6. SUMMARY

BALB/c strain of white Swiss mice, has been found to be susceptible to *P. berghei* (NK-65). Asynchronicity of the parasite is confirmed due to presence of rings, trophozoites and schizonts in the blood of infected mice at the same time. Infected mice die within a week of inoculation of parasite, except the cases, where parasite migrates from red blood cells to reticulocytes. Migration of parasites to reticulocytes increases the survival of infected mice up to two weeks. The maximum parasitaemia has been calculated to be around 50% before the death of mice.

In the present study, *in vitro* and *in vivo* antiplasmodial efficacy of three traditional medicinal plants (*Ajuga bracteosa*, *Xanthium strumarium* and *Berberis aristata*) with folklore reputation has been evaluated in order to provide relevant scientific evidences for their clinical use and to establish their potential role for the development of new antiplasmodial components.

The leaves of *Ajuga bracteosa* (voucher No. 8895) and *Xanthium strumarium* (voucher No. 17920) and root bark of *Berberis aristata* (voucher No. 17919) were collected from Mandi District of Himachal Pradesh and identification was confirmed from Dr. Y.S. Parmar Horticulture University, Nauni, Solan and Department of Botany, Panjab University, Chandigarh respectively. Ethanolic leaves extract of *A. bracteosa* (ELEAB) and *X. strumarium* (ELEXS) were prepared by Soxhlet extraction method. Dried and powdered leaves (500 g) yielded 42 g ELEAB and 18 g ELEXS, dried residue after evaporation of solvent in rota evaporator, whereas, dried root bark of
Berberis aristata (500 g) yielded 20 g dried residue after aqueous extraction. All residues were stored at -4°C and subjected to phytochemical screening to detect the presence of various secondary plant metabolites. ELEAB and ELEXS have shown positive detection results for the presence of alkaloids, saponins, phenolic compounds, steroid and cardiac glycosides. Anthraquinones were not detected in either of extracts. In AREBA extract, phenolic compounds and anthraquinones were not detected.

Dried residues of all the three plant extracts were subjected to schizont maturation inhibition assay to test their *in vitro* antimalarial efficacy. Concentrations of extract/drugs corresponding to 50% schizont maturation inhibition (IC$_{50}$) was obtained by plotting linear curve dose-response of extract (10-100 µg/ml) used and percentage of schizont maturation inhibition obtained. Short term *in vitro* culture of *P. berghei* in RPMI-1640 was maintained to check antimalarial efficacy of different extracts. All plant extracts have been found to inhibit *P. berghei* schizont maturation in dose dependent manner with IC$_{50}$ of 10 µg/ml (ELEAB), 5 µg/ml (ELEXS) and 40 µg/ml (AREBA). Chloroquine (10 µM) has shown more than 90% schizont maturation inhibition *in vitro*. All the results have been expressed as mean ± S.E.M. of three independent experiments and each concentration was run in triplicate every time.

Acute toxicity of plant extracts was determined by the limit test of Lorke. Maximum concentration of 5 g/kg/mouse was orally administered to female BALB/c mice, fasted for 4 hrs. LD$_{50}$ of ELEAB and AREBA has been determined more than 5 g/kg which is the upper testing limit of any compound to be administered orally in rodents. No mortality and significant histopathological changes were observed in mice of these groups after two weeks. However, ELEXS exhibited LD$_{50}$ of 1.5 g/kg concentration in mice. The histopathological examination of organs showed splenomegaly after two weeks. ELEAB and AREBA extracts showed clinical safety in BALB/c mice as compared to ELEXS.
Peters 4-day test was performed to check suppressive antiplasmodial efficacy of plant extracts in early *P. berghei* infection. After inoculation of $1 \times 10^7$ *P. berghei* infection mice were divided into different groups and were administered with different concentration of extracts ELEAB (250-1000 mg/kg), ELEXS (150-500 mg/kg) and AREBA (150-650 mg/kg). All the extracts exhibited dose dependent chemosuppressive effect. Maximum chemosuppression of 86.6% (p < 0.001), 88.6% (p < 0.001) and 67.1% (p < 0.001) was recorded with 1000 mg/kg ELEAB, 500 mg/kg ELEXS and 350 mg/kg AREBA respectively, whereas, chloroquine (5 mg/kg/day) exhibited more than 85% chemosuppression, in positive control.

Repository activity of plant extracts was assessed by Peter's method using pyrimethamine (1.2 mg/kg/day) as positive control. All plant extracts exhibited dose dependent chemosuppression effect showing 90.4% (350 mg/kg ELEXS), 58.8% (750 mg/kg ELEAB) and 53.9% (350 mg/kg AREBA) chemosuppression respectively, however, pyrimethamine (1.2 mg/kg/day) caused around 90% chemosuppression.

Curative (schizontocidal) activity of plant extracts in established infection of *P. berghei* was determined by Rane test, using chloroquine (5 mg/kg/day) as positive control. Plant extracts exerted 85.1% (750 mg/kg ELEAB), 91.1% (150 mg/kg ELEXS) and 30.6% (350 mg/kg AREBA) chemosuppression respectively with significant p value (< 0.01 to 0.001). The mean survival time was 27.4 ± 0.46 (750 mg/kg ELEAB), 29.8 ± 0.19 (150 mg/kg ELEXS) and 12.8 ± 0.46 (350 mg/kg AREBA) days respectively. All mice of negative control group died with in 7 days and positive control group (CQ) survived for one month study period.

Concentrations of the extracts exhibiting maximum schizontocidal activity were compared for its schizontocidal activity with quinine (10 mg/kg), artesunate (100 mg/kg), china (1 : 2 $\phi$) and *Eucalyptus* (1 : 2 $\phi$) along with negative control group. Eight groups
of mice (G-2 to G-9) having ten mice each were infected with $1 \times 10^7$

*P. berghei* infected cells on day 0. G-1 was kept as normal control
group during one month study period. All mice of G-2 died within first
week. G-3 (750 mg/kg ELEAB), G-4 (250 mg/kg ELEXS), G-5 (350
mg/kg AREBA) exhibited 90.6%, 92.5% and 60.8%
chemosuppression with significant p value (< 0.001) on day 7. G-6
(china 1 : 2 $\phi$) and G-7 (*Eucalyptus* 1 : 2 $\phi$) exhibited 91.4 and 86.5%
chemosuppression as compared to G-2 on day 7. G-8 (10 mg/kg
quinine) and G-9 (100 mg/kg artesunate) showed 97.6 and 98.2%
chemosuppression with significant p value (< 0.001) on day 7.

Mononuclear (MN) and polymorphonuclear (PMN) white blood
cells were isolated from all the groups of mice (G-1 to G-9) on day 7
by double density gradient centrifugation. All the cells were subjected
to fluorescent staining using AO/EB to detect their mortality in each
group. Percentage of live PMN cells was maximum in G-9 followed by
G-8, G-3, G-6, G-5, G-7 and G-4. The number of live MN cells was
between 96.4% and 97.5% in G-3, G-5, G-6, G-8 and G-9. The
maximum number of dead white blood cells were observed in G-4
group.

Red blood cells and white blood cells obtained from the blood
of various groups (G-1 to G-9) were subjected to Scanning Electron
Microscopy to observe the changes in the surface of cells under the
influence of various plant extracts/antimalarials. Scanning Electron
Micrographs of normal mice (G-1) exhibited biconcave normal red
cells measuring 3.2-3.6 $\mu$m. *P. berghei* infected (G-2) red cells (3.8-
4.0 $\mu$m) appeared as deformed cells with knobs and depressions on
the surface of cells due to the presence of parasite inside the cells.
Scanning Electron Micrographs of G-3 to G-5 groups exhibited
regular biconcave cells with few cells showing knobs on the surface
due to infection. Size of RBCs was smallest in G-3 (1.1 to 1.6 $\mu$m)
followed by G-4 (2.3-3.0 $\mu$m) and G-5 (3.0-3.4 $\mu$m). RBCs of G-6 to
G-9 groups have been observed to have some irregularities in their
surface which was due to the presence of parasite. Average size of RBCs in these groups is between 3.6-4.2 μm.

Scanning electron micrographs of G-1 group exhibited three types of mononuclear cells. Smooth surface cells (3.0 μm), slightly ruffled surface cells (4.0 μm) and cells with villous surface (2.8 μm). G-2 group exhibited MN cells with various types of depressions on the smooth surface (2.8-3.8 μm). G-4 exhibited both smooth (3.3 μm) as well as villous surfaced (3.8 μm) cells, while G-5 exhibited only smooth surface MN cells (2.5-3.0 μm) with various depressions, on the cell surface. MN cells of G-6 mice exhibited ruffled surface MN cells (3.1-3.8 μm), while G-7 exhibited smooth surface MN cells (3.2 μm) with various depressions.

Polymorphonuclear cells separated from the blood of G-1 to G-9 groups exhibited finger-like projections on the surface of cells in scanning electron micrographs. The size and shapes of PMN cells was observed to be different in various plant extracts/antimalarials treated groups. G-1 exhibited finger-like knobs with blunt ends having 3.2 μm diameter of cells. P. berghei infected PMN (2.8 μm) cells, exhibited pointed finger-like projections all over the cell surface. Projections on surface of PMN cells obtained from G-3 and G-5 were blunt and less in number, whereas, PMN cells isolated from G-4 exhibited more pointed projections as seen in G-2 group. Group G-6 to G-9 exhibited PMN cells with pointed projection over the surfaces which was higher in number in G-6 and G-7 and lesser in G-8 and G-9. PMN cells of G-9 were comparable to G-1 in size and shape.

Liver, spleen and kidney were obtained by sacrificing mice from various groups (G-1 to G-9) on day 7 and processed for histological examination after fixation in Bouin’s fluid. Haematoxylin/eosin stained transverse sections of normal mouse liver exhibited hexagonal units. Hepatocytes radiating from central vein to periphery and portal triads on the periphery of lobule were also visible. Sections of P. berghei infected liver exhibited reticuloendothelial cell proliferations,
haemozoin deposition and increased number of Kupffer cells with infiltration of infected RBCs in the portal circulation.

Sinusoidal dilation was observed in G-3, slightly basophilic cytoplasm and condensation of chromatin of hepatocyte was observed in G-4. Deposition of haemozoin and less acidophilic cytoplasm of hepatocytes was observed in G-5. Sinusoidal dilation with kupffer cells hyperplasia and reticuloendothelial cell proliferation was observed in G-6 and G-7. Slightly basophilic cytoplasm of hepatocytes and reduction in sinusoidal spaces were observed in G-8. No haemozoin deposition or kupffer cell hyperplasia was observed in G-9. The histology of artesunate treated group and ELEAB treated group was comparable to normal mouse liver.

Haematoxylin/eosin stained transverse sections of normal mouse spleen exhibited normal histology with intact capsule, differentiation of red pulp and white pulp separated by marginal zones and central arteriole surrounded by white pulp area. *P. berghei* infection leads to enlargement of white pulp area and less demarcation in red pulp and white pulp area along with hyperplasia of lymphatic cells. Haemozoin deposition has also been observed. Histology of spleen in ELEAB, quinine and artesunate treated mice has been observed to be comparable to normal spleen histology. In G-4 and G-5 widening of white pulp with lymphatic cell proliferation has been observed. Sinusoidal dilation and lymphatic cell proliferation along with haemozoin depositions were evident in G-7.

Histology of normal mouse kidney reveals the presence of outer cortex and inner medullary region. Convoluted tubules, medullary rays and glomeruli are visible in cortex. Renal pyramids can be seen in medulla. *P. berghei* infected mouse kidney exhibits abnormal proliferation of mesangial cells and cytoplasmic vacuoalization of tubular cells G-3, G-8 and G-9 groups revealed normal architecture of kidney, whereas little shrinkage was observed in inner and outer membrane of Bowman's capsule in G-4. Abnormal
mesangial cell proliferation was evident in G-5. Glomerular constriction was observed in G-6 and G-7.

Some mice were sacrificed after light anaesthesia on day 7. Liver and spleen were dissected out and homogenized in cold tris HCl buffer. The supernatant obtained was used for various biochemical estimations.

In *P. berghei* infected mice, 41% decrease in hepatic protein concentration was recorded as compared to normal group of mice (G-1). Among plant extracts/drug treated mice, G-6 showed maximum increase in hepatic protein concentration followed by G-4, G-8 and G-9 as compared to G-1. G-7 exhibited 36% decrease in hepatic protein concentration.

In contrast to hepatic protein concentration, spleen registered a significant increase in its protein concentration as compared to G-1. All treated groups (G-3 to G-9) showed significant increase in protein concentration as compared to G-1, while all groups except G-7 showed a significant decrease (p < 0.001) in total protein concentration as compared to infected control (G-2).

Alkaline phosphatase activity was found to be significantly increased (p < 0.0001) in *P. berghei* infected mice (G-2) as compared to G-1. Specific activity of ALP was expressed as nmol p-nitrophenol/min/mg protein. Among treated groups G-7 showed maximum ALP activity (72.10 ± 1.37) followed by G-5 (55.16 ± 3.97), G-6 (98.28 ± 1.34), G-4 (45.96 ± 3.06), G-8 (33.72 ± 1.76), G-3 (30.86 ± 2.22) and G-7 (29.56 ± 1.64) with significant (p < 0.001) as compared to G-1. G-3 (750 mg/kg ELEAB) and G-9 (100 mg/kg artesunate) exhibited ALP activity comparable to G-1 (normal) mice. In *P. berghei* infected spleen significant increase (p < 0.0001) in ALP activity was recorded as compared to normal mice G-1. Maximum ALP activity was registered in *Eucalyptus* treated mice (G-7), followed by G-6, G-8 and G-9 with significant (p < 0.001) value as compared to G-1.
Specific activity of acid phosphatase (ACP) was expressed as nmol p-nitrophenol/min/mg protein. Infected mouse liver (G-2) exhibited significant increase in ACP activity (p < 0.001) as compared to normal mice (G-1). Minimum ACP activity in treated group was recorded in G-9 followed by G-3, G-8, G-6, G-4, G-5 and G-7 in increasing order.

ACP activity was significantly increased (p < 0.0001) in infected mouse spleen as compared to normal mouse. Maximum ACP activity was recorded in G-7 (74.38 ± 4.12) followed by G-5 (66.9 ± 3.49), G-4 (62.99 ± 4.6), G-6 (58.98 ± 2.65), G-8 (55.64 ± 4.91), G-3 (53.60 ± 3.26) and G-9 (50.52 ± 3.32) as compared to positive control. ACP activity in liver and spleen of G-3 and G-9 was found to be comparable to G-1.

Specific activity of aspartate aminotransferase (AST) was expressed as nmol pyruvate (min/mg/protein). AST activity in P. berghei infected mice (G-2) was increased significantly (p < 0.0001) as compared to normal mice (G-1). In treated groups, maximum enhancement in AST activity was recorded in G-7 followed by G-5, G-6, G-4, G-8, G-3 and G-9 as compared to G-1. Specific activity of AST in infected spleen was enhanced significantly as compared (p < 0.001) to G-1. G-3 and G-9 exhibited AST activity comparable to normal mice.

Specific activity of alanine aminotransferase (ALT) in infected mice liver (G-2) was increased (p < 0.0001) as compared to G-1. Maximum increase in ALT activity was recorded in liver of G-7 followed by G-5, G-6, G-4, G-8, G-3 and G-9 as compared to G-1. Similar trend was observed in ALT activity in spleen of various groups.

Lactate dehydrogenase (LDH) activity of infected mice liver (G-2) was increased significantly (p < 0.0001) as compared to G-1. LDH activity was increased in liver of all treated mice as compared to G-1, but it was significantly less (p < 0.001) as compared to G-2.
Maximum increase in LDH activity of spleen was observed in G-7 (481.08 ± 10.14) followed by G-5 (476.50 ± 11.13), G-4 (471.44 ± 8.40), G-6 (463.36 ± 6.76), G-8 (455.26 ± 4.26), G-3 (422.18 ± 7.41) and G-9 (416.12 ± 3.39) as compared to G-1, however, LDH activity was recorded to decline significantly (p < 0.001) in all treated groups as compared to infected mice (G-2).

Following conclusions can be drawn from the present work:-

- Evaluation of antiplasmodial efficacy of *Ajuga bracteosa*, *Xanthium strumarium* and *Berberis aristata* has been found to exert significant schizont maturation inhibition of *P. berghei* isolates *in vitro* with an IC50 of 10, 5 and 40 μg/ml respectively. It shows moderate and active *in vitro* antiplasmodial efficacy of ELEAB and ELEXS respectively, while weak antiplasmodial efficacy of AREBA.

- The phytochemical screening of ELEXS and ELEAB has showed the presence of alkaloids, saponins, phenolic compounds, steroids and cardiac glycosides while anthroquinone was not detected. In AREBA, anthroquinone and phenolic compounds, both were not detected.

- LD50 of ELEAB and AREBA has been found to be more than that of 5 g/kg which showed the clinical safety of plant extracts, while LD50 of ELEXS has been found to be 1.5 g/kg, which indicates the moderate toxicity of plant extract.

- ELEXS has been found to show higher *in vivo* suppressive, preventive and curative effect than that of ELEAB, however, the higher concentration of extract has been found to be toxic, as observed in histopathological and biochemical parameters, while ELEAB has been found to be safe as evident from the results. Thus, it exhibits better antiplasmodial efficacy. AREBA has been found to show weak *in vitro* as well as *in vivo* antiplasmodial efficacy when compared with ELEXS and ELEAB.
Among China and *Eucalyptus*. China (1 : 2 φ) showed better results as compared to *Eucalyptus* (1 : 2 φ) in terms of antiplasmodial efficacy and clinical safety. *Eucalyptus* (1 : 2 φ) has been found to exert side effects on the tissue (liver, spleen and kidney) and seen to elevate all liver function enzymes of host.

Among quinine and artemisunate treated mice artemisunate showed better results in terms of clinical safety as evident from the histological and biochemical study carried out in the present investigation.