinaria and P. discoideus.
Olukoya et al. (1993) reported potent activity of ethanol extract of *P. discoideus* against *Staph. aureus, E. coli* and moderate activity against *Streptococcus* group D strains.

In the present study, methanol extract of *P. wightianus* exhibited potent inhibition against most of the bacterial strains which was more than that of the standard drugs (given in parentheses), such as 31 mm for *P. aeruginosa* (25 mm), 33 mm for *E. coli* (30 mm), 30 mm for *Pr. vulgaris* (20 mm), 25 mm for *Aeromonas hydrophila* (20 mm), 22 mm for *S. typhi* (20 mm), 23 mm for *V. vulnificus* (16 mm) and 27 mm for *V. parahaemolyticus* (14 mm). In the case of fungal strains, it was more potent such as 40 mm against *M. gypseum* (30 mm), 32 mm against *T. metagrophytes* (30 mm), 30 mm against *A. niger* (28 mm) and 30 mm against *C. albicans* (24 mm; Table 11). However, the MIC and MBC of the methanol extract for most of the bacterial strains and MFC of the fungal strains were recorded as 10 mg/ml and 20 mg/ml, respectively (Tables 10 & 12). This is followed by hexane extract exhibiting maximum activity and chloroform extract expressed moderate activity against almost all the bacterial and fungal strains.

Among the fungal strains, the test extracts were most active against dermatophytes which cause skin infections. Antidermatophytic activity has been reported by Agrawal et al. (2004) for chloroform fraction of *P. amarus* against *M. gypseum* and Ahmad et al. (1998) and Rani and Khullar (2004) for alcohol and aqueous extracts of *Emblica officinalis*.

Antimicrobial properties of the secondary metabolites in plants have been reported by several researchers such as tannins by Chung et al. (1988), Machado et al. (2003), Singh et al. (2005), Lim et al. (2006) and Chattopadhyay et al. (2007), phenols by Jurd et al. (1971), Alberto et al. (2006) and Rao et al. (2006), flavonoids
by Pepeljnjak et al. (2005) and Singh et al. (2006), coumarins by Cowan et al. (1999), triterpenoids by Rojas et al. (1992), saponins by Tyler (1993), Wang et al. (1998), Turker and Camper (2002) and Wallace (2004) and catechins by Toda et al. (1989), Alberto et al. (2004) and Bendini et al. (2006). In the present study, the preliminary phytochemical screening of the *P. wightianus* revealed the presence of glycosides, steroids, triterpenes, flavones and phenols in the hexane, chloroform and methanol extracts and catechins, coumarins, sugars, saponins and tannins in the methanol extract alone. Furthermore, the phytochemical evaluation (Section 4.1.8) also revealed the presence of lupeol, friedelin, β-sitosterol, campesterol and stigmasterol in the hexane and chloroform extracts and bergenin, gallic acid, ellagic acid, corillagin and geranin in the methanol extract.

Goyal and Rani (1989) reported *in vitro* antimicrobial activity of lupeol against both gram-positive bacteria such as *S. albus*, *S. aureus* and *B. subtilis* and gram-negative bacteria such as *E. coli*, *K. pneumoniae*, *Shigella dysenteriae*, *P. vulgaris* and *P. pyocyanea*. β-sitosterol has been shown to have a variety of antibacterial activity against *Staphylococcus*, *Streptococcus* and *E. coli* antiviral and antifungal activities ([www.enerex.ca](http://www.enerex.ca)) and against *E. coli* by Singh and Singh (2003).

Prithiviraj et al. (1997) reported antifungal activity of bergenin against some plant pathogenic fungi and proved its monosodium salt was effective against the plant pathogenic fungi. In the present study also, the test extracts, especially the methanol extract had a potent activity against all the tested human pathogenic fungi. It may be due to the presence of bergenin in it.

Tannins serve as a natural defense mechanism against microbial infections. They inhibited the growth of many bacteria, fungi, yeasts and viruses (Chattopadhyay et al., 2007). They have been traditionally in use for protection of inflamed surfaces
of the mouth and treatment of catarrh, wounds, haemorrhoids and diarrhea and as an
antidote in heavy metal poisoning (Ogunleye and Ibitoye, 2003). Medicinal plants
containing phenolic compounds including tannins as major constituents are used
topically for care and repair of skin wounds (Dweck, 2002). Their mode of
antimicrobial action may be related to their ability to inactive microbial adhesions,
enzymes, cell envelope transport of proteins, etc. (Cowan, 1999). They are complex
with polysaccharide (Ya et al., 1988) and can also directly inactivate microorganisms.
Condensed tannins have been determined to bind cell walls of ruminal bacteria,
preventing growth and protease activity (Jones et al., 1994). A high concentration of
tannins coagulates the bacterial cell wall protein resulting in bactericidal activity
while in low concentration it is bacteriostatic (Robinson, 1975; Trease and Evans,
1996). According to Scalbert (1991), tannins can be toxic to filamentous fungi, yeast
and bacteria. Thus, in the present study, the high content of tannins in the methanol
extract may be responsible for its better antimicrobial action.

Ndukwe and Zhao (2007) reported antibacterial activity of 2, 3, 8 – tri – O-
methyl ellagic acid against S. pneumoniae (19 mm), V. cholerae (24 mm), Staph.
aureus (25 mm), K. pneumoniae (20 mm), Ps. aeruginosa (20 mm), B. cereus (21
mm), E. coli (25 mm) and S. typhi (22 mm). In the case of present study, methanol
extract (containing ellagic acid) showed more activity such as 28 mm against B.
cereus, 17 mm against Staph. aureus, 33 mm against E. coli, 18 mm against K.
pneumoniae, 31 mm each against Ps. aeruginosa and S. typhi and 30 mm against V.
cholerae at 100 mg/ml. The results also corroborate these findings.

Reddy et al. (2007) reported potent antibacterial activity of ellagic acid and
gallic acid against E. coli and Ps. aeruginosa. However, these compounds were
inactive against fungi such as A. fumigatus and C. albicans at the tested

The phenolic compounds are generally toxic to microorganisms due to the enzymatic inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman, 1987). Polyphenols are known to form with proteins soluble complexes of high molecular weight. Thus after being adsorbed, the polyphenols will react with the protein moiety of cell enzymes (oxidoreductases) in the cytoplasm and cell wall. They may also bind to bacterial adhesions and so, interfering with the availability of receptors on the cell surface (Machado et al., 2003). Flavones, flavonoids and catechins have the ability to complex with extra-cellular and soluble proteins and also with bacterial cell walls, thus disrupt the microbes. Some of them disrupt and damage microbial membranes (Tsuchiya et al., 1996). Generally, flavonoids are phenolic in nature (disinfectant) as they cause cell membrane damage, thus release cell contents and cause lysis, mostly bactericidal. Probable targets in the microbial cell for the coumarins are surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes (Cowan, 1999). Antibacterial (Fernandez et al., 1996) especially gram-positive and antifungal (Hoult et al., 1996) nature of coumarins were well-studied.

Saponins also are surface agents which lead to the lysis of cell surface (Robinson, 1975; Trease and Evans, 1996) and are high in antimicrobial action than flavonoids and tannins. Wang et al. (1998) and Wallace (2004) have reported antimicrobial activity of saponins against bacteria and fungi. Saponins are also effective in removing protozoa from the rumen (Wallace, 2004). Numerous studies have demonstrated that saponins and saponin – containing plants have toxic effects on
protozoa (Navas-Camacho et al., 1993; Diaz et al., 1994; Newbold et al., 1997; Odenyo et al., 1997). So, it can be possibly implied for the role of saponins present in the methanol extract against the antimicrobial activity.

According to Irobi et al. (1994) and Tereschuk et al. (1997), plant extract with antioxidant activity frequently display antimicrobial activity. In the present study, the test extracts of *P. wightianus* exhibited potent *in vitro* antioxidant activity. In DPPH assay at 100 μg/ml methanol extract exhibited 48.60%, followed by hexane extract 41.05% and chloroform extract exhibited 25.54% scavenging activity. In nitric oxide radical scavenging assay, methanol extract exhibited maximum % of inhibition (70.09%), followed by hexane extract (54.59%). Chloroform extract was inactive to scavenge the nitric oxide radical generation at 100 μg/ml. This may also be responsible for the observed antimicrobial activity of the respective test extracts.

In the present study, the polarity of the solvent seems to be played an important role in exhibiting potential antibacterial activity. Methanol extract of *P. wightianus* showed remarkable activity against most of the bacterial and fungal strains. This view has been supported by Rabe and van Staden (1997), Grierson and Afolayan (1999) and Parekh and Chanda (2006, 2007).

It was observed from the present study that the methanol extract was potent against almost all the bacterial and fungal strains tested, however, it was more active against bacterial strains such as *Ps. aeruginosa*, *E. coli*, *S. paratyphi*, *V. cholerae*, *Pr. mirabilis*, *Pr. vulgaris*, *B. cereus* and *B. subtilis*. Among the fungal strains, it was potent against *M. gypseum*, *T. mentagrophytes*, *C. albicans* and *Aspergillus* species. These agents commonly cause skin infections, especially *Staph. aureus* and *Ps. aeruginosa* are predominant organisms in both leg ulcers and superficial wounds and showed increased resistance to commonly used antibiotics (Valencia et al., 2004).
The potential of *P. wightianus* extract against the standard strains of *Staph. aureus* and *Ps. aeruginosa* may be explored in order to develop a topical antimicrobial therapy to promote skin wound healing. In addition, the anti-pseudomonal and anti-staphylococcal activities of the plant extracts are considered important because the test extract has expressed activity against in nosocomial infections. Further, the anti-dermatophytic and anti-candidal roles of the test extracts strongly support the efficacy of the test extracts against the agents of both bacterial and fungal organisms causing skin diseases.

The group of fungi causing skin, hair and nail infections are collectively called dermatophytes. The superficial mycotic infection is the series of infection of the skin caused by dermatophytic organisms and its related fungi. They invade the keratinized portion of the skin, hair and nail and use keratin as nitrogen source (Joklik et al., 1992). Dermatophytosis is one of the most prevalent infections in the world. Though they are extremely annoying and millions of dollars are spent annually in their treatment with few exceptions they are not debilitating or life threatening. The genus *Epidermophyton* infects skin and nail only, the genus *Trichophyton* infects skin, nail and hair and the genus *Microsporum* infects skin and hair (Jagdish Chander, 1996). The clinical forms of dermatophytosis were erroneously termed tinea or ring worm, depending on their anatomical site involved. For example, Tinea corporis affects body and Tinea pedis affects foot called athlet's foot (Chakrabarti, 2001.) For all dermatophytes, the first step in infection is colonization of the horny layer of tissue, and then the fungus spreads in a centrifugal pattern forming the ring that gives the infection to the common name “Ring Worm” (Fisher and Cook, 1998).

*Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Microsporum canis* were frequently isolated from the arms and legs of the clinical form Tinea corporis
whereas *Trichophyton mentagrophytes* and *Trichophyton rubrum* are also responsible for the clinical form Tinea pedis, which affect feet. *Epidermophyton floccosum* mainly produces the clinical form Tinea cruris (Forbes *et al.*, 1998).

In the present study, it was clearly observed that the test extracts have potential activity against the all the tested dermatophytes which strongly support the use of test extracts against skin infection causing agents.

The causative agents of urinary tract infections are *P.s. aeruginosa, E. coli, Pr. mirabilis, Pr. vulgaris* and *K. pneumoniae*. The potent inhibition of the test extracts against them is an important finding in the present study expressing the possibility to use *P. wightianus* for developing drugs against urinary tract infections. *Ps. aeruginosa* is the commonest and most serious cause of infection in burns (Ananthanarayan and Jayaram Paniker, 1996). *E. coli* is also responsible for pyogenic infections apart from its role in urinary tract infections and diarrhea. *Vibrio vulnificus* is responsible for wound infection and septicemia. In the present study, a strong and potent inhibition recorded against *P.s. aeruginosa* (31 mm), *E. coli* (33 mm), *V. vulnificus* (23 mm) at 100 mg/ml for methanol extract, organisms reveal the potentiality of the test extracts against the respective skin infection causing agents. Moreover, the extracts exhibited activity against the enteric agents such as *E. coli* - 30 mm (hexane), 21 mm (chloroform) and 33 mm (methanol), *S. typhi* - 16 mm (hexane) and 22 mm (methanol), *S. paratyphi* - 17 mm (hexane) and 31 mm (methanol), *V. cholerae* - 24 mm (hexane) and 30 mm (methanol) and *V. parahaemolyticus* - 27 mm (methanol) at 100 mg/ml (Table 9). Thus, the results in the present study validate the ethnotherapeutic claim of the plant as an antidiarrheal agent.
5.3 PHARMACOLOGY

5.3.1 Analgesic Activity

Analgesia is a state of reduced awareness to pain and analgesics are substances which decrease pain sensation by increasing threshold to painful stimuli.

There are two types of analgesic drugs such as narcotic and non-narcotic agents. Narcotic analgesic drugs act through their interaction with opioid receptors and at spinal level, they inhibit the transmission nociceptive impulses through the dorsal horn and suppress nociceptive spinal reflexes. The non-narcotic analgesic agents (NSAIDs-Non steroidal anti-inflammatory drugs) act through the inhibition of arachidonate cyclooxygenase, which in turn lead to the decreased production of prostaglandins, sensitize nociceptive nerve endings to inflammatory mediators such as bradykinin and 5 hydroxy tryptamine. In order to distinguish between the central and peripheral analgesic action of the test drugs both hot plate method (Thermal stimulation by radiant heat) as well as acetic acid-induced (chemical stimulation) writhing reflex were carried out. The acetic acid- induced writhing is a non-specific model, i.e., it will not distinguish between opioid and non-opioid type activities while pain induced by thermal methods indicates narcotic involvement (Turner, 1965; Besra et al., 1996). Acetic acid-induced writhing assay is used for detecting both central and peripheral analgesics whereas the hot plate and other thermal methods are more sensitive in centrally acting analgesics (Dewey et al., 1970; Fukawa et al., 1980).

Acetic acid causes inflammatory pain by inducing capillary permeability (Amico-Roxas et al., 1984) and creates increase in peritoneal fluids of prostaglandin E$_2$ (PGE$_2$) and prostaglandin F$_{2a}$ (PGF$_{2a}$) (Deraedlt et al., 1980; Bentley et al., 1983). Several mediators such as kinin substance (Chapman and Dickenson, 1992; Correa et al., 1996; De Campos et al., 1996), acetylcholine and prostaglandins (Chapman and
Dickenson, 1992) also take part in the visceral pain model nociception (Vogel and Vogel, 1997) and transmission of nociception from the viscera (Cervero and Laird, 1999).

The hot plate method in the present study, hexane (4.30±0.63 at 60 min and 4.43 ±0.77 at 120 min; 4.30±0.95 at 60 min and 4.26±0.48 at 120 min), chloroform (3.90±0.87 at 60 min and 4.28 ±0.76 at 120 min; 4.26±1.60 at 60 min and 4.36±0.76 at 120 min) and methanol (4.23±1.30 at 60 min and 4.28±0.79 at 120 min; 4.28±0.95 at 60 min and 4.36±0.87 at 120 min) extracts of *P. wightianus* failed to increase the latency at the dose levels of 100 and 200 mg/kg/bw respectively when compared to the standard drug morphine (12.1±0.5 at 60 min and 18.3± 0.63 at 120 min; P<0.001) at dose level 5 mg/kg s.c., respectively (Table 13; Fig. 30). Almost all the species of *Phyllanthus* have shown very similar results so far (Calixto *et al.*, 1998) and have been virtually inactive against tail flick and hot plate tests (Gorski *et al.*, 1993; Santos *et al.*, 1994, 1995 a, b, 1999; Perianayagam *et al.*, 2004).

The extracts of several species of *Phyllanthus*, including *P. corcovadensis* (Gorski *et al.*, 1993), methanolic extract of callus culture of some species of *Phyllanthus* (Santos *et al.*, 1994), *P. niruri, P. sellowianus, P. tenellus* and *P. urinaria* (Santos *et al.*, 1995 c) and *P. carolinensis* (Filho *et al.*, 1996), *P. amarus, P. fraternus, P. orbiculatus* and *P. stipulatus* (Santos *et al.*, 2000) were all effective in preventing the pain response induced by acetic acid, formalin and capsaicin-induced neurogenic pain in mice. The results in the present study also support the same.

In acetic acid writhing test, all the extracts of *P. wightianus* exhibited significant and dose-dependent reduction in writhings (Table 14; Fig. 31). All the test drugs showed superior activity at the dose level of 200 than 100 mg/kg/b.w. Methanol extract recorded maximum % protection of analgesia such as 59.11% and 52.40% at
200 and 100 mg/kg/b.w. respectively. This is followed by hexane extract such as 45.59% and 37.35% and chloroform such as 43.59% and 35.18% at similar doses. The analgesic effect produced by these extracts may be peripheral in nature and may not involve any central analgesic action. The findings of Gorski et al. (1993) and Santos et al. (1995 a, b, 1999) also support this observation. The effect could be due to the inhibition of capillary permeability (Amico – Roxas et al., 1984) by decreasing prostaglandin E₂ (PGE₂) and prostaglandin F₂α (PGF₂α) (Deraedt et al., 1980; Bentley et al., 1983) in peritoneal fluids and inhibition of histamine. Many classes of naturally occurring secondary metabolites have been isolated and characterized in *Phyllanthus* species such as steroids, flavonoids, alkaloids, terpenes, lignans, tannins, and phenols (Ueno et al., 1988; Bachmann et al., 1993; Miguel et al., 1995 a, b, 1996; Calixto et al., 1998). Some of them include β-sitosterol, stigmasterol, geraniin, furosin and quercetin, which produce significant and dose-related antinociception when assessed in several chemical models of nociception in mice (Miguel et al., 1995 a, b, 1996; Santos et al., 1995 c; Filho et al., 1996). Further, the presence of ellagic acid, gallic acid and rutin in this genus could also be responsible for antinociceptive properties (Ihantola – Vormisto et al., 1997). Gorski et al. (1993) and Santos et al. (1995 b) have reported antinociceptive property of the hydro-alcoholic extract of *P. carolinensis* and other species of *Phyllanthus* and postulated that antinociception could be contributed by phytosterols, quercetin, gallic acid ethyl ester, geraniin and the flavonoid mixture.

Ellagitannin and geraniin have been reported in several species of *Phyllanthus* such as *P. urinaria* (Okuda et al., 1980), *P. niruri* (Ueno et al., 1988), *P. amarus* (Foo, 1993 a, b) and *P. sellowianus* (Miguel et al., 1995 a). Ellagitannin is known for its analgesic effect (Miguel et al., 1996). In addition, it has the capacity to reduce
systemic blood pressure by inhibiting nor-adrenaline release (Cheng et al., 1994). Geraniin also inhibits the formation of 5 – lipooxygenase and cyclooxygenase products derived from arachidonic acid path way in rat peritoneal polymorpho nuclear leukocytes (Kimura et al., 1986).

Miguel et al. (1996) reported the antinociceptive effect of geraniin isolated from P. sellowianus. Geraniin expressed approximately eight times more active than acetaminophen and aspirin when tested against abdominal constriction induced by acetic acid. Perianayagam et al. (2004) reported antipyretic and analgesic activities and the presence of alkaloids, tannins, phenolic compounds, carbohydrates and amino acids in Emblica officinalis. This observation provides support to the presence of active constituents and their analgesic effect.

The results in the present study strongly provide the basis to P. wightianus to the presence of different classes of constituents such as β-sitosterol, campesterol and stigmasterol under the group of sterols in the hexane and chloroform extracts and tannins such as ellagic acid, gallic acid and geraniin in the methanol extract and their synergistic antinociceptive action. However, the plant may contain many other constituents that would have contributed analgesic activity.

### 5.3.2 Anti-inflammatory Activity

The test extracts produced significant inhibition of carrageenan-induced rat paw inflammation, the test that has significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Olajide et al., 1999).

Carrageenan induces an inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 24 h after injection of carrageenan, has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability (Vinegar et al., 1987). The oedema maintained during the plateau phase
is presumed to be due to kinin-like substances (van Arman et al., 1965; Di Rosa and Sorrentino, 1968). Inflammation volume reaches its maximum approximately 3 h post-treatment after which it begins to decline (Garcia et al., 2004). The late phase, which is also a complement-dependent reaction, has been shown to be due to over production of prostaglandins in tissues (Di Rosa et al., 1971; Malpure et al., 2006).

In the present study at 100 and 200 mg/kg/p.o., methanol extract showed maximum protection such as 54.68 and 60.93%, followed by hexane extract such as 43.75 and 50.00% and by chloroform extract such as 35.93 and 42.18%, respectively.

Anti-inflammatory activities of various species of Phyllanthus have been studied by several researchers following different models such as inhibitory activity of PMNs and platelets by the leaves of P. emblica (Ihantola-Vormisto et al., 1997), COX-1 and COX-2 assays by P. amarus (Chukwujekwu et al., 2005), carrageenan-induced paw oedema and neutrophils influx model in P. amarus (Kassuya et al., 2005), carrageenan-induced paw oedema in P. amarus (Mahat and Patil, 2007), in P. debilis (Chandrasekhar et al., 2005) and in P. singampattiyana (Maridass et al., 2005) with significant protection. The present findings also corroborate the same.

In the present study, the inhibition of the oedema by the test extracts may be acting on late phases (Table 15) and suggested that anti-inflammatory activity of the test extracts may be mediated by inhibiting the over production of prostaglandins, i.e. PGE2 and nitric oxide.

Most currently used anti-inflammatory agents inhibit cyclooxygenase and therefore synthesize prostaglandins. Free radical scavenging agents also play a role in the treatment of inflammation because reactive oxygen radicals produced by neutrophils and macrophages are implicated in tissue damage during the inflammatory process in some conditions.
Traditional medicinal preparations are in wide use for inflammatory disease (Crellin and Philpott, 1990; Bohlin, 1995; Miller and Murray, 1998). Eight out of 16 species used for anti-inflammatory or anti-histamine in nature belong to the subgenus Phyllanthus (Unander et al., 1995). Methanol extract of Phyllanthus amarus significantly inhibited acute, sub-acute and chronic pain produced in different models of inflammatory pain (Mahat and Patil, 2007). In vitro and in vivo studies have reported inhibition of induction of cytokines, NOS and COX – 2 (Kiemer et al., 2003). COX-2 is responsible for the production of the prostanoid mediators of inflammation (Vane and Botting, 1996). Most NSAIDs in current use are inhibitors of both iso enzymes (COX-1, COX-2) even though they vary in their degree of inhibition (Griswold and Adams, 1996).

During the inflammatory phase from the macrophages, the reactive free radical nitric oxide is synthesized by inducible NO syntheses iNOS (Rao et al., 2006). Excessive production of NO plays a pathogenic role in both acute and chronic inflammation (Clancy et al., 1998). NO is responsible for the vasodilatation, increase in vascular permeability, oedema formation and synthesis of prostaglandins at the site of inflammation (Moncada et al., 1991; Grisham, 1999). Selvemini et al. (1996) reported the role of NO in relation to carrageenin-induced paw oedema. Manipulation of NO free radical can be a potential and promising therapeutic area to treat inflammations. Approaches being currently used for inflammatory disorders include NO scavengers as well as NO inhibition (Mittal et al., 2003). In the present study, the test extracts of P. wightianus exhibited significant in vitro – NO free radical scavenging activity such as hexane extract 54.59%, chloroform extract – inactive and methanol extract 70.09% at 100 µg/ml respectively (Table 16). NO free radical scavenging activity of the test extracts might be in part attributed to the observation of
anti-inflammatory effect. A large number of plant-derived compounds are principally phenolics and terpenes which have anti-inflammatory effects (Polya et al., 2003a) wherein a number of them are variously used as antioxidants and analgesics (Harborne and Baxter, 1993).

Swarnalakshmi et al. (1984) reported the anti-inflammatory activity of bergenin against carrageenin-induced rat paw oedema. Its potency is dose-dependent and maximum at higher doses. Li et al. (2004) reported racemosic acid from the bioassay-guided fraction of the ethanol extract of Ficus racemosa and its potential inhibitory activity against COX-1 and 5-COX in vitro. In addition, they isolated bergenin along with racemosic acid from the active fractions. Lupeol has been shown to possess anti-arthritic activity (Chronic inflammatory disorder) by its possible suppression of the T-lymphocyte (Bani et al., 2006). Hasmeda et al. (1999) reported the inhibition of protein kinase in the anti-inflammatory effects of triterpenes such as lupeol and other ones.

In the present study, in chronic tests using adjuvant-induced polyarthritis model, the test extracts and bergenin demonstrated superior activity compared to the acute test model. Calixto et al. (1998) reported anti-inflammatory activity of β-sitosterol isolated from Phyllanthus flexuosus. As lipooxygenase inhibitors possess significant anti-inflammatory effect (Singh and Majumdar, 1997). Geraniin may be in part attributed to the anti-inflammatory effect of the methanol extract. Therefore, it has the potential to inhibit both the cyclooxygenase and lipooxygenase pathways of arachidonate metabolism (dual inhibition property).

The presence of lupeol and sterols (β-sitosterol, campesterol and stigmasterol) in the hexane and chloroform extracts and bergenin and geraniin in the methanol extract may be either individually or synergistically responsible for the anti-
inflammatory activity. The results seem to support the traditional use of this plant in relieving inflammation.

5.3.3. In vitro Antioxidant Activity

Many antioxidant compounds, naturally occurring in plant sources, have been identified as a free radical or active oxygen scavengers (Zheng and Wang, 2001). These plant products exert antioxidative effects by quenching various free radicals and the singlet form of molecular oxygen. Higher levels of the antioxidant enzymes have been correlated with decreased susceptibility to cell damage (Kinsella et al., 1993; Khan et al., 1997; Aruoma, 1998; Lai et al., 2001).

Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito et al., 1983). In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers (Kumaran and Karunakaran, 2007).

Effect on DPPH

In the present study, all the extracts had very good action on DPPH free radical and maximum inhibition was exhibited by methanol extract (48.60%), followed by hexane extract (41.05%) and chloroform extract (25.54%) at 100 µg/ml. Vitamin C was used as the reference standard and exhibited maximum inhibition of 72.07%.

DPPH is relatively a stable free radical and the assay determines the ability of the test extract to reduce DPPH radical to the corresponding electrons to paired ones. Antioxidants can act by converting the unpaired electrons to paired ones. The
reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenyl picryl hydrazine. DPPH react stoichiometrically with antioxidants which are good hydrogen donors (Blois, 1958; Gardner et al., 1998; Schwarz et al., 2001). Thus, the inhibition of DPPH radical indicates that the test extracts cause reduction of DPPH radical in a stoichiometric manner. This observation has already been reported by Vani et al. (1997), Sanchez-Moreno et al. (1999) and Sanchez-Moreno (2002). The experimental data reveal that all the extracts of P. wightianus are likely to have the effect of scavenging free radical (Table 16).

The high potent DPPH radical scavenging activity of extracts of P. niruri (IC50 values at 10-30 μg/ml) has been reported by Harish and Shivanandappa (2006). Kumaran and Karunakaran (2007) reported the antioxidant potentials of methanol extract and standard with the DPPH radical effect of some of the Phyllanthus species in the following order: P. debilis (87.24%) > Ascorbic acid (77.75%) > P. urinaria (73.18%) > BHT (72.20%) > P. virgatus (63.21%) > P. maderaspatensis (48.9%) > P. amarus (38.67%) at the dose of 25 μg/ml. In the present study, the methanol extract exhibited better activity of 48.60% at 100 μg/ml.

Effect on Inhibition of Nitric Oxide Radical Generation

In the present study, methanol extract exhibited better NO scavenging activity (70.09%) compared with the standard Vitamin C (77.07%), followed by hexane extract (54.59%). This observation reveals that these two extracts competed with oxygen to react with nitric oxide and thus inhibited the generation of the anions. Chloroform extract was inactive to scavenge the nitric oxide radical generation at 100 μg/ml (Table 16).
A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom (Jayaprakash et al., 2001; Khanam et al., 2004). The reducing property of the test extracts implies that it is capable of donating hydrogen atom. The presence of phenolic compounds in the test extracts may be a contributing factor towards antioxidant activity because the phenolic compounds are known to have direct antioxidant property due to the presence of hydroxyl groups, which can function as hydrogen donor (Duh et al., 1999; Dreosti, 2000).

The preliminary phytochemical analysis reveals the presence of flavonoids in the test extracts and tannins such as gallic acid, ellagic acid, corillagin and geraniin in the methanol extract. Polyphenols particularly flavonoids and tannins are well-known natural antioxidants (Arnason et al., 1981; Duh et al., 1999; Dreosti, 2000). Flavonoids are highly effective scavengers of all types of oxidizing radicals (Halliwell, 1996 a, b; Bors et al., 1997). The concentration of hydrogen peroxide in water may vary according to the phenolic compounds. Since phenolic compounds present in the extract are good electron donors, they may accelerate the conversion of \( \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} \) (Ruch et al., 1984). Nakagawa and Yokozawa (2002) reported that green tea can directly scavenge NO and \( \text{O}_2 \) and that its action is attributable to its phenolic components. Phenolic compounds of plant materials have been shown to neutralize free radicals in various model systems (Zhang et al., 1996). The potent antioxidant activity exhibited by Amala is due to its polyphenols such as ellagic acid, gallic acid, tannin, etc. (Ihantola - Vormisto et al., 1997; Bhattacharya et al., 1999; Santos et al., 1999). Kumaran and Karunakaran (2006 a) reported the potent activity of polyphenols such as geraniin, corillagin, furosin and gallic acid and rutin from *Phyllanthus debilis* against the DPPH radical scavenging antioxidant activity. When up to 1.0 g is daily ingested from a diet rich in fruits and vegetables (Tanaka et al.,
1988 b, c) polyphenolic compounds present in them inhibit mutagenesis and carcinogenesis in humans. The DPPH radical scavenging activity of *Phyllanthus* extracts may be mostly related to the hydroxyl group in phenolics (Kumaran and Karunakaran, 2007). In the present study, the methanol extract of *P. wightianus* showed strong antioxidant activity in both the models of assays such as DPPH (48.60%) and nitric oxide (70.09%) scavenging. It is concluded that the superior activity exhibited by methanol extract might be due to the presence of the phenolic compounds.

It is found out that the antioxidant property of the plants possesses hepatoprotective activity (Gupta *et al.*, 2004; Mondal *et al.*, 2005; Pal *et al.*, 2006) and hypoglycemic property (Garg and Bansal, 2000; McCune and Johns, 2002; Mazumder *et al.*, 2005; Mondal *et al.*, 2006). In the present study, the various test extracts of *P. wightianus* possess antidiabetic (Section 4.3.6) and hepatoprotective (Section 4.3.9) activities. It explains the antioxidant potential of the test extracts for the hepatoprotective and antidiabetic activities.

### 5.3.4 Wound Healing Activity

The term "wound" has been defined as a disruption of normal anatomical structure and more importantly function. Therefore, "healing" is the complex and dynamic process that results in the restoration of anatomical continuity and function (Lazarus *et al.*, 1994). Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct but overlapping phases such as hemostasis, inflammation, proliferation and remodeling (Diegelmann and Evans, 2004). These steps are orchestrated in a controlled manner by a variety of bioactive molecules like growth factors, cytokines, their receptors and matrix molecules (Shukla *et al.*, 1999). Such a controlled phenomenon can be disrupted in diseases like diabetes, immuno-
compromised persons, ischemia, etc., thus leading to the development of a chronic wound. Prolonged or incomplete wound healing is then a troublesome complication (Ingold, 1993).

Apart from that, secondary infections by microbes in the wounds may further aggravate the conditions. Some of the important organisms include \textit{Staph. aureus}, \textit{Str. pyogenes}, \textit{Corynebacterium} species, \textit{E. coli} and \textit{Ps. aeruginosa} wherein the most common are \textit{Staph. aureus} and \(\beta\)-hemolytic \textit{Str.} species (Mertz and Ovingotn, 1993) which are considered “transient flora” of the skin (Bikowski, 1999). \textit{P. aeruginosa} is the predominant organism, which causes air born infection and its frequency of infection is more in burn patients. Infected wounds heal more slowly and have an increased incidence of scarring (Robson, 1997). Mycotic infections are also an important etiology of these infections, most of them are caused by dermatophytes and other related fungi. A wide range of antibiotics are being used at present for healing wounds and for treating wound infections but they are now proved to have adverse effects in the human body. In view of these developments, so much of attention has been paid recently to extracts of biologically active compounds isolated from plant species used in herbal medicinal system (Essawi and Srour, 2000).

The results in the present study suggest that topical application of the test drugs in animals significantly enhanced the rate of wound healing as assessed by the wound contraction (The time of wound closure for the ointments of standard drug and methanol extract was observed as 14±2 days and 16±2 days for hexane and 18±2 days for chloroform extract) and tensile strength (420±10.1 g, 670±12.3 g, 659±13.1 g, 562±12.6 g, 480±11.6 g - for control, standard, methanol, hexane and chloroform extracts respectively).
Treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control group. The increased tensile strength in the test extract ointment-treated groups (659±13.1 g, 562±12.6 g and 480±11.6 g - for methanol, hexane and chloroform extracts respectively) and the standard ointment treated (670±12.3 g) group in the incision model also confirms this observation (Table 17; Figs. 33 & 34).

It is well-accepted that several local growth factors help in the wound healing process. It is possible that the test extracts may have a growth factor like activity or have the ability to stimulate the expression of growth factors like the basic fibroblast growth factor (bFGF). The bFGF has the broadest range of target cells such as endothelial cells, fibroblasts, myoblasts, etc. (Schweigerer, 1988). Wound contraction is mediated by specialized myofibroblasts found in the granulated tissue (Moulin et al., 2000). The increase in tensile strength of treated wounds may be due to increase in collagen concentration and stabilization of the fibers (Udupa, 1994 a; Udupa et al., 1995).

Pro-inflammatory cytokines have been implicated to stimulate the synthesis of platelet activating factors by the recruited monocytes which in turn induce several angiogenic factors and chemokines (Lupia et al., 1996). Moon et al. (1999) reported the pronounced improvement of type - I collagen material invasion by β-sitosterol which acts as an angiogenic factor in wound healing. Angiogenesis is the growth of new vascular capillary channels from pre-existing vessels and is of fundamental importance in a number of physiological processes such as embryonic development, reproduction, wound healing and bone repair (Maheswari et al., 2006).

When wounding occurs it is accompanied within quite a short time by pain, reddening and oedema of the surrounding tissue. These are all classical symptoms of
inflammation and are caused by the release of the eicosanoids, prostaglandins and leukotrienes and of reactive oxygen species (ROS).

The release of other factors such as the cytokines is also important. The release of these substances is caused by neutrophils aggregating at the wound site and producing proteolytic enzymes and ROS as antimicrobial defenses and as aids to the debridement of dead tissue. As the test extracts of *P. wightianus* exhibited potent anti-inflammatory (Section 4.3.3) and antioxidant (Section 4.3.4) activities these properties could have contributed wound healing in part.

Apart from the role of antioxidants in removing products of inflammation, they are also beneficial in wound healing in some other means. Antioxidants counter the excess proteases and ROS often formed by neutrophils accumulation in the wound area and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and skin lipids will be made less flexible. So, antioxidant substances will reduce the possibility of occurrence of these adverse events. Because of these factors, overall antioxidant effects appear to be important in the successful treatment of wounds (Houghton *et al.*, 2005). In the present study, the test extracts were shown to be strongly antioxidant due to the presence of flavonoids and polyphenols.

Open wounds are particularly prone to infection, especially by bacteria and superficial mycotic agents and also provide an entry point for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced with concomitant killing of regenerating cells (Houghton *et al.*, 2005). *Staph. aureus, Str. pyogenes,* and *Ps. aeruginosa* are the most common wound pathogens with $\geq 10^3$ CFU/g tissue which has been classified as infection (Bergstrom *et al.*, 1994).
In vitro antimicrobial studies (Section 4.2) of the present study showed that the test extracts were active against both gram-positive and gram-negative bacteria and Candida albicans. They are especially active against the superficial skin infection causing keratinophilic fungi dermatophytes. The presence of saponins, flavonoids and other phenolics in the test extracts could contribute to wound healing because of their detergent ability to remove grease, dirt and bacteria from tissue and act as antimicrobials (Houghton and Mensah, 1997).

The potent antioxidant and antimicrobial activities exhibited by the test extracts also contribute for the wound healing activity. This observation agrees with the opinion expressed by Singh et al. (2006).

The results in the present study reveal that the test extracts demonstrated polyvalent activity due to their anti-inflammatory, antioxidant and antimicrobial properties to heal wounds.

Among the test extracts, the methanol extract exhibited better activity than other extracts. It may be due to the presence of several active constituents and their synergistic activity. The significant burn wound healing activity (ED\textsubscript{50} =190\textmu g/wound; Kimura et al., 2006) and antiulcer activity (In aspirin-induced gastric ulcers in rats bergenin reduced the ulcer index as 6.3±2.1 at 50 mg/kg when compared to control-18.3±3.3) (Goel and Maiti, 1997). These reports on bergenin also support the present findings.

5.3.5 Antidiabetic Activity

The ethnopharmacological use of herbal remedies for the treatment of diabetes mellitus is an area of study ripe with potential as a starting point in the development of alternative, inexpensive therapies for treating the disease. Several plants are used in folk medicine as antidiabetic agents (Bailey and Day, 1989; Ivorra et al., 1989;
Swanston- Flatt et al., 1990; Alarcon-Aguilar et al., 1998; Bnouham et al., 2006). Despite the availability of different types of oral hypoglycemic agents, there is a growing trend towards using natural products as adjuncts to conventional therapies (WHO, 1980). Species of *Phyllanthus* reported for their potent and promising antidiabetic activity include *P. amarus* (Sridhya and Perival, 1995; Adeneye et al., 2006; Ali et al., 2006), *P. niruri* (Chopra et al., 1956; Perry, 1980), *P. sellowianus* (Hnatyszyn et al., 1997, 2002), *P. urinaria* (Higashino et al., 1992), *P. fraternus* (Hukeri et al., 1988), *P. maderaspatensis* (Prashanth et al., 2001), *P. emblica* (*Emblica officinalis*) (Anila and Vijayalakshmi, 2000; Sabu and Kuttan, 2002; Babu and Stanely Mainzen Prince, 2004; Rao et al., 2005).

The results in the present study showed that the plasma glucose levels in diabetic- treated animals were significantly decreased (36.73 and 42.49% for hexane extract; 32.38 and 41.29% for chloroform extract; 43.50 and 52.00% for methanol extract at 100 and 200 mg/kg respectively; and glibenclamide (10 mg/kg) produced a maximum of 61.80%) in comparison with diabetic control animals (Table 19). The body weight of the treated animals was also restored. A pronounced reduction of cholesterol, triglycerides, alkaline phosphatase, ASAT and ALAT was recorded in the extract-treated animals and glibenclamide-treated animals (Table 20). It is known that glibenclamide stimulates insulin secretion but also reduces glucose levels acting at the liver and other tissues (Luzi and Pozza, 1997). The drug reduces the potassium permeability of β-cells by blocking the ATP sensitive potassium channels, causing depolarization, Ca²⁺ entry and hence insulin secretion (Rang et al., 1999). The results in the present study indicate that the hypoglycemic activity of the test extracts may be due to a stimulating effect on the remnant β-cells, an improvement in insulin action or due to insulin-like effect. This view is in agreement with Hnatyszyn et al. (2002).
Diabetes mellitus has been shown to be associated with atherosclerotic and cardiovascular disease and cholesterol is involved in atherosclerosis (Kannel and McGee, 1979).

Insulin deficiency leads to various metabolic aberrations in the animals such as increased levels of blood glucose, cholesterol, alkaline phosphatase and transaminase and decreased level of protein content (Felig et al., 1970; Begum and Shanmugasundaram, 1978; Shanmugasundaram et al., 1983 a, b). A marked increase in serum TGL (183.6±4.8 mg/dl) and serum cholesterol (181.5±3.20 mg/dl) was observed in diabetic rats in the present study. This is in agreement with the findings of Nikkhila and Kekki (1973) and Dhanabal et al. (2004). Elevation of plasma lipid concentration in diabetic rats is well-documented. In the present study, the test extracts-treated groups of *P. wightianus* reduced cholesterol (99.2±7.4 mg/dl and 81.8±2.2 mg/dl for hexane extract; 102.3±6.3 mg/dl and 85.5±2.5 mg/dl for chloroform extract; 81.4±4.7 mg/dl and 75.5±2.6 mg/dl for methanol extract at 100 and 200 mg/kg respectively) and TGL levels (121.2±2.6 mg/dl and 113.8±2.9 mg/dl for hexane extract; 130.6±6.3 mg/dl and 122.7±3.9 mg/dl for chloroform extract; 110.5±9.3 mg/dl and 108.7±4.7 mg/dl for methanol extract at 100 and 200 mg/kg respectively). Several investigators have demonstrated that near normalization of blood glucose level resulted in the significant reduction of plasma cholesterol, free fatty acids and plasma apoprotein (Anila and Vijayalakshmi, 2000).

It is known that hypercholesterolemia, diabetes mellitus and obesity are closely associated to hypertension and stroke. Adeneye et al. (2006) reported dose-dependent decrease in the fasting plasma glucose and cholesterol and hypolipidemic potential of *P. amarus*. Jahromi et al. (1992) reported hypolipidemic and significant decrease in atherogenic index by bergenin isolated from the leaves of *Flueggea*
microcarpa. Anila and Vijayalakshmi (2000) reported the significant reduction of cholesterol, triglycerides, phospholipids and fatty acids by flavonoids isolated from Emblica officinalis and its inversely related action of hyperglycemia and hyperlipidemia. Thakur and Mandal (1984) reported the anticholesterolemic and antiatherogenic effect of the fruits of Emblica officinalis.

In diabetic animals, the change in the levels of serum enzymes is directly related to changes in the metabolism in which the enzyme is involved (Dhanabal et al., 2004). Several researchers have reported increase in transaminase activity in the liver and serum of diabetic animals which is active in the absence of insulin because of increased availability of amino acids in diabetes. This mechanism is responsible for the increased gluconeogenesis and ketogenesis observed in diabetes (Felig et al., 1970). In the present study, the test extracts significantly decreased ASAT (99.7±1.8 U/L and 94.7±6.38 U/L for hexane extract; 113.5±7.2 U/L and 102.3±6.8 U/L for chloroform extract; 93.9±3.4 U/L and 84.3±7.1 U/L for methanol extract at 100 and 200 mg/kg respectively) and ALAT (59.6±1.6 U/L and 53.7±9.1 U/L for hexane extract; 62.6±1.3 U/L and 61.5±5.0 U/L for chloroform extract; 53.1±1.4 U/L and 48.7±1.6 U/L for methanol extract at 100 and 200 mg/kg respectively) enzyme activities. Hence, the improvements recorded in the levels of ASAT and ALAT are as a consequence of improvement in the carbohydrate, fat and protein metabolism due to the treatment by the test extracts. Restoration of ASAT and ALAT to their normal levels after the treatment also indicates revival of insulin secretion to near normal levels.

Among the parameters of protein metabolism, the present study showed an overall reduction in serum total protein (3.9±1.5 g/dl) in diabetic rats and the test extracts showed an elevation (5.9±0.2 g/dl and 7.0±0.2 g/dl for hexane extract;
5.8±0.4 g/dl and 6.2±0.8 g/dl for chloroform extract; 7.2±0.2 g/dl and 7.7±0.6 g/dl for methanol extract at 100 and 200 mg/kg respectively) of total protein. The increased levels of ALP in diabetic rats (279.2±12.6 U/L) were found to be significantly reversed by the action of the test extracts (202.8±12.4 U/L and 189.2±12.8 U/L for hexane extract; 221.2±12.6 U/L and 201.6±13.3 U/L for chloroform extract; 181.3±12.3 U/L and 171.1±11.6 U/L for methanol extract at 100 and 200 mg/kg respectively) (Table 20).

Treatment of the test extracts in the present study exhibited a significant increase in the body weight of the diabetic animals. This may be due to the improvement in glycemic control.

There are several reports explaining hypoglycemic property of ellagic acid. Shimizu et al. (1989) reported the aldose reductase inhibitory activity of it isolated from *P. niruri*. Sabu and Kuttan (2002) studied the anti diabetic and anti-oxidant activities of *Emblica officinalis*, *Terminalia bellerica* and *Terminalia chebula* and reported the presence of gallic acid or gallic acid-derivatives and ellagic acid responsible for their observed antidiabetic and antioxidant activities. Mankil et al. (2006) also reported ellagic acid as an anti diabetic agent. Their observations support the present study that the test extracts may decrease the effect of inflammatory cytokine release in diabetics which in turn might reduce insulin resistance.

Huang et al. (2005) reported that there is an improved sensitivity of the insulin receptor exhibited by *Punica granatum* flower and attributed responsibility for the presence of gallic acid in it. Steroid containing plants are known to exhibit antidiabetic activity. Oral administration of β-sitosterol dramatically brought down high blood sugar levels. The antihyperglycemic effect of β-sitosterol is believed to be by the increase of insulin level which is attributable to a stimulation of insulin
secretion from pancreatic β-cells. β-sitosterol given to diabetic rats orally improved diamine oxidase (DAO) levels. The results in the present study indicate a possible role of antihyperglycemic use for it in the prevention and treatment of diabetes (www.enerex.ca). It also helps to lower the cholesterol level and is believed to reduce the serum cholesterol by inhibiting the intestinal re-absorption of circulating cholesterol which is secreted in the bile. Human liver microsome studies show that it inhibits cholesterol absorption and people given by it were lowered their cholesterol and triglyceride levels (www.enerex.ca).

A study showed that vegetarians to be protected from fat loading diets by their high intake of β-sitosterol from plants. It is known that certain flavonoids exhibit hypoglycemic activity (Hukeri et al., 1988; Pathak et al., 1991; Geetha et al., 1994; Ahmed et al., 2000; Anila and Vijayalakshmi, 2000).

Other phytochemical constituents such as triterpenoids and glycosides (Mankil et al., 2006), polyphenols (Orhan et al., 2006; Aslan et al., 2007), tannins (Teotia and Singh, 1997) and saponins (Sui et al., 1994; Murakami et al., 1996) have been reported as antidiabetic agents. Ali et al. (2006) reported α-amylase inhibitory property of P. amarus and attributed credit to the presence of three pure pentacyclic triterpenoids such as oleanolic acid, ursolic acid and lupeol. Prashanth et al. (2001) reported in vitro α-amylase inhibition by P. maderaspatensis and explained its therapeutic use to control obesity and diabetes.

Oliver (1980) and Mankil et al. (2006) have enumerated glycosides, alkaloids, flavonoids, terpenoids, phenolics and steroidal compounds as active ingredients in the plants reported for hypoglycemic property.

There is no doubt that herbs may be effective due to their fiber, vitamin and mineral content. Diet is the basic control of diabetic disorders. Addition of natural
fiber in the diet is widely encouraged. Vitamins and minerals are helpful to exacerbate the formation of insulin resistance due to working as co-factors in the signaling of insulin action and/or the glucose metabolism (Polya et al., 2003 b). Plants may provide certain necessary elements like calcium, zinc, magnesium, manganese and copper to the β-cells (Akhtar and Iqbal, 1991). Supplement of minerals such as manganese, selenium and zinc has been used for long time. Due to the presence of such minerals in *P. wightianus*, it could be possible to achieve collective antidiabetic effect. So, it is concluded that the presence of different types of phytochemical constituents in the test extracts is synergistically responsible for the hypoglycemic activity. However, contribution of other unidentified constituents can not be ruled out for the activity. In future, the extracts of *P. wightianus* can be used to find out the antimicrobial activity of the diabetes mellitus - II patients against microbial diseases as people with diabetes are more prone to microbial infections than the normal healthy persons.

### 5.3.5.1 Diabetes Prone to Microbial Infections

Diabetes is associated with increased susceptibility to a number of infections but it manifests only when it is uncontrolled. It is attributable to impaired functioning of the polymorphonuclear leucocytes, defective chemotaxis, phagocytic uptake and probably intracellular killing also. Infections known to be associated with diabetes mellitus include those of the female urinary tract infections, staphylococcal infections, skin sepsis, perinephric abscess, tuberculosis, pulmonary infections, malignant otitis externa due to *Ps. aeruginosa* and a variety of fungal infections such as athlete's foot, ringworm and vaginal infections. The IDDM group has more of congenital anomalies and viral infections prone to ketosis (Abraham and Geevarghese, 1990).
Acute pyelonephritis, asymptomatic bacteriuria (Harding et al., 2002), acute cellulitis and lymphangitis (Lee et al., 2003), *Staph. aureus* bacteremia (Akbar et al., 2000), osteomyelitis, gangrenous infection and anaerobic cellulitis (Lipsky et al., 2005), bacterial pneumonia caused by *Staph. aureus* (Boyko et al., 1989) and gram-negative organisms such as *K. pneumoniae*, *E. coli*, *Enterobacter* species, *Pseudomonas* species and *Acinetobacter* species (Johanson et al., 1979), *H. influenzae* (Levinson and Kaye, 1985), pulmonary tuberculosis (Koziel and Koziel, 1995; Bashar et al., 2001; Perez – Guzman et al., 2001), pulmonary infections caused by the mycotic agents include Mucor mycosis (Bigby et al., 1986), *A. fumigatus*, *A. niger*, *A. flavus* (Bouter et al., 1991), *Cryptococcus neoformans* and *Coccidioides immitis* (Baker et al., 1992). Parasitic infections include *Pneumocystis carinii* (Kovacs et al., 1988) and viral infections such as influenza (Bouter et al., 1991), diabetes and human immunodeficiency virus (Murray and Lumpkin, 1997) and polio virus types 1–3.

### 5.3.6 Antiarthritic Activity

Adjuvant-induced arthritis in rat model leads to a severe inflammatory joint disease primarily affecting synovial membrane of affected joints, with clinical and laboratory features representing a valid model for human rheumatoid arthritis (Pearson, 1956; Pearson and Wood, 1963, 1964; Barbier et al., 1984; Taurog et al., 1988; Billingham et al., 1990). Rheumatoid arthritis, one of the commonest autoimmune diseases, is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for the deformity and disability. Experimental adjuvant-induced arthritis model develops swelling, warmth, erythema and tenderness
in the distal joints and tendons of the animals with a maximum severity between 16
and 21 days (Escandell et al., 2006).

As in the human pathology, the process is mediated by various inflammatory
cells and mediators. Neutrophils produce reactive oxygen species and the activated
macrophages generate reactive oxygen and nitrogen species (Eicosanoids through
cyclooxygenase – 2 and nitric oxide through nitric oxide synthase – 2). Along with
cytokines such as interleukin – 1 β and tumor necrosis factor - α (Park et al., 2004 a,
b), they have destructive effects on the cartilage.

Although non-steroidal anti-inflammatory drugs (NSAIDs), steroidal agents
and immuno-suppressants have been developed and used in the treatment of
rheumatoid arthritis over the past few decades there remains an ideal strategy to
alleviate the symptoms for the longer term because of their side effects including
gastrointestinal disorders, immunodeficiency, humoral disturbances, etc. Therefore,
therapeutic agents that could be used for long-term administration (Agnello et al.,
2002) with lower side effects (Badger and Lee, 1997) are highly desirable.

The present study shows that the adverse physical, biochemical and
radiological changes in arthritic animals were reversed to a considerable extent by
oral administration of the test extracts of P. wightianus and its bergenin. Changes in
body weight in arthritic animals were found to occur in response to the incidence and
severity of arthritis. Less in body weight has also been reported in arthritic rats (Walz
et al., 1971; Besson and Guilbaud, 1988).

Increase in serum aminotransferase is due to liver impairment which is a
feature of adjuvant arthritis (Whitehouse et al., 1974). In the present study also, there
was a significant increase in serum aminotransferase of the arthritic rats (0.99±0.03)
which was brought back to near normalization after treatment with the test extracts
and bergenin (0.73±0.08, 0.75±0.06, 0.69±0.01 and 0.68±0.06 for hexane, chloroform, methanol extracts at 200 mg/kg and bergenin at 50 mg/kg respectively; Table 25). There is a correlation between the development of inflammatory process and the release of lysosomal enzymes into the extra-cellular compartment in arthritic rats (Weissman, 1972). Acid phosphatase also seems to be important index for the examination of the integrity of the lysosomal membrane and is responsible for the tissue damage and necrosis of hepatic tissue (Yasuda et al., 2000). Increased activities of cathepsin-D and acid phosphates (Olsen et al., 1990; Geetha, 1993) have been observed in arthritic rats. Hydrolytic enzymes are released by the rupture of the lysosomal membrane which in turn initiates the synthesis of inflammatory mediators such as thromboxanes, prostaglandins and leucotrienes. This may be attributed towards persistent inflammation. These changes are in agreement with the decreased lysosomal stability in adjuvant-induced arthritis. Drugs capable of stabilizing the lysosomal membrane can reduce inflammation (Agha and Gad, 1995).

Denaturation of proteins as one of the causes of rheumatoid arthritis is well-documented (Singhal and Patterson, 1993). Igdoura et al. (1995) reported the role of cathepsin-D in the intracellular degradation of exogenous and endogenous proteins and its proteolytic activity is increased during various pathogenic processes leading to injury of lysosomes (Kominami et al., 1991). In the present study, the activity of lysosomal enzymes in both serum and body tissues was markedly increased in the adjuvant-induced arthritic rats and significantly reduced after treatment with the test extracts and bergenin (Table 26). The marked decrease in serum and tissue lysosomal enzyme activity in the treated group indicated that the test extracts and bergenin may have an enhancing effect in membrane stabilization. An important
mechanism of anti-inflammatory activity has been found to be the membrane stability modulating effect (Subrata et al., 1994).

Inhibition of paw oedema in adjuvant arthritic rat is a hallmark of anti-inflammatory drug action (Ramprasath et al., 2005). Treatment of adjuvant arthritic rats with bergenin and the test extracts showed decline in inflammation. The oral administration of the test extracts showed a remarkable inhibition on both primary and secondary inflammations of adjuvant-induced arthritis in rats. The inhibition of inflammation by bergenin and methanol extract at 200 mg/kg/p.o. was higher than other test extracts and comparable to indomethacin-treated group (Tables 23 & 24).

It has been accepted that the adjuvant injection may not only cause the rat arthritic inflammation in the injected site as two phases of primary and secondary swellings but also in non-injected hind paw as well as other diarthrodial joints and tail synarthroses (Jiang et al., 1997). The initial reduction of oedema and soft tissue thickening at the depot site is probably due to the effect of adjuvant whereas the late occurring disseminated arthritis and flare in the injected foot are presumably immunological events (Ward and Sidney Cloud, 1966).

The pathogenesis of rheumatoid arthritis is perpetuated by the activity of a complex network of cytokines (Choy and Panayi, 2001). As a consequence of the inflammatory process a large number of cytokines and growth factors with overlapping biological effects are found in the synovium. A marked hyperplasia of synoviocytes and blood vessels in the synovium, and a mononuclear cellular infiltrate consisting of macrophages, T and B cells are found. CD4+ T helper cells (Th) can differentiate into two distinct subsets designated Th1 and Th2 type cells, which are characterized by different cytokine production profiles and effector functions.
Th1 cells produce interleukin-2 (IL-2) and interferon gamma (IFN-γ), support macrophage activation and are involved in delayed type hypersensitivity responses (van der Graaff et al., 1999). Th2 cells, on the other hand, secrete IL-4, IL-5 and IL-13 and provide efficient help for β-cell activation, antibody production and down modulate the production of pro-inflammatory cytokines by macrophages. From animal experiments, it has become clear that balance between Th1 and Th2 cells or their cytokines is important in the induction or prevention of organ specific autoimmune disease (Liblau et al., 1995). Several reports have been published on the detection of Th1 and Th2 cytokines in rheumatoid arthritis (Milterburg et al., 1992; Chen et al., 1993; Quayle et al., 1993; Simon et al., 1994). Biological agents that specifically inhibit the effects of TNF-2 or IL-1 represent a major advancement in the treatment of rheumatoid arthritis (Shanahan et al., 2003).

In the present study, the immunomodulatory activity of the test extracts (Section 4.3.8) has also confirmed the strong inhibition by SRBC-induced DTH reaction. The test extracts suppressed the DTH reaction mainly against the effector phase of DTH without inhibition of SRBC-induced humoral immune response. The suppression of DTH reaction positively correlates the suppression of Th1 inhibition. At the same time, the test extracts also showed a remarkable inhibition on the carrageenin-induced paw edema (Section 4.3.3) Therefore, the inhibition of the test extracts against adjuvant-induced arthritis might include both direct anti-inflammatory and anti-DTH mechanisms. This view is in agreement with Jiang et al. (1997). Further, Swarnalakshmi et al. (1984) evaluated the anti-inflammatory activity of bergenin. Nazir et al. (2007) also reported the immunomodulatory effect of bergenin in the adjuvant-induced arthritis rats using Flow cytometric study. They have proved the mechanism of action of bergenin and norbergenin as Th1 inhibition as
well as Th2 induction/production, i.e. the possible modulation of Th1/Th2 cytokine balance – to support the anti-arthritic activity of bergenin and norbergenin. Bani et al. (2006) also reported the anti-arthritic activity of lupeol through possible suppression of the immune system, i.e. the cytokines IL-2, IFN (γ) (Th1) and IL4-(Th2).

β-sitosterol is also known to boost the function of the T-cells and “prime” the immune system to function and operate more efficiently. If the immune system is over reacting as in the rheumatoid arthritis it can return to normal by decreasing the inflammatory response while helping to control the B-cell activity or antibody production (www.enerex.ca).

The human skeleton consists of 80% cortical bone and 20% trabecular bone. Trabecular bone having a large surface area is metabolically more active and more affected by factors that lead to bone loss. The main bone minerals are calcium and phosphates. More than 99% of the calcium in the body are in the skeleton, mostly as crystalline hydroxyapatite, but some as non-crystalline phosphates and carbonates; together, these make up half the bone mass phosphates which are also a major constituent of bone and are important in modifying the calcium concentration in bone and other tissues, in part by an effect on the synthesis of calcitriol (Rang et al., 1999). In the present study also, qualitative analysis of the test extracts and estimation of calcium, cobalt and other metals by atomic absorption spectroscopy revealed the presence of calcium in larger amounts (6.300 mg in 1 g plant material; Section 4.1.3). Radiological analysis in arthritic rats strongly supports the ethnobotanical efficacy such as the topical application of the crude plant paste on fractured bones by the Malayali tribes for bone setting.

The role of free radicals in inflammatory diseases like rheumatoid arthritis can not be ruled out. The increase in lipid peroxidation can be attributed to weakening or
failure of the antioxidant defense system in rheumatoid arthritis (Barbar and Harris, 1994). In the present study also, severe tissue damages and increased biochemical markers were observed in the serum and tissues of the arthritic rats which was brought back to the near normalization level with the treatment of test extracts and bergenin. So, we can not rule out the role of the antioxidant nature of the test extracts and bergenin for the observed antiarthritic effects (Section 4.3.4).

In all aspects of the present study right from physical, biochemical, immunological to radiological aspects, the improvement of the arthritic animals treated with the test extracts and bergenin strongly supports the ethnobotanical use of the plant as an antiarthritic and an agent for using bone fractures. The results suggest that the test extracts and bergenin might be useful for the treatment of clinical rheumatoid arthritis because of the similarities of this model to human rheumatoid arthritis and the sensitivity of this model to anti-inflammatory and immune suppressing agents (Walz et al., 1971; Baimgartner et al., 1974) as suggested by several authors.

5.3.7 Immunomodulatory Activity

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases. Several Phyllanthus species have been reported for their Immunomodulation potentials such as P. debilis (Thabrew et al., 1991), P. emblica (Suresh and Vasudevan, 1994), P. tenellus (Ignacio et al., 2001) and P. sellowianus (Fernandez et al., 2002).

In the present study, the test extracts of P. wightianus showed significant inhibition on the delayed type hypersensitivity reactions induced by SRBC with an anti-inflammatory action but without inhibiting the humoral immune response which shows the selective suppression of cellular immune response (CIR) by the test
extracts. This is in agreement with the view of Xu et al. (1993). Among the test extracts, methanol extract exhibited better inhibition than hexane and chloroform extracts. Bergenin, one of the active constituents of methanol extract was reported for its potent immuno modulating activity in chronic immuno inflammatory disease (adjuvant arthritis) by Nazir et al. (2007). They reported the balancing potentials of bergenin and norbergenin in Th1/Th2 cytokines. Th1 cells produce interleukin - 2 (IL-2) and interferon gamma (IFN-γ), which support macrophage activation are involved in delayed type hypersensitivity responses (van der Graaff et al., 1999). Th2 cells, on the other hand, secrete IL-4, IL-5 and IL-13 and provide efficient help of B cell activation, antibody production and down modulate the production of pro-inflammatory cytokines by macrophages. According to Nazir et al. (2007), bergenin effectively suppresses the production of Th1 cells and promotes the production of Th2 cells as an anti-inflammatory agent.

In the present study, better inhibition showed by the methanol extract may be due to the presence of bergenin in it. However, it can not be ruled out the role of other active constituent(s) present in the methanol extract for its immuno modulating potential. Further, the phytochemical evaluation revealed the presence of lupeol in hexane and chloroform extracts and the preliminary phytochemical screening reveals the presence of flavonoids in the test extracts. The immuno suppressive role of lupeol in chronic immune inflammatory reaction was also supported by Bani et al. (2006). Okoli et al. (2003) reported lupeol as an anti-inflammatory agent. The presence of lupeol in the hexane and chloroform extracts may be responsible for its anti-inflammatory action in DTH response. In the humoral antibody response to SRBC, there was a dose-dependent increase in HA titers perhaps showing the stimulation of B-lymphocytes for the production of antibodies. The methanol and hexane extracts
exhibited significant increase in HA titer such as 8.80±0.62, 7.40±0.32 for methanol and 7.01±0.16, 6.6±0.31 for hexane at 200 and 100 mg/kg respectively, whereas in the chloroform extract such as 5.70±0.31, 5.30±0.26 at 200 and 100 mg/kg respectively at both dose levels, it was less, which showed the ineffectiveness of the stimulation of B-lymphocytes (Table 28). Increase in HA titer by methanol and hexane extracts indicates enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978). According to Makare et al. (2001) and Dash et al. (2006), this could be due to the presence of flavonoids which augment the humoral response by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis.

In the non-specific immunity determined by survival rate against fungal infection, there was a significant increase in survival rate of all the test extracts. The increase in survival rate is a general marker exhibiting potency of the test extracts to overcome infectious condition. Better inhibition of methanol extract (59.95%) indicates its high potency to overcome infectious condition than hexane (54.02%) and chloroform extracts (45.96%).

The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b, leading to more rapid clearance of foreign particulate matter from the blood (Furthvan and Bergvanden, 1991). Increased carbon clearance is an indicator of enhanced in vivo phagocytic activity and competency of granulopoetic system in removal of foreign particle, thereby an indicator of enhanced immunological response (Thakur et al., 2007). Phagocytic defects are associated with varied pathological condition in humans (Jo White and Gallin, 1986). When the carbon particles are injected intravenously the rate of clearance of carbon from blood by macrophages is governed by an exponential
equation. This observation seems to be the general way in which inert particulate matter is cleared from the blood (Gokhale et al., 2003). However, in the present study, the test extracts were found to be moderately stimulating the phagocytic activity of the macrophages as evidenced by the slight increase in the rate of carbon clearance when compared to the control animals.

In the cyclophosphamide-induced myelosuppression assay, the test extracts significantly increased the total white blood cell count such as 7.52±0.21 and 7.23±0.11 to methanol extract; 5.56±0.28 and 5.23±0.19 to chloroform extract; and 6.96±0.23 and 6.63±0.21 to hexane extract at 200 and 100 mg/kg respectively (Table 31) and also restored the myelosuppressive effects induced by cyclophosphamide. The total WBC count was significantly increased in all test extracts and among them, methanol and hexane extracts exhibited better increase in WBC count. According to Hartwell (1969), plant phenolics having anti-inflammatory potential showed B-cell stimulation to a significant extent. On the whole, the results indicated that the test extracts of *P. wightianus* hold promising immunomodulatory potential.

5.3.8 Hepatoprotective Activity

5.3.8.1 *In vitro* Inactivation of HBsAg

More than 2 billion people have been infected with HBV worldwide and about 350 million people are chronically infected carriers of the virus (Merle et al., 2001). HBV contains numerous antigenic components including hepatitis surface antigen (HBsAg). HBV Infected cells secrete HBsAg and complete virion particles. Although HBsAg is not infectious complete particles are infectious (Dane particles). When HBsAg is in the blood the complete virus is also present (Brind et al., 1997). Therefore, compounds inhibiting the production or secretion of HBsAg may be used as therapeutic agents against HBV (Kwon et al., 2005).
Apart from the development of specific hepatoprotective drugs, many of *Phyllanthus* species have been reported for the activities of HBsAg inhibition such as *P. amarus* (Thyagarajan et al., 1988, 1990; Blumberg et al., 1989; Jayaram et al., 1990, 1997; Jayaram and Thyagarajan, 1996; Mehrotra et al., 1991; Liu et al., 2001), *P. chamaecristoides* (Alonso et al., 1995), *P. niruri* (Thyagarajan et al., 1982; Jayaram et al., 1987; Venkateswaran et al., 1987; Mehrotra et al., 1990).

In the present study both the methanol extract and bergenin exhibited potent inhibition of anti-HBs binding activity and the activity was dose-dependent. At the dose level of 200μg/ml, bergenin showed its maximum activity as 59% inhibition while it was 50% at 400μg/ml. This showed that maximum activity could be realized at 200μg/ml itself therefore further increase in concentration reduced the percentage of activity. In the case of methanol extract, increase in concentration such as 8 mg/ml exhibited maximum inhibition up to 56%.

The dose-dependent inhibition of HBsAg activity by the plant extracts has been reported by Thyagarajan et al. (1982), Venkateswaran et al. (1987), Mehrotra et al. (1990, 1991), Alonso et al. (1995), Jayaram and Thyagarajan (1996) and Kwon et al. (2005).

Alonso et al. (1995) reported *in vitro* inactivation of HBsAg in three species of *Phyllanthus* and attributed its inactivation in *P. chamaecristoides* to the presence of flavonoids. Lim et al. (2000 c) reported *in vitro* effect of bergenin (100μ M) isolated from *Mallotus japonicus* in galactosamine-intoxicated primary cultured rat hepatocytes showing the inhibition by the release of glutamic pyruvic transaminase and sorbitol dehydrogenase and the increase of RNA synthesis. Kim et al. (2000) also reported similar activity by the model of CCl4 - intoxicated primary cultured rat
hepatocytes and proved its antihepatotoxicity through glutathione-mediated detoxification and free radical suppressing activity.

However, there has been no published literature available for *in vitro* inactivation of HBsAg by bergenin so far. The present study proved the efficacy of bergenin against *in vitro* inactivation of HBsAg for the first time in science. Further, detailed study with different incubation timings at different temperatures may be useful to fix the exact dose level to treat the clinical conditions. So, it can be concluded that the potent inhibiting activity of methanol extract against HBsAg is due to the presence of bergenin in it.

5.3.8.2 Isoniazid (INH) and Rifampicin (RMP)-induced Hepatic Injury in Rats

Isoniazid (INH) was the first effective bactericidal drug used to treat tuberculosis and is an important part of most anti-tubercular drug regimens till far. Rifampicin (RMP), which is another effective bactericidal drug added to the regimen in 1962 has remained the most effective combination along with isoniazid (Snider *et al.*, 1984). However, these drugs are also well known as hepatotoxic agents (Steele *et al.*, 1991). Hepatitis has been reported to occur in 0.46% of patients receiving these anti-tubercular drugs (Alexander *et al.*, 1982). Toxic neuropathy and hepatitis are the most common adverse reactions to isoniazid (Nolan *et al.*, 1999; Blumberg *et al.*, 2003; Yee *et al.*, 2003; Shakya *et al.*, 2004). Rifampicin has produced severe immunoallergic reactions along with hepatocellular carcinoma (Blumberg *et al.*, 2003; Shakya *et al.*, 2004) and the rate of hepatotoxic reaction reported was much higher in the Indian patients (Ramachandran, 1980) compared to that of the developed countries at similar doses (O'Brien *et al.*, 1983; Mindie and Gabriel, 2002). Nelson *et al.* (1976) postulated that one of the isoniazid metabolites acts as an acetylat ing agent causing injury to the macromolecules of hepatocytes. Santhosh *et al.* (2006) reported that
rifampicin, a powerful inducer of drug metabolizing enzymes in man and rats, contributes to the hepatotoxicity of isoniazid by enhancing the rate of the production of toxic metabolites.

So, management of hepatic disorders has become a matter of serious concern worldwide and there is a great lack in modern medicines to treat hepatitis, cirrhosis, liver damage and hepatic carcinoma produced by toxins or for biliary tract disorders. However, from time immemorial, physicians practicing different principles including herbs have been trying various plant products to alleviate these disorders and diseases, e.g. silymarin, a natural flavonoid derived from *Silybum marianum*. The drug is recommended in the management of clinical cases of hepatic disorders. It is well-known that species of *Phyllanthus* (Euphorbiaceae) have been in wider use by the traditional medical practitioners to treat liver disorders with promising results (Syamsundar *et al*., 1985; Venkateswaran *et al*., 1987; Thyagarajan *et al*., 1988).

Hepatoprotective nature of bergenin isolated from the cortex of *Mallotus japonicus* was reported both *in vivo* and *in vitro* against the toxic substances such as CCl₄ by Lim *et al.* (2000 b) and Kim *et al.* (2000) and D-galactosamine by Lim *et al.* (2000 c, 2001).

Hepatotoxicity of INH is thought to be initiated by cytochrome P450 (CYP)-mediated metabolism of INH to acetylhydrazine and hydrazine (Jenner and Timbrel, 1994; Sarich *et al*., 1996, 1999; Chowdhury *et al*., 2006).

Rifampicin generally co-administered with INH in the treatment of tuberculosis enhances hydrazine production by enzyme induction (Pessayre *et al*., 1997). The high reactivity of hydrazine with sulfhydryl groups results in glutathione (GSH) depletion within the hepatocytes, which leads to cell death (van den Dobbelsteen *et al*., 1996; Macho *et al*., 1997; Tasduq *et al*., 2005 b). Sodhi *et al*.
(1997) and Attri et al. (2000) have also demonstrated the critical role of GSH in anti-tubercular drugs-induced hepatotoxicity. According to Tasduq et al. (2005 b), the hepatic injury by anti-tubercular drugs is due to membrane damage (as indicated by increased serum markers), suppression of antioxidant defense mechanisms accompanied by enhanced lipid peroxidation and stimulation of metabolic activation by CYP 2E1 and modulation of [Ca2+] ions. Chowdhury et al. (2001) demonstrated oxidative stress in patients having anti-tubercular drugs-induced hepatotoxicity. Oxidative damage by these drugs is generally attributed to the formation of highly reactive oxygen species, which acts as stimulator of lipid peroxidation and source for destruction and damage to the cell membrane (Georgieva et al., 2004). Apart from increased oxidative stress in the liver, reported that occurrence of increased oxidative stress in the liver mitochondria, associated with mitochondrial permeability alterations and increased apoptosis of the hepatocytes was an important mode of liver injury (Chowdhury et al., 2006). Increased GSH depletion as well as oxidation in the liver of mice co-treated with INH + RMP could be either due to its consumptive utilization by the drugs metabolites or an inability of the GSH synthetic machinery in the liver to cope up with the increase demand of synthesis or both resulting in the imbalance in GSH homeostasis.

Aminotransferases are an important class of enzymes linking carbohydrate with amino acid metabolism thus they clearly establish the relationship between the intermediates of the citric acid and amino acids. Alanine aminotransferase and asparate aminotransferase are well-known diagnostic indicators of liver diseases. In case of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream. Elevated levels of these enzymes in serum are presumptive markers of drug-induced
necrotic lesions in the hepatocytes (Amr and Alaa, 2005). In turn, the damage in hepatocytes increased the bilirubin release (Man–Fung et al., 2003). Increased level of AST, ALT, ALP and bilirubin has been reported in INH+RMP-induced hepatotoxicity in rats (Tasduq et al., 2005 b; Pal et al., 2006; Santhosh et al., 2006). Enhanced susceptibility of hepatocytes in cell membrane might have resulted in increased release of these diagnostic marker enzymes in systemic circulation (Santhosh et al., 2006). In the present study also, it was observed that the administration of INH + RMP elevated the levels of serum marker enzymes such as AST (200.41±5.3 U/L), ALT (212.92±3.2 U/L), ALP (40.55±1.6 KA units) and bilirubin (2.63±0.34 mg/dl) in group–2 animals.

The co-treatment with the test extracts and bergenin 200 mg/kg attenuated the condition and elevated serum levels of AST (98.71±2.3, 101.72±2.96, 89.61±4.682 and 15±6.8 U/L to hexane, chloroform, methanol and bergenin at 200mg/kg), ALT (69.14± 6.4, 88.05±1.5, 53.51±3.9, 48.32±6.3 U/L to hexane, chloroform, methanol and bergenin at 200 mg/kg) and ALP (23.14±6.3, 28.59±1.5, 19.16±1.7, 17.02±1.8 KA units to hexane, chloroform, methanol and bergenin at 200 mg/kg), bilirubin (1.41±0.26, 1.56± 0.34, 1.12±0.3, 0.99±0.4 mg/dl to hexane, chloroform, methanol and bergenin at 200 mg/kg) were significantly decreased towards normalization (Table 33). This was in agreement with the reports of Lim et al. (2000 b). The stabilization of serum bilirubin, AST, ALT and ALP by the drug treatment is a clear indication of the improvement of the functional status of the liver cells (Table 33). Histopathological studies also support these findings.

The improved histology of liver co-treated with the test extracts and bergenin (Fig 51) as compared to that observed in animals administered with only INH + RMP indicates the possibility of the test extracts being able to induce accelerated
regeneration of liver cells by reducing the leakage of AST, ALT and ALP in the blood, and thereby lowering their values in the serum. Serum transaminase returns to normal with the healing of liver parenchyma and regeneration of liver cells.

Alterations of protein metabolism have been considered for decades to be one of the conditions associated with hepatic dysfunction. In the present study also there was a significant decline in protein of animals treated with INH + RMP alone (4.52±0.12 g/dl) when compared to that of control animals (9.23±0.14 g/dl). The disaggregation of polyribosomal profiles induced by anti-tubercular drugs is also associated with the inhibition of protein synthesis, which may be partially responsible for the fatty liver, probably not necrosis, although it contributed to disabling of the cells. Co-treatment with bergenin and the test extracts brought back the protein levels towards normalization (6.7 ±0.16, 5.81±0.13, 7.57±0.13, 8.70±0.11 g/dl to hexane, chloroform, methanol and bergenin at 200 mg/kg; Table 33) that is parallel with the report of Santhosh et al. (2006).

The major disorder encountered in anti-tubercular drugs induced hepatitis is fatty accumulation in the liver which develops either due to excessive supply of lipids to the liver or interference with lipid deposition (Santhosh et al., 2006). In the present study also the levels of total cholesterol and triglycerides were significantly higher in group–2 INH+RMP administered rats (Table 33) to that of normal control animals indicating anti-tubercular drugs induced hypercholesterolemic condition, which also indicates that hepatic injury related alterations in lipid composition of liver tissue appears to occur due to destruction of hepatocytes. Co-administration with bergenin and the test extracts of P. wightianus significantly reduced the anti-tubercular drugs-induced elevation in the levels of total cholesterol and triglycerides compared to that of group–2 animals indicating hypolipidemic nature of bergenin and the test extracts.
Jahromi et al. (1992) reported that oral administration of bergenin isolated from *Flueggea microcarpa* significantly decreased (lowered) the serum total cholesterol and triglycerides. Cholesterol lowering effect of *Phyllanthus* species has been reported by Mishra et al. (1981), Thakur (1985), Jacob et al. (1988), Mathur et al. (1996) and Adeneye et al. (2006).

The phytochemical evaluation of the present study reveals the presence of sterols in the hexane and chloroform extracts. Although β-sitosterol is very similar in its chemical composition to serum blood cholesterol it is completely different in its biological function. It interferes with cholesterol absorption, prevents the rise in serum cholesterol and inhibits the intestinal re-absorption of circulating cholesterol which is secreted in the bile. Therefore, hepatoprotective effect of bergenin and the test extracts of *P. wightianus* is probably related to its ability to inhibit lipid accumulation in the liver tissue by its antilipidemic property. The findings of the histopathological evidences are positive and support the hepatoprotective effect.

Combination of INH+RMP treatment in experimental animals enhanced lipid peroxidation indicating increased oxidative stress in liver (Skakun and Slivaka, 1992; Chowdhury et al., 2001). It is well-known that drugs with antioxidant activity are effective in the treatment of hepatotoxicity (Roy et al., 2006).

Antioxidants can interfere with the oxidation process by reacting with the free radicals, checking the free catalytic metals and also by acting as oxygen scavengers (Gulcin et al., 2002). All the test extracts exhibited significant *in vitro* antioxidant activity against DPPH and nitric oxide free radical assay (Section 4.3.4). The antioxidant capacity of bergenin has been well-documented (Takahashi et al., 2003; Rana et al., 2005). The antioxidant capacity of the test extracts and bergenin would have contributed hepatoprotection.

Tasduq *et al.* (2005 a) reported the protective effect of 50% hydro-alcoholic extract of *Emblica officinalis* against anti-tubercular drug-induced liver toxicity and the activity was found to be due to its membrane stabilizing, antioxidative and CYP 2E1 inhibitory effects.

Mankani *et al.* (2006) and Prasad *et al.* (2006) have reported lupeol as a hepatoprotective agent whereas the latter commented that it might be due to its combating effect on oxidative stress.

Shin *et al.* (2005) reported that ellagic acid isolated from *P. urinaria* showed strong hepatoprotection by inhibiting HB e Ag secretion in the HePG 22.2.15 cell line. Lim *et al.* (2000 b, 2001) studied CCl₄ and D-galactosamine-induced toxicity *in vivo* to assess hepatoprotection of bergenin. Similarly, in the present study, bergenin demonstrated hepatoprotective activity *in vivo* against liver injury induced by INH + RMP (Table 33; Fig. 51).

Bergenin has shown consistent and better hepatoprotection. However, methanol extract of *P. wightianus* offered more or less related or comparable protection. This could be due to the presence of bergenin in it or the synergistic activity of other polyphenols in the methanol extract such as gallic acid and or ellagic acid or a combination of other compound(s) in it. The overall hepatoprotective effect may probably due to a counteraction of free radicals by its antioxidant nature/or to its ability to inhibit lipid accumulation by its antilipidemic property.