3 DISCUSSION
3.1 ANTIHEPATOTOXIC ACTIVITY OF INDIAN COMMERCIAL HERBAL FORMULATIONS

The survey of Indian market revealed that about 40 commercial polyherbal formulations (Appendix) were available for treating liver disorders. Only 18 formulations (Table 1) could be procured, and were tested for antihapatotoxic activity in rats intoxicated with CCl₄, as per schedule given in Table 2. Serum transaminases - GOT and GPT, were estimated in the experimental animals for assessing the antihapatotoxic activity. Among the 18 formulations tested, A. paniculata is present as an ingredient in 16 formulations, E. alba in 9, P. zeylanica and T. purpurea each in 3 formulations. Each of the formulations was tested at a dose of 0.8ml/kg by intraperitoneal route. This dose was selected on the basis of recommended human dose which, for most of the preparations, is 15ml three time a day, i.e., 45ml per day by oral route. Considering average adult human weight to be 60kg, the recommended daily dose of the formulation for human use turns out to be 0.75ml/kg,p.o. Thus, it was considered appropriate to administer each formulation at single daily dose of 0.8ml/kg,i.p. All the formulations except Kalmegh Compound have syrupy or plain aqueous base. Kalmegh Compound contains 40%v/v ethanol.

For ease of administration, all formulations except Kalmegh Compound were diluted with an equal volume of water prior to their use. The control group of rats were treated with normal saline. In case of Kalmegh Compound, the preparation was diluted with double its volume of water, and the control group of animals received 13.33%v/v ethanol instead of normal saline.
Kalmegh Compound®, Livergen® and Stimuliv® were found to be highly active as indicated by 0% proportion of abnormal values of serum transaminases in the rats treated with each of these preparations (Figure 1). Among these, Kalmegh Compound® is most active since the mean values of both SGOT and SGPT in the rats treated with Kalmegh Compound® (Group C, Table 8) do not differ significantly from the corresponding values in the control group (Group A, Table 8). Livarin®, Livomyn®, Livotrit® and Vimliv® (Table 5) were found to possess moderate antihepatotoxic activity as evident from 50% proportion of abnormal values (Figure 1) of SGOT and SGPT in the rats treated with each of these preparations. Proportion of abnormal values in Livosin-treated group of rats was 58.3% indicating that Livosin® exerted 41.7% protection against CCl₄ intoxication. The rats treated with Hepa-10® and Liverin® exhibited 66.7% proportion of abnormal values suggesting that both Hepa-10® and Liverin® cause 33.3% overall reduction in CCl₄-induced increase in the levels of SGOT and SGPT. Adliv-75® and M-Liv® provided 25% protection against CCl₄ intoxication as evident from 75% proportion of abnormal values in the rats treated with each of these preparations. Biligen® afforded only marginal protection (8.3%) as is obvious from 91.7% proportion of abnormal values of the transaminases in the rats treated with Biligen®. The remaining preparations viz., Liv. 52®, Livokin®, Livol®, Livotone® and Tefroll® (Table 5), did not exert significant inhibitory effect on CCl₄-induced increase in the levels of SGOT and SGPT as evidenced by 100% proportion of abnormal values (Figure 1) of SGOT and SGPT in the rats treated with each of these preparations.
3.2 ANTIHEPATOTOXIC ACTIVITY OF EXTRACTS OF THE PLANTS UNDER INVESTIGATION

Pet. ether, chloroform, methanol, aqueous and total aqueous extracts of *A. paniculata* (whole plant), *E. alba* (whole plant), *P. zeylanica* (roots) and *T. purpurea* (roots) were prepared as per scheme given in Figure 2. The extracts were dried taking precautions to preserve thermolabile constituents, if any, present in the extracts. Selection of the whole plant or the root for extraction was based upon the part recommended for use in the indigenous system of medicine (169,246,271, 325).

Antihepatotoxic activity of all the five extracts obtained from each of the plants viz., *A. paniculata*, *E. alba*, *P. zeylanica* and *T. purpurea* was evaluated in rats intoxicated with CCl₄ which is a four-day schedule (Table 2). A dose of 500mg/kg, i.p., was selected arbitrarily to test the activity of each extract and, in order to check the safety of this dose level, a pilot study was conducted. Separate groups of 5 rats each were treated with 500mg/kg, i.p., of each extract for four days and were observed for mortality on the fifth day (Tables 11-13). The extracts showing mortality during the course of the treatment or on the fifth day were tested again at lower dose levels, and the dose showing no mortality was selected. The doses thus selected for various extracts of *A. paniculata*, *E. alba*, *P. zeylanica* and *T. purpurea* are given in Section 2.3.2.2 (page 93).

Antihepatotoxic activity of all the extracts was assessed by estimating GOT, GPT, ALP and BRN in the serum of experimental
animals. The levels of these biochemical parameters in different
groups of rats treated with the extracts of the plants under investigation
are shown in Table 14 (A. paniculata), 15 (E. alba), 16 (P. zeylanica)
and 17 & 18 (T. purpurea). Statistically significant difference at
95% confidence level between various groups in each set of experiment
has been shown in the respective tables. Figure 3 shows the overall
effect, expressed as percentage proportion of abnormal values (PAV%),
of all the extracts from the four plants.

A critical analysis of Tables 14-18 and of Figure 3 shows
that the methanolic extract of A. paniculata at a dose of 500mg/kg.i.p.,
exhibited maximum antihepatotoxic activity against CCl₄ intoxication
in rats. The PAV% for the methanolic extract of A. paniculata is
only 38 indicating 62% overall reduction in CCl₄-induced increase in
the levels of SGOT, SGPT, SALP and SBRN. The mean levels of SGOT,
SALP and SBRN in the rats treated with the methanolic extract of
A. paniculata (Group E, Table 14) do not differ statistically from
the mean levels of these parameters in the rats of the control group
(Group A, Table 14) indicating complete normalization of these biochemical
parameters. Though there was significant lowering of the SGPT but
complete normalization could not be achieved with the methanolic
extract of A. paniculata.

The pet. ether extract (500mg/kg) of A. paniculata (Table 14),
the pet. ether extract (500mg/kg) and the methanolic extract (500mg/kg)
of E. alba (Table 15), the pet. ether extract (0.3mg/kg), the chloroform
extract (3mg/kg), the aqueous extract (500mg/kg) and the total aqueous extract (500mg/kg) of *P. zeylanica* (Table 16), the pet. ether extract (2mg/kg) and the chloroform extract (0.5mg/kg) of *T. purpurea* (Table 17) did not exhibit statistically significant inhibition of CCl$_4$-induced increase in the levels of SGOT, SGPT, SALP and SBRN. PAV% of the four biochemical parameters in the groups of rats treated with these extracts was 100 (Figure 3) indicating complete lack of antihepatotoxic activity in these extracts at the dose levels tested.

Antihepatotoxic activity of other extracts as evident from PAV% (Figure 3) was in the following decreasing order. Values in parentheses indicate PAV%.

- *T. purpurea*: aq. ext., 200mg/kg (47.6)
- *T. purpurea*: total aq. ext., 200mg/kg (52.4)
- *A. paniculata*: CHCl$_3$ ext., 500mg/kg (66.7)
- *E. alba*: Total aq. ext., 500mg/kg (79.1)
- *E. alba*: aq. ext., 500mg/kg (87.5)
- *T. purpurea*: MeOH ext., 100mg/kg (90.4)
- *P. zeylanica*: MeOH ext., 500mg/kg (91.6)
- *A. paniculata*: aq. and total aq. ext., 500mg/kg each (95.2)
- *E. alba*: CHCl$_3$ ext., 500mg/kg (95.8)

Thus, the methanolic extract of *A. paniculata* exhibiting maximum hepatoprotective activity in rats intoxicated with CCl$_4$ was selected for in-depth investigation.
3.3 ISOLATION AND CHARACTERIZATION OF ANDROGRAPHOLIDE

Since the methanolic extract of *A. paniculata* was found to possess significant antihepatotoxic activity, it was considered worthwhile to isolate the major constituent of this extract and examine its antihepatotoxic activity. The major constituent of the methanolic extract is the diterpenoid lactone, andrographolide (172). This lactone was isolated from the methanolic extract prepared from the marc left after successive extraction of powdered *A. paniculata* (whole plant) with pet. ether and chloroform. The methanolic extract was treated with activated charcoal to adsorb colouring matter, and filtered. The filtered methanolic extract, on concentration, deposited crystals which were purified by recrystallization from methanol to get andrographolide in an yield of 0.78% w/w. The isolate having melting point 228°-229°C (with decomposition) appeared as single spot on TLC with three different solvent systems (Section 2.4.2, page 105). UV and IR spectra of the isolate were found to be superimposable with those of the reference sample of andrographolide. Co-TLC of the isolate and the reference andrographolide gave single spot (RF 0.44; solvent system - chloroform-benzene-ethanol, 7:1.5:1.5). Mixed melting point of the isolate and the reference andrographolide was found to be 228°-229°C. Thus, the identity and purity of the isolate was confirmed as andrographolide.

In order to ascertain if the hepatoprotective activity of the methanolic extract was due to andrographolide or attributed to other constituents, andrographolide-free-methanolic extract was prepared
by chromatographing the methanolic extract over silica gel column packed in chloroform (Table 19). Fraction number 8-10 eluted in chloroform-methanol (99.5:0.5) gave a crystalline compound identity of which was confirmed as andrographolide by co-TLC with the reference andrographolide. All the remaining fractions (1-7 and 11-32) were combined and evaporated to dryness to get andrographolide-free-methanolic extract as evidenced by the absence of the spot of andrographolide on TLC plate.

3.4 QUANTITATIVE DETERMINATION OF ANDROGRAPHOLIDE

The TLC-UV method reported by Talukdar and Datta (234) is considered to be most sensitive among all the methods reported (230-236) for estimating andrographolide in *A. paniculata*. Sensitivity of this method has been examined and since it was found to be limited, an attempt has been made to use HPLC for quantitation of andrographolide.

In order to check the sensitivity of TLC-UV method, different amounts (varying from 10 μg to 100 μg) of andrographolide were loaded on TLC plates. A set of three spots of each amount of andrographolide was applied on TLC plates and developed in chloroform-benzene-ethanol (7:1.5:1.5). The developed spots were scrapped off, and andrographolide was extracted from the scrapped silica gel using ethanol. The solution was finally made up to 20 ml and its absorbance was recorded at 223 nm. Average absorbance (Table 20) was plotted against the amount loaded (Figure 4). As is obvious from Figure 4, the plot of absorbance versus amount of andrographolide loaded showed linearity up to 30 μg loading.
Below this amount, the plot is not linear indicating that a minimum of 30 μg of andrographolide has to be loaded on TLC plate for accurate quantitative estimation of andrographolide.

For the quantitative determination of andrographolide by HPLC, different amounts (varying from 5ng to 75ng) of andrographolide were injected in Resolve™ 5μ Spherical Silica column and were eluted isocratically with chloroform-methanol (9:1) at a flow rate of 0.7ml/minute. Detector was adjusted at a range of 0.1 AUFS and detection was done at 254nm. Chart speed was maintained at 1cm/minute. Under these conditions of the HPLC run, the peak of andrographolide was detected at Rf 2.90 minutes. Peak height obtained with different amounts of andrographolide is shown in Table 21. Standard plot of peak height versus amount of andrographolide injected is shown in Figure 5. It is obvious from the plot that as low a quantity as 10ng is sufficient for accurate quantitation of andrographolide by HPLC. Thus, HPLC technique for estimating andrographolide is 3000 times more sensitive than the TLC-UV technique.

With the standardized HPLC technique, andrographolide content in A. paniculata (whole plant) from four commercial sources viz., M/s Ambika Aushdhalaya (Calcutta), M/s Zandu Pharmaceutical Works Ltd. (Bombay), M/s Himachal Drug Company (Dehradun) and M/s Vijay Kumar and Sons (Haridwar), was estimated with a view to ascertain variations, if any, in the content of andrographolide in the commercial samples. Andrographolide content was also estimated by HPLC in different parts viz., root, stem, leaf,
pericarp and seed, and in the biologically active methanolic extract of *A. paniculata*.

For estimating andrographolide content in the four commercial samples of *A. paniculata* and in different parts of the plant, the powdered plant material dried to constant weight was extracted exhaustively with methanol in a soxhlet extraction assembly and the methanolic extract thus obtained was evaporated to dryness under reduced pressure. Accurately weighed amount of the dry extract was dissolved in a known volume of the mobile phase (chloroform-methanol, 9:1). An aliquot of this solution was injected in the HPLC column. A standard solution of andrographolide was used as an external standard to quantitate andrographolide in different extracts and the results integrated on the basis of area under the curve by the Data Module of the HPLC system are shown in Tables 22 (commercial samples) and 23 (different plant parts). The commercial samples of the crude drug from four different sources showed slight variations in the content of andrographolide. The plant procured from M/s Ambika Aushdhalaya was found to have highest amount (0.81% w/w) and the one procured from M/s Vijay Kumar and Sons contained the lowest amount (0.65% w/w) of andrographolide. Quantitative estimation of andrographolide in different parts (Table 23) of *A. paniculata* (M/s Ambika Aushdhalaya) revealed that highest concentration (2.39% w/w) is present in the leaves followed by the root (0.44% w/w), the stem (0.20% w/w), the pericarp (0.18%) and the seed (0.13%).

The methanolic extract of *A. paniculata* (M/s Ambika Aushdhalaya)
exhibiting maximum antihapatotoxic activity was found to contain 11.61\%w/w of andrographolide.

3.5 ANTIHEPATOTOXIC ACTIVITY OF ANDROGRAPHOLIDE, THE METHANOLIC EXTRACT AND THE ANDROGRAPHOLIDE-FREE-METHANOLIC EXTRACT OF A. PANICULATA

Antihapatotoxic activity of andrographolide at an arbitrarily selected dose of 100mg/kg, i.p., was compared with 861.33mg/kg, i.p., of the methanolic extract (equivalent to 100mg/kg of andrographolide) and 761.33mg/kg, i.p., of the andrographolide-free-methanolic extract (equivalent to 861.33mg/kg of the methanolic extract) using rats intoxicated with CCl\textsubscript{4} as per schedule given in Table 2. The mean levels of SGOT, SGPT, SALP, SBRN and HTG estimated in different experimental groups of rats for assessing hepatoprotective activity of the materials under test are given in Table 24. The overall effect of treatment of rats with andrographolide, the methanolic extract and the andrographolide-free-methanolic extract on the five biochemical parameters is depicted in Figure 6.

Andrographolide was found to exert maximum inhibitory effect against CCl\textsubscript{4}-induced increase in the levels of various biochemical parameters as is clearly evident from Figure 6. PAV\% in the rats treated with andrographolide was 51.4 in contrast to 68 and 85 in the rats treated with the methanolic extract and the andrographolide-free-methanolic extract respectively. This observation is further confirmed by the statistical analysis presented in Table 24. The mean levels of all the five biochemical parameters in the andrographolide-
treated group of rats (Group C, Table 24) are significantly different from the mean levels of the corresponding parameters in the \( \text{CCl}_4 \) group of rats (Group B, Table 24). There is complete normalization of \( \text{CCl}_4 \)-induced elevation in SGOT in the rats treated with andrographolide as is evident from the fact that the difference in the mean levels of SGOT in the control group (Group A, Table 24) and in the andrographolide-treated group (Group C, Table 24) of rats is insignificant which is statistically confirmed. Further, the mean levels of all the biochemical parameters except the SALP in andrographolide-treated group of rats are significantly different from the levels of the corresponding parameters in the rats treated with the methanolic extract (Group D, Table 24), and the mean levels of all the five biochemical parameters in the andrographolide-treated group of rats differ from the corresponding levels in the rats treated with the andrographolide-free-methanolic extract (Group E, Table 24). These statistically analysed observations coupled with the fact that the mean levels of SGOT, SGPT, SALP, SBRN and HTG in andrographolide-treated group of rats are lower than the corresponding levels in either the methanolic extract-treated group or the andrographolide-free-methanolic extract-treated group of rats suggest that the antihepatotoxic activity of andrographolide against \( \text{CCl}_4 \) intoxication is certainly better than either the methanolic extract or the andrographolide-free-methanolic extract.

The methanolic extract and the andrographolide-free-methanolic extract of \( \textit{A. paniculata} \) appear to be rather toxic as is evident from the mortalities observed in the groups of rats receiving them. To begin with, number of rats in all the experimental groups was seven.
But two rats in the methanolic extract-treated group and three in the andrographolide-free-methanolic extract-treated group died during the course of experiment (Table 24). Further, the mean level of SBRN in the methanolic extract-treated group of rats (Group D, Table 24), and the mean levels of SALP and SBRN in the rats treated with the andrographolide-free-methanolic extract (Group E, Table 24) do not differ significantly from the corresponding levels of these biochemical parameters in the CCl₄-group (Group B, Table 24) suggesting that the methanolic extract and the andrographolide-free-methanolic extract did not exert any inhibitory effect on CCl₄-induced increase in the levels of SBRN, and SALP & SBRN respectively.

Though the methanolic extract showed PAV% of 38 (Figure 3) at 500mg/kg, i.p., dose but it has less inhibitory effect at higher dose (861.33mg/kg, i.p.) as the PAV% at the latter dose was 68 (Figure 6).

On examining the histopathological profile of the liver of the rats of all the experimental groups (Plate 1) it was observed that in the liver of CCl₄-treated rats (Plate 1,B) there was intense centrilobular necrosis and vacuolization. Fatty degeneration was observed in the areas other than the centrilobular ones. There was mononuclear infiltration in the areas of fatty change. Livers of the rats treated with andrographolide (Plate 1,C) showed significant signs of amelioration of CCl₄-induced liver injury as is obvious from the presence of normal hepatic cords, absence of necrosis and vacuoles, and less degree of infiltration by inflammatory cells. Livers of the rats treated with the methanolic extract (Plate 1,D) also exhibited certain degree
of protection against $\text{CCl}_4$-induced injury but no protection could be observed in the livers of rats treated with the andrographolide-free-methanolic extract (Plate 1,E).

Biochemical and histopathological observations clearly demonstrate that andrographolide is the major antihepatotoxic principle present in the methanolic extract of *A. paniculata*. Removal of this diterpenoid lactone from the methanolic extract leads to a significant fall in the activity of the extract. Thus it was considered worthwhile to study the antihepatotoxic activity of andrographolide at length using different models of hepatotoxicity.

3.6 DETERMINATION OF ACUTE AND SUBACUTE TOXICITY OF ANDROGRAPHOLIDE

Acute toxicity was determined by administering andrographolide in doses of 7, 9, 11, 13 or 15g/kg, i.p., to separate groups of 10 mice (male) each. The animals were observed for mortality over a period of 24h. $\text{LD}_{50}$ of andrographolide calculated (Table 25) by the method of Pizzi (359) was found to be 11.46g/kg by intraperitoneal route with 10.08 to 13.03g/kg as the 95% confidence limits.

When administered to mice at a dose of 100mg/kg, i.p., for four days, andrographolide (Group D, Table 26) did not alter liver function as is evident from the observation that it did not exert statistically significant effect on pentobarbitone induced sleep time with respect to control group of mice (Group A, Table 26). It is well known that barbiturates are metabolised in the liver (360).
Any type of liver malfunction delays the metabolism of barbiturates thereby slowing their excretion rate leading to increase in the duration of barbiturate induced hypnosis (361). Since andrographolide did not alter pentobarbitone induced sleep time, it is an indirect evidence for lack of liver toxicity of andrographolide per se. This was further confirmed by administering andrographolide at single daily dose of 50mg/kg,p.o., for fourteen days to rats and determining SGOT, SGPT, SALP, SBRN and HTG in the treated rats on the 15th day. Results are shown in Table 33 and Figure 11. No significant effect of andrographolide (Group D, Table 33) on these biochemical parameters with respect to the control group of animals (Group A, Table 33) was observed. Proportion of abnormal values of various biochemical parameters in the rats treated with andrographolide was 0% (Figure 11) indicating that andrographolide does not cause hepatotoxicity per se. This finding was further confirmed by histopathological examination of the liver of the rats treated with andrographolide (Plate 4,D). Hepatic architecture was perfectly intact in the rats treated with 50mg/kg/day,p.o., andrographolide for 14 days.

Thus, andrographolide is safe as far as its effect on liver function, and LD_{50} are concerned.

3.7 ANTIHEPATOTOXIC ACTIVITY OF ANDROGRAPHOLIDE

Antihepatotoxic activity of andrographolide was evaluated using CCl_{4}, GalN, PcmL and EtOH models of liver toxicity.
3.7.1 CCl₄ Model

Effect of andrographolide (100mg/kg, i.p.) on pentobarbitone sleep time in mice intoxicated with CCl₄ as per schedule given in Table 2 was studied. Mean sleep time of various experimental groups of mice is shown in Table 26. Andrographolide treatment completely normalised CCl₄-induced increase in the pentobarbitone sleep time as evidenced by the observation that there is no statistical difference (p = 0.001) between the mean sleep time of the control (Group A, Table 26) and that of the andrographolide-treated groups of mice. This observation coupled with those discussed under Section 3.5 (page 165) suggest that andrographolide provides significant protection to rats against CCl₄ hepatotoxicity.

3.7.2 GalN Model

Intraperitoneal administration of andrographolide (50, 100, 200 or 400mg/kg) to rats 48h, 24h and 2h prior to GalN challenge exhibited a dose dependent inhibition of GalN-induced increase in the levels of SGOT, SGPT, SALP, SBRN and HTG (Table 27). Pretreatment with 400mg/kg, i.p., of andrographolide was found to exert highly significant (p = 0.001) protective effect. There was complete normalization of all the five biochemical parameters in the rats treated with 400mg/kg andrographolide as evident from the observation that the mean levels of all the five biochemical parameters in the rats treated with this dose of andrographolide (Group F, Table 27) do not differ statistically from the levels of the corresponding parameters in the rats of the control group (Group A, Table 27). The protective effect of pretreatment
with 400mg/kg, i.p., dose of andrographolide is further confirmed by histopathological examination of the livers of the control, the GalN-treated and the andrographolide-treated group of rats (Plate 2). The livers of the GalN-treated rats (Plate 2,B) showed focal necrosis especially in periportal area. Predominantly microvesicular fatty change was observed and there was infiltration by lymphocytes and neutrophils. In contrast, andrographolide-treated rats (Plate 2,C) retained hepatic architectural pattern quite close to the liver of the control group of rats (Plate 2,A).

While comparing the intraperitoneal route of administration with the oral route it was observed that the latter required 800mg/kg andrographolide for complete normalization of various biochemical parameters (Table 2B). Incomplete absorption of andrographolide from the gastro-intestinal tract could be responsible for requirement of higher dose by oral route to achieve the same results as are obtained by lower dose (400mg/kg) administered intraperitoneally. Histopathological profile of the livers of the rats treated with 800mg/kg, p.o., andrographolide is shown in Plate 2,D. Rats receiving andrographolide were found to possess livers with histological pattern quite close to the livers of the rats of the control group (Plate 2,A) suggesting that protective effect is exerted by andrographolide against GalN-induced hepatic lesions.

The overall effect of pretreatment with different doses of andrographolide by intraperitoneal or oral route on SGOT, SGPT, SALP, SBRN and HTG in rats intoxicated with GalN is depicted in Figure 7. It is obvious from this figure that 400mg/kg of andrographolide administered
intraperitoneally or 800mg/kg administered orally exert protective
effect of the same order. PAV% in both cases is 12.5 indicating
87.5% reduction in GalN-induced increase in the levels of various biochemical parameters.

Significant observations have been made on the effect of pre-
and post-treatment of GalN-intoxicated rats with andrographolide.
Since maximum hepatoprotective activity of andrographolide was observed
at a dose of 400mg/kg, i.p., this dose was employed for studying
the effect of andrographolide administration at different time intervals
with respect to GalN challenge. The mean levels of SGOT, SGPT,
SALP, SBRN and HTG determined 48h after GalN administration are
given in Tables 29 and 30. Highly significant antihepatotoxic activity
was exerted by andrographolide when administered '48h, 24h and
2h' prior to or '2h before and 1h after' GalN challenge. PAV% in
rats treated with these two modes of administration was 11.4 and
14.3 (Figure 8) indicating 88.6% and 85.7% protection respectively.
The mean levels of various biochemical parameters in the rats pretreated
with three doses (Group C, Table 29) or pre- and post-treated with
one dose each of andrographolide (Group C, Table 30) do not differ
(p = 0.001) with the mean levels of the corresponding parameters
in the control group of rats suggesting complete normalization of GalN-
induced increase in the levels of various biochemical parameters.

Significant lowering of various biochemical parameters was
also observed with andrographolide treatment 2h before (Group D,
Table 29), 1h after (Group D, Table 30) or 1h, 4h and 7h after (Group E,
Table 29) GalN administration to rats but the effect in these three
modes of administration was less than that observed after pretreatment with three doses (group C, Table 29) or pre- and post-treatment with one dose each (Group C, Table 30). Rats receiving andrographolide 4h and 7h after GaIN challenge (Group F, Table 29) did not experience any inhibition of GaIN-induced increase in the levels of various biochemical parameters as evident from the fact that the mean levels of various biochemical parameters in the rats in this group do not differ statistically from the mean levels of the corresponding parameters in the rats treated with GaIN (Group B, Table 29).

Overall antihepatotoxic effect with different modes of administration of andrographolide (400mg/kg,i.p.) against GaIN intoxication may be summarised on the basis of PAV% of various biochemical parameters in different experimental groups (Figure 8). Antihepatotoxic activity of different modes of andrographolide administration was found to be in the following decreasing order. Values in parentheses indicate PAV%: '-' sign indicates that andrographolide was administered before GaIN challenge; '+' sign indicates that the treatment was given after GaIN challenge.

-48h, -24h, -2h : (11.4)
-2h, +1h : (14.3)
-2h : (51.4)
+1h, +4h, +7h : (57.1)
+1h : (62.9)
+4h, +7h : (100.0)

3.7.3 PcML Model

Pretreatment of rats with 50, 100, 200 or 400mg/kg,i.p., andrographolide
48 h, 24 h and 2 h before Pcml administration exhibited significant reduction in Pcml-induced increase in the levels of SGOT, SGPT, SALP and HTG but no effect was observed with respect to the level of SBRN (Table 31). Hepatoprotective effect of pretreatment with 200 mg/kg, i.p. (Group E, Table 31) or 400 mg/kg, i.p. (Group F, Table 31) andrographolide was of the same order as the mean levels of various biochemical parameters do not differ statistically in the rats receiving these two doses. Further, PAV% of various biochemical parameters in the rats treated with 200 or 400 mg/kg, i.p., andrographolide is also the same, 62.9 (Figure 9). Thus, protective effect of andrographolide against Pcml intoxication is significant even at 200 mg/kg, i.p., in contrast to 400 mg/kg, i.p., in the case of GalN intoxication. Statistical equivalence (p = 0.001) between the mean levels of SGOT and SALP in the rats treated with 200 mg/kg, i.p., andrographolide (Group E, Table 31) and the corresponding mean levels in the rats of the control group (Group A, Table 31) suggests complete normalization of these biochemical parameters. Plate 3 shows liver histomorphology of the control, the Pcml-treated and the andrographolide-treated (200 mg/kg, i.p.) groups of rats. Pcml administration caused gross necrosis of the centrilobular hepatocytes as evidenced by nuclear pyknosis, karyolysis and eosinophilic infiltration (Plate 3,B). Treatment with andrographolide reversed to a large extent the hepatic changes produced by Pcml. This is obvious from the absence of eosinophilia and presence of fewer necrotic zones (Plate 3,C) in the slides of livers of rats treated with andrographolide.

Unlike GalN hepatotoxicity, the Pcml toxicity was significantly
countered by treatment with 200mg/kg,i.p., andrographolide 1h, 4h and 7h after PcmL challenge. All the biochemical parameters including SBRN were completely normalized by post-treatment with three doses of andrographolide (Group E, Table 32) as evident from statistical equivalence (p = 0.001) between the mean levels of various biochemical parameters in the rats of this group and the mean levels of the corresponding parameters in the rats of the control group (Group A, Table 32). PAV in the rats receiving andrographolide 1h, 4h and 7h after PcmL challenge was 34.3 (Figure 10) indicating 65.7% protection. In contrast to GalN hepatotoxicity, PcmL toxicity was also ameliorated significantly when andrographolide was administered 4h and 7h after PcmL challenge (Group F, Table 32), PAV being 62.9. Rats pretreated with three doses (Group C, Table 32) or single dose (Group D, Table 32) of andrographolide were also protected against PcmL intoxication, PAV being 65.7 and 71.4 indicating 34.3% and 28.6% protection respectively. All these observations indicate that andrographolide (200mg/kg,i.p.) exerts maximum protective effect against PcmL hepatotoxicity in rats when administered 1h, 4h and 7h after PcmL challenge.

3.7.4 EtOH Model

Andrographolide, when administered to rats concurrently with EtOH, exhibited highly significant protective activity against EtOH intoxication. Hepatotoxicity was induced by administering EtOH to rats at a dose of 5ml/kg/day by oral route for 14 days. Andrographolide was administered daily at an arbitrarily selected dose of 50mg/kg,p.o., 2h after EtOH administration. The mean levels of SGOT, SGPT, SALP,
SBRN and HTG determined on the 15th day in the control, the EtOH treated and the andrographolide-treated groups of rats are shown in Table 33. The rats treated with andrographolide exhibited PAV% equal to 12.5 while PAV% in rats of the EtOH-treated group was 75.0 (Figure 11). Proportion of abnormal values was not 100% after EtOH intoxication because EtOH did not elevate the level of SBRN as is obvious from insignificant F ratio in case of SBRN (Table 33). Statistical equivalence (p = 0.001) between the mean levels of SGOT, SGPT, SALP and HTG in the rats treated with andrographolide (Group C, Table 33) and the mean levels of the corresponding parameters in the control group (Group A, Table 33) suggests highly significant protection of rats against EtOH intoxication. Histopathological profile of the livers of the control, the EtOH-treated and the andrographolide-treated groups of rats is represented in Plate 4. The liver of the EtOH intoxicated rats (Plate 4, B) shows abundant microvesicular fatty change. Inflammatory zones in the central areas consisting of necrosing hepatocytes and Mallory bodies surrounded by polymorphs were found. The liver of andrographolide-treated rats (Plate 4, C) showed absence of Mallory bodies and inflammatory zones but microvesicular fatty change could be observed. These histopathological observations confirm the hepatoprotective activity of andrographolide administered concurrently with EtOH.

Since SBRN level in rats showed no significant change after EtOH intoxication it was not estimated while studying the effect of post-treatment of EtOH-intoxicated rats with andrographolide. The rats were treated with single dose (50 or 100mg/kg, p.o.) of andrographolide 2h
after EtOH intoxication (5ml/kg/day, p.o., for 14 days). The mean levels of SGOT, SGPT, SALP and HTG in various experimental groups estimated on the 15th day are shown in Table 34. Highly significant \( (p = 0.001) \) inhibitory activity was exerted by 100mg/kg, i.p., dose of andrographolide. EtOH-induced increase in the levels of SGOT, SGPT and SALP was normalized completely after post-treatment with single dose (100mg/kg) of andrographolide (Group D, Table 34) as evidenced by statistical equivalence between the mean levels of these biochemical parameters and the mean levels of the corresponding parameters in the control group of rats (Group A, Table 34). Andrographolide (50mg/kg, p.o.) exhibited significant protective effect with respect to SGOT but the inhibition of SGOT was less than that elicited by 100mg/kg, p.o., dose (Table 34).

Since post-treatment of rats with single dose of 100mg/kg, p.o., of andrographolide showed maximum protective effect against EtOH intoxication, this dose level was employed for examining the effect of post-treatment of EtOH-intoxicated rats with three or five repeated doses of andrographolide. Rats, after intoxication with 5ml/kg/day, p.o., EtOH for 14 days, were treated with either three doses, one each on 14th, 15th and 16th day, or five doses, one each from day 14 to day 18, and were sacrificed on the 17th or the 19th day respectively to estimate the levels of SGOT, SGPT, SALP and HTG. Results are shown in Tables 35 and 36 respectively. Significant lowering of SGOT, SGPT and HTG was observed after post-treatment with three doses of andrographolide (Table 35). While there was complete normalization of EtOH-induced increase in SGPT and HTG levels but SALP was not inhibited. In the set of experiment involving post-treatment of rats with five doses of
andrographolide, the mean levels of the four biochemical parameters estimated on the 19th day in all the experimental groups viz., control, EtOH-treated and andrographolide-treated, did not differ statistically from one another (Table 36) indicating self recovery of liver function, 6th day after stopping EtOH administration.

The overall effect of post-treatment with andrographolide at different time intervals on SGOT, SGPT, SALP and HTG in rats intoxicated with EtOH is depicted in Figure 12. The figure clearly demonstrates self recovery in rats receiving EtOH for 14 days followed by no treatment afterwards. PAV% of various biochemical parameters in the EtOH-treated rats sacrificed on 15th, 17th or 19th day is 96.9, 75.0 and 3.1 respectively. PAV% in EtOH-intoxicated rats treated with one or three doses of andrographolide was 37.5 and 12.5 respectively.

The above observations lead to the conclusion that andrographolide can completely reverse EtOH hepatotoxicity in rats if administered concurrently with EtOH, or when administered 2h or '2h, 24h and 48h' after intoxication for 14 days.

3.8 COMPARATIVE ANTIHEPATOTOXIC ACTIVITY OF ANDROGRAPHOLIDE AND SILYMARIN

Silymarin isolated from *Silybum marianum* (Compositae) is the most thoroughly investigated of all the plant products known to have hepatoprotective activity (67), and is being used clinically (8). Encouraged by the observations that andrographolide exerts significant antihepatotoxic activity against CCl<sub>4</sub>, GalN, PcmL and EtOH intoxication in rats, it
was considered appropriate to compare the activity of this diterpenoid lactone with that of silymarin. Three models of hepatotoxicity viz., CCl₄, GalN and PcML, were employed for comparing hepatoprotective activity of andrographolide and silymarin. Since 20mg/kg, i.p., of silymarin is reported (362) to exert pronounced protective effect against CCl₄ and GalN liver toxicity, both silymarin and andrographolide were administered to rats at this dose level in all the three models of hepatotoxicity. Antihapatotoxic activity of silymarin and andrographolide was also compared at 100mg/kg (CCl₄ model), 200mg/kg (PcML model) and 400mg/kg (GalN model) because andrographolide was found to exert significant hepatoprotective action at these dose levels during the course of present investigations.

In case of CCl₄ model, andrographolide and silymarin were administered to rats as per schedule given in Table 2. In GalN model, comparison was done after treatment of rats with the test substance 48h, 24h and 2h prior to GalN challenge (Table 3) while in PcML model, effect of treatment 1h, 4h and 7h after PcML challenge (Table 4) was compared. SSDH, SGLDH, HGN and/or SGPT were estimated in the experimental animals in order to evaluate antihapatotoxic activity. The mean levels of various biochemical parameters in different groups of experimental animals are shown in Tables 37, 38 (CCl₄ model), 39, 40 (GalN model) and 41, 42 (PcML model). The mean levels of the biochemical parameters in different groups of rats in each set of experiment have been compared by LSD procedure (p = 0.001).

In CCl₄ model, andrographolide and silymarin exhibited significant antihapatotoxic activity at 20mg/kg, i.p., dose and the level of activity
of both the compounds was of the same order as evident from statistical equivalence between the mean levels of SGPT, SSDH, SGLDH and SBRN in the andrographolide-treated (Group C, Table 37) and the silymarin-treated rats (Group D, Table 37). Same level of hepatoprotective activity for both the compounds has also been observed at 100mg/kg, i.p. dose (Table 38). It is of particular interest to note that the level of HGN depressed by CCl₄ was normalized to the control level with 100mg/kg, i.p., dose of both andrographolide and silymarin (Table 38).

Treatment of rats with 20mg/kg, i.p., of either andrographolide or silymarin 48h, 24h and 2h prior to GalN challenge did not exhibit any protective effect with respect to SGPT, SSDH and SGLDH as evident from the observation that there is no statistical difference in the mean levels of these parameters between the GalN-treated group and the andrographolide/silymarin-treated group (Table 39). However, GalN-induced depression in the HGN level was completely normalized with the administration of andrographolide and silymarin at 20mg/kg, i.p., dose. Pretreatment with 400mg/kg, i.p., of andrographolide and silymarin exhibited significant protection against GalN hepatotoxicity. SGLDH and HGN were completely normalized by both the compounds (Table 40). Complete normalization of SSDH could not be achieved by either of the compounds but the level of SSDH was lowered significantly. Further, the mean level of SSDH in the rats treated with andrographolide (Group C, table 40) differs significantly from the corresponding level in the rats treated with silymarin (Group D, Table 40) indicating better inhibitory activity of silymarin with respect of SSDH.
Treatment of rats with 20mg/kg, i.p., each of andrographolide and silymarin 1h, 4h and 7h after PcmL challenge indicated that andrographolide is devoid of hepatoprotective activity at this dose level as evident from the statistical equivalence between the mean levels of the four biochemical parameters in the rats treated with andrographolide (Group C, Table 41) and the mean levels of the corresponding parameters in the rats treated with PcmL (Group D, Table 41). Silymarin significantly inhibited PcmL-induced increase in the levels of SGPT, SSDH and SGLDH but did not have any inhibitory effect on HGN (Table 41). Post-treatment with both andrographolide and silymarin, each at a dose of 200mg/kg, i.p., exhibited significant protection of rats against PcmL intoxication (Table 42). The levels of SSDH and SGLDH were significantly lowered by both the compounds and those of HGN were completely normalized. However, the mean level of SSDH in the rats treated with andrographolide (Group C, Table 42) differs significantly from the corresponding level in the rats treated with silymarin (Group D, Table 42) indicating better inhibitory activity of andrographolide (200mg/kg, i.p.) with respect to SSDH.

3.9 POSSIBLE MECHANISM OF ACTION OF ANDROGRAPHOLIDE

The present investigations reveal that andrographolide exerts hepatoprotective effect against CC14, GalN, PcmL and EtOH - the four well known hepatotoxins. It is well established that injury to liver by CC14 is due to free radical formation during its metabolism by hepatic microsomes (24,25) which in turn causes peroxidation of cellular membranes leading to necrosis. The damage caused by GalN in rats has been considered to be much akin to the damage in viral hepatitis in humans (27).
Hepatotoxicity of GalN is due to its metabolism in the liver which causes lowering of the levels of uracil nucleotides (UTP, UDP-glucose and UDP-galactose) resulting in inhibition of RNA synthesis leading to necrosis (363). PcmL is metabolised in the liver primarily to glucuronide and sulphate conjugate (364,365). Hepatotoxicity of PcmL has been attributed to the formation of toxic metabolites when a part of PcmL is metabolised by cytochrome P-450 (366). Induction of cytochrome P-450 or depletion of hepatic glutathione is a pre-requisite for PcmL-induced hepatotoxicity (35). Fatty liver produced by EtOH is due to increased biosynthesis of fatty acids, increased esterification of fatty acids to triglycerides, and decreased oxidation of fatty acids in mitochondria (52).

As the cell membrane degeneration is a terminal feature in all the four types of liver cell necrosis, it is quite likely that andrographolide brings about its protective effect by a variety of actions such as alteration of plasma membranes or a direct stabilizing effect in addition to an inhibitory effect on microsomal enzymes (239) or inhibitory action against lipid peroxidation (238). In the present investigations it is quite interesting to note that protective effect of andrographolide has been observed in all the four types of intoxications all of which differ in their induction mechanism of liver injury. Protective mechanisms not specific to CCl₄, GalN, PcmL or EtOH toxicity may be responsible for hepatoprotective activity of andrographolide. Thus, the stimulation of hepatic regeneration known to cause the liver to become more resistant to damage by poisons (367) could explain the hepatoprotective effect.
of andrographolide. Likewise, activation of the functions of reticulo-endothelial system (368) or an inhibition of protein biosynthesis (369) are some of the known mechanisms which can reduce the hepatotoxicity of CCl₄, GaIN, PcmL or EtOH. The present investigations have not been aimed at allowing a choice to be made among such various possibilities and, thus, further work in this area may unfold specific mechanism of hepatoprotective action of andrographolide against all the four hepatotoxins used.